

Transport Studies of Component Proteoglycan Molecules through Cartilage

by

Michelle Chi Jen

Submitted to the Department of Electrical Engineering and
Computer Science

in Partial Fulfillment of the Requirements for the degree of

Master of Engineering in Electrical Engineering

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September 1995

© Michelle Chi Jen, MCMXCV. All rights reserved.

The author hereby grants to MIT permission to reproduce and
distribute publicly paper and electronic copies of this thesis
document in whole or in part, and to grant others the right to do so.

Author

Department of Electrical Engineering and Computer Science

June 21, 1995

Certified by

Alan J. Grodzinsky

Professor

Thesis Supervisor

Accepted by

Frederic R. Morgenthaler

Chairman, Department Committee on Graduate Theses

MASSACHUSETTS INSTITUTE
OF TECHNOLOGY

AUG 10 1995

LIBRARIES *Walker Eng*

Transport Studies of Component Proteoglycan Molecules through Cartilage

by

Michelle Chi Jen

Submitted to the Department of Electrical Engineering and Computer Science
on June 21, 1995, in partial fulfillment of the
requirements for the degree of
Master of Engineering in Electrical Engineering

Abstract

This paper describes a study of the transport properties of rat chondrosarcoma proteoglycan monomers and GAG chains through the extracellular matrix of adult bovine cartilage. Cartilage disks were harvested from the femoropatellar groove of freshly slaughtered adult cows and mounted in a two compartment transport chamber. Radiolabelled monomers or GAG chains were added to the upstream compartment; the downstream concentration of the solutes were monitored during passive diffusion and field applications. Reasonable diffusion coefficients were calculated based upon these experiments and partition experiments. Electromigration appeared to play the dominant role in solute flux during field applications.

Thesis Supervisor: Alan J. Grodzinsky

Title: Professor

Acknowledgments

First and foremost, I would like to thank my advisor Alan Grodzinsky for his unending patience and encouragement. I feel very lucky to have gotten a chance to work in his lab.

There are not enough words to express my appreciation for all the time and advice Minerva Garcia has contributed to helping me along with my work in this lab. She's played a critical part in all aspects of the work I've done, from the trips to the slaughter house to repacking dried out columns to providing tips on experiments and data interpretation. I am truly amazed with your unbounded patience and generosity, and I am very fortunate to have had you as my mentor. Thank you, thank you, thank you.

Thanks to Jane Murray for introducing me to all the laboratory basics. Thanks to Eliot Frank for solving all those esoteric software problems that no one else could. Thanks to Larry Bonassar for his Athena tips. Thanks to everyone else in the lab who have helped me out by providing answers to random lab trivia and for making my experience in this lab a pleasant one.

Many thanks to Dr. Anna Plaas of the Shriner's Hospital in Tampa for providing the radiolabelled rat chondrosarcoma aggrecan and GAG chains. Thanks also to Dr Jim Kimura, Dr. Vince Hascall, and Dr. John Sandy for very helpful comments on column chromatography.

My friend Victor has made my years at MIT really great ones. I cannot thank you enough for all that you've done for me through the years. You are truly one of a kind! I wish you all the best for the future.

Thanks to Julia and all the rest of the gals of McCormick "5th West" for all the silly times. Thanks to all the other friends I've met over the years here for making these past five years at MIT a truly unforgettable experience.

Finally, thanks to my family for all your support throughout the years.

This research was funded in part by NIH grant AR33236.

Contents

1	Introduction	8
1.1	Cartilage Composition and Structure	8
1.2	Motivation	9
1.3	Previous Research	11
1.3.1	Transport	11
1.3.2	Partitioning	11
1.3.3	Concentration Dependent Molecular Shrinkage	12
1.4	Objectives	12
1.5	Additional Background	14
2	Materials and Methods	15
2.1	Preparative Steps	15
2.1.1	Solute Preparation	15
2.1.2	“Cold” Chondroitin Sulfate Preparation	16
2.2	Cartilage Dissection	17
2.3	Transport Experiments	18
2.3.1	Equipment	18
2.3.2	Protease Inhibitors	20
2.3.3	General Protocol	20
2.3.4	GAG Chains Transport	22
2.3.5	Aggrecan Transport	22
2.4	Partition Experiments	22
2.5	Auxiliary Procedures	23

2.5.1	GAG assay	23
2.5.2	DNA assay	23
2.5.3	Scintillation Counting	24
2.5.4	Weights	24
2.5.5	Chromatography	24
3	Theory and Calculations	27
3.1	Flux Equation	27
3.1.1	Donnan Equilibrium Partitioning	27
3.1.2	Calculation of Diffusion Coefficient	28
3.2	Electroosmotic Convection	29
4	Results	31
4.1	Partition Coefficients	31
4.1.1	GAG Chains	31
4.1.2	Aggrecan Monomers	33
4.2	Transport	35
4.2.1	GAG Chains	35
4.2.2	Aggrecan Monomers	41
4.3	Chromatography	46
4.3.1	Free ³⁵ S in GAG Chain Preparation	46
4.3.2	Characterization of “Cold” Chondroitin Sulfate	48
4.3.3	Free ³⁵ S in Aggrecan Preparation	49
5	Discussion	51
5.1	Experimental Difficulties	51
5.2	Interpretation Within Context of Current Literature	52
5.3	Summary	53
5.4	Future Research	53
A	Tables	55

List of Figures

1-1	Proteoglycan	8
2-1	Superose 6 chromatograph of GAG chains	16
2-2	Transport Chamber	18
3-1	Electroosmotic Convection in Cartilage	30
4-1	GAG Partition Measurements: Experiment #1	32
4-2	GAG Partition Measurements: Experiment #2	34
4-3	Aggrecan Partitioning	35
4-4	CS Transport Experiment #1	36
4-5	CS Transport Experiment #2	38
4-6	CS Transport Experiment #3	40
4-7	PG Transport Experiment #1	42
4-8	PG Transport Experiment #1: Aliquots	43
4-9	PG Transport Experiment #2	45
4-10	PG Transport Experiment #2: Aliquots	46
4-11	CL6B on GAG chains	47
4-12	CL6B on “cold” GAG chains	48
4-13	CL2B on aggrecan	49
4-14	PD10 Fractionation	50

List of Tables

4.1	GAG Partition Measurements: Experiment #1	32
4.2	GAG Partition Measurements: Experiment #2	33
4.3	PG Partition Measurements	34
A.1	Auxiliary Data for GAG Chain Transport: Experiment #1	55
A.2	Auxiliary Data for GAG Chain Transport: Experiment #2	56
A.3	Auxiliary Data for GAG Chain Transport: Experiment #3	56
A.4	Auxiliary Data for Aggrecan Transport: Experiment #1	56
A.5	Auxiliary Data for Aggrecan Transport: Experiment #2	56
A.6	Auxiliary Data for GAG Chain Partition: Experiment #1	57
A.7	Auxiliary Data for GAG Chain Partition: Experiment #2	57
A.8	Auxiliary Data for Aggrecan Partition	57

Chapter 1

Introduction

1.1 Cartilage Composition and Structure

Healthy articular cartilage is essential to proper joint operation, distributing loads and providing a low frictional surface for motion. The functional properties of cartilage

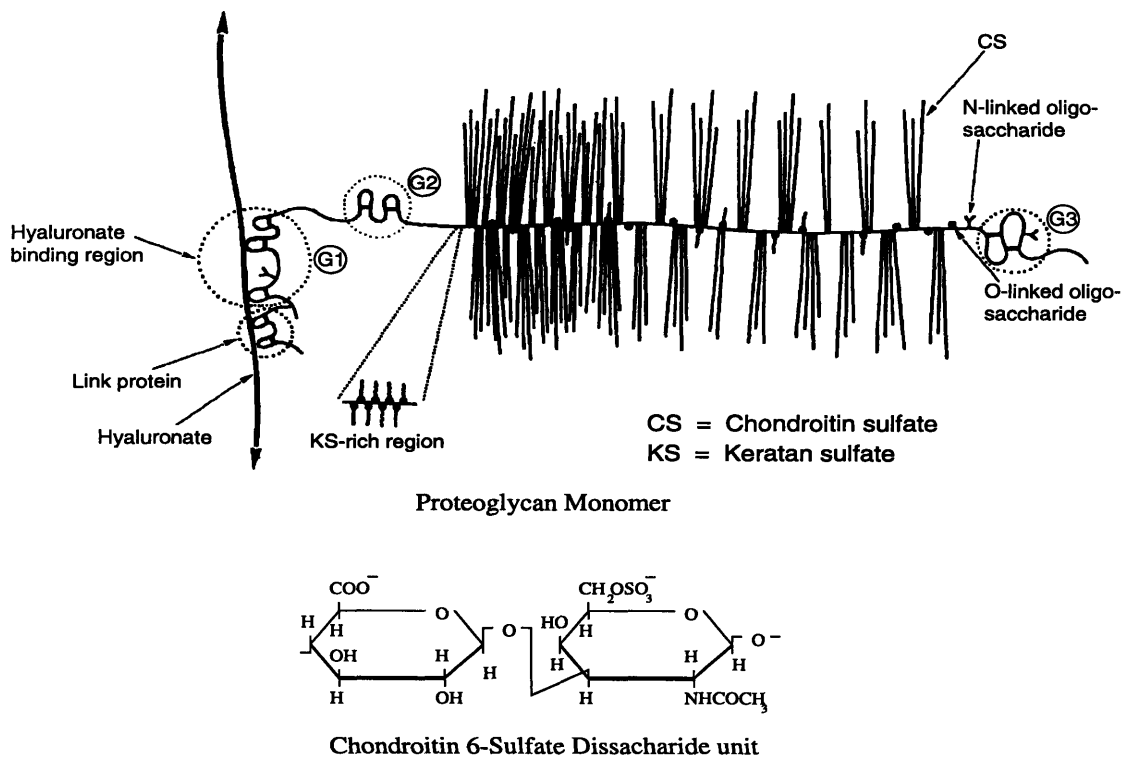


Figure 1-1: Proteoglycan Structural Representation adapted from [6][1]

depend largely on the proteoglycan and collagen structural macromolecules, which make up much of the solid component of a dense extracellular matrix. The proteoglycans, composed of negatively charged polysaccharides known as glycosaminoglycans (GAGs) on a protein core, expand due to repulsive forces and interact with tissue fluid to give cartilage its equilibrium stiffness. The collagen forms a network through extensive crosslinking of fibrils which restrains proteoglycan expansion and gives cartilage its tensile strength. In addition, a sparse population of cells, chondrocytes, whose density declines with age, is responsible for the synthesis of the matrix macromolecules.

A conceptual representation of proteoglycan structure is provided in Figure 1. A variable length protein core consists of three domains G1, G2, and G3. The region between the G2 and G3 domains is the site of attachment of a 100 or so linear GAG chains, of which chondroitin sulfate (CS) chains predominate. The CS chains are comprised of repeating disaccharide units which possess on average two negative charges of which one is a sulfate group located in either the 4 or the 6 position. The G1 domain allows the proteoglycan to associate reversibly with hyaluronic acid. The association (or aggregation) of 300 or more monomers with hyaluronic acid plays a key role in the immobilization of the proteoglycans in the cartilage matrix. Average molecular weight of CS chains is 20,000 while that of the aggrecan monomer is 1×10^6 . GAG concentration may vary from 17 to 85 mg ml⁻¹ in articular cartilage[2].

1.2 Motivation

Although the level of synthesis of matrix macromolecules by the chondrocytes declines as cartilage matures, it never completely diminishes[1]. Even in adult cartilage, ongoing synthesis activity occurs as part of normal matrix turnover. This turnover consists mainly of proteoglycan degradation and synthesis[10] (as collagen molecules are much more slowly replaced in mature cartilage[10][11]). Estimated time for all the GAGs in a matrix to be replaced ranges from about 280 days in adult dog to 600-1000

days in adult humans[10]. This level of metabolic activity can become elevated due to variations in environmental conditions, e.g. increased dynamic loading[11]. Furthermore, various distinct pools of proteoglycans may have turnover rates that deviate from the rate mentioned above[10]. Newly synthesized proteoglycan molecules must be transported from the chondrocytes to their ultimate destination in the matrix where they become immobilized. Transport of the newly-synthesized proteoglycans is an important part of a process leading to their final incorporation into the matrix. Additionally, proteoglycan fragments resulting from matrix degradation must be transported out of the matrix as waste products. While the estimated proteoglycan turnover rates mentioned above require only relatively slow movement of the proteoglycans through the cartilage matrix, the mechanism(s) responsible for proteoglycan transport are not yet well characterized. Proteoglycan transport measurements would give us a more complete picture of the physiologic processes that occur in the matrix.

Aside from contributing to our understanding of *in vivo* processes, one of the most valuable returns of characterizing bulk transport of proteoglycans through the cartilage matrix is the potential to shed more light on the interpretation of experiments. This refers in particular to experiments that involve measurement of proteoglycan content released from the matrix of tissue subjected to certain degradative treatments or conditions. Specifically, knowledge about proteoglycan transport rate would enable the identification of a certain fraction of the time lag between start of treatment and appearance of measurable levels of released proteoglycan as a transit period during which the proteoglycans are actually moving through the matrix. Indeed, this would eliminate some of the ambiguity involved in determining how long it takes for various treatments (e.g. enzymatic) to make an impact in the matrix.

1.3 Previous Research

1.3.1 Transport

Thus far, there have been no reported measurements of bulk proteoglycan transport through cartilage (although there is ongoing research focussing on localized transport). Previous studies on bulk proteoglycan transport focus on proteoglycan flow properties in solution. In one study, Comper and coworkers found that the diffusion coefficient of rat chondrosarcoma proteoglycan monomers ($MW \sim 2.6 \times 10^6$) in solution varies linearly with concentration from $< 1 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ for a concentration of $< 5 \text{ mg ml}^{-1}$ to $\sim 2.3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for a concentration of $\sim 60 \text{ mg ml}^{-1}$ [2]. Furthermore, they propose that the movement of the monomers is dominated largely by the interaction of the CS chains with the surrounding water[2]. In fact, for concentrations above 10 mg ml^{-1} diffusion coefficients for proteoglycan monomers and CS chains ($MW \sim 30,000$) were found to be very similar[2].

1.3.2 Partitioning

A possible pathway for the movement of proteoglycans through the dense extracellular matrix of cartilage (as mature cartilage lacks a vascular supply) may be provided by a small number of large pores proposed to exist by Maroudas[9]. In studies on transport of large solutes through cartilage, Maroudas found that partition coefficients of large solutes, in addition to being highly sensitive to GAG content, show a sharply decreasing trend with increasing Stokes radius up to 35.5 \AA of serum albumin; above this size, she found that the partition coefficient of IgG ($MW \sim 160,000$) with Stokes radius of 56 \AA nearly coincides with that of serum albumin[9] (0.01 at a fixed charge density of 0.08 mEq g^{-1} (34.1 mg ml^{-1}) and 0.001 at a fixed charge density of 0.16 mEq g^{-1} (68.3 mg ml^{-1}))[10]. Average effective pore size of cartilage is estimated to be around 90 \AA [10]. Maroudas accounts for the lack of further drop off in partition coefficient for solutes larger than serum albumin by suggesting that the transport of these larger solutes occurs mainly through a hypothetical small pop-

ulation of pores much larger than the average pore, which she further postulates may be key in enabling movement of proteoglycans through the cartilage matrix[9].

1.3.3 Concentration Dependent Molecular Shrinkage

An interesting study on the molecular shrinkage of proteoglycans was carried out by Harper and Preston[5]. Under high ionic strength conditions that minimize charge interactions, they found that proteoglycans shrank in various solutions of linear flexible polymers with the amount of shrinkage dependent upon the concentration of added polymers[5]. Using a light scattering technique, they found that the apparent molecular weight of proteoglycans decreased with increasing concentration of chondroitin sulfate up to 20 mg/ml[5], a phenomena they attributed to excluded volume interactions between the proteoglycan and chondroitin sulfate molecules[5]. The results of this study suggest that an increased background concentration of chondroitin sulfate might enhance the partitioning of PG monomers into cartilage, thus also improving its flux through cartilage.

1.4 Objectives

The purpose of this thesis is to characterize the bulk transport of proteoglycans through cartilage. In particular, this involves the following:

- Quantifying the steady-state, diffusive flux of proteoglycan monomers and their component chondroitin sulfate chains through cartilage.
- Measuring the effect of an applied electric field and convective fluid flow on the above diffusive fluxes.
- Measuring partition coefficients of the proteoglycan monomers and CS chains.

Diffusive Flux While the size of a solute plays an important role in determining its diffusive properties through tissue, the shape of the solute may play an equally important role. In the case of long proteoglycan monomers (aggrecan), one might

expect the monomer to be able to bend and deform as it makes its way through the matrix, while a more globular solute with a similar molecular weight might not be expected to penetrate the matrix. With this frame of mind, one might predict similar diffusivities for monomers (minus their G1 binding domain) and their component CS chains as found by Comper and coworkers for the case of a proteoglycan solution.

Fields and Convection in the Matrix The application of an electric field introduces both electrophoretic and convective (electroosmotic) effects in cartilage. The physiologic analog of this is when cartilage undergoes compression due to loading. The induced convection of tissue fluid as it is squeezed out of the cartilage and then allowed to flow back in introduces internal fields known as streaming potentials. The origin of the streaming potentials comes from the equilibrium requirement of electroneutrality in the cartilage. The negative charges of the proteoglycan aggregates fixed in the matrix result in a majority of mobile positive ions in the tissue fluid. Fluid convection during compression carries away the positive ions leaving behind the fixed negative charges of the proteoglycan aggregates, thus inducing internal electric fields. One can imagine that these electric fields would have a substantial impact on the transport rate of freely moving, negatively charged proteoglycan monomers and GAGs. In addition, one would suspect convection to enhance the transport rate of these proteoglycan subunits, which are normally hindered in their movement through the matrix because of their large size. An important issue here is to separate the opposing convective and electrophoretic effects.

Partition Coefficients The partitioning of proteoglycans between the cartilage and the outside solution is used in combination with flux measurements to determine diffusivity. One would predict an extreme downward partitioning of freely moving proteoglycans from the outside solution into the cartilage both due to their high negative charge density and their large size.

1.5 Additional Background

Although not specifically addressed in this study, the fixed charge density (directly related to the GAG content) of cartilage may have a significant impact on transport and partitioning of solutes into cartilage. GAG content varies considerably from joint to joint. The porous structure of the GAG-water gel in the matrix is much finer than the 60 to 200 nm gaps separating collagen fibrils[2], thus GAG concentration is the limiting factor which determines permeability of cartilage to various solutes[9]. Especially for large solutes whose size is on the order of the average pore size of the matrix, one would expect variations in GAG content to have a significant impact on their ability to penetrate and move through the matrix. Studies by Maroudas have shown that a threefold increase in fixed charge density decreases by a hundredfold the partition coefficient of such large solutes as serum albumin and IgG[10]. Variations in GAG content might thus be expected to have an even more pronounced effect on the movement through the matrix of an average proteoglycan monomer with effective hydrodynamic radius ranging from 50 to 80 nm[2].

Chapter 2

Materials and Methods

2.1 Preparative Steps

2.1.1 Solute Preparation

The radiolabelled rat chondrosarcoma aggrecan and free GAG chains were all prepared and generously provided by Dr. Anna Plaas. The following are the protocols that Dr. Plaas used in preparing the solutes.

Aggrecan Monomer The aggrecan monomers were obtained using D1 preparations. Rat chondrosarcoma cells were labelled with $60\mu\text{Ci/ml}$ of $^{35}\text{S-SO}_4$ in DMEM/10% FCS for 18 hours. The cells and medium were removed with a cell-scraper and extracted in 4M Gdm·HCl/50mM sodium acetate at 4°C for 48 hours in the presence of protease inhibitors. CsCl was added to a concentration of 1.45g/ml; the preparation was subsequently centrifuged at 38000 r.p.m. at 10°C for 48 hours. The D1 fraction (density ~ 1.58 g/ml) was dialyzed against distilled water (to remove the free label) and lyophilized. The final GAG content was approximately 4 mg, and the specific activity was approximately 50×10^6 cpm/mg GAG.

Free GAG chains The free GAG chains were prepared by β -elimination of the D1 fraction prepared as described above. The D1 fraction was dissolved in 50 mM NaOH/1M NaBH₄ and treated at 45°C for 24 hours to eliminate the chains. The

preparation was neutralized on ice with acetic acid, washed with an equal volume of methanol, and speedvac concentrated. The residue was washed twice with acidified methanol. The final washed/dried sample was resuspended in 500 μ l of 500 mM ammonium acetate at pH 7.3 and chromatographed on Superose 6 in 500 mM ammonium acetate at pH 7.3. The peak fractions were pooled (as shown in Figure 2-1), lyophilized, and washed twice with distilled water. The final preparation had a GAG content of approximately 4 mg with a specific activity of approximately 330×10^6 CPM.

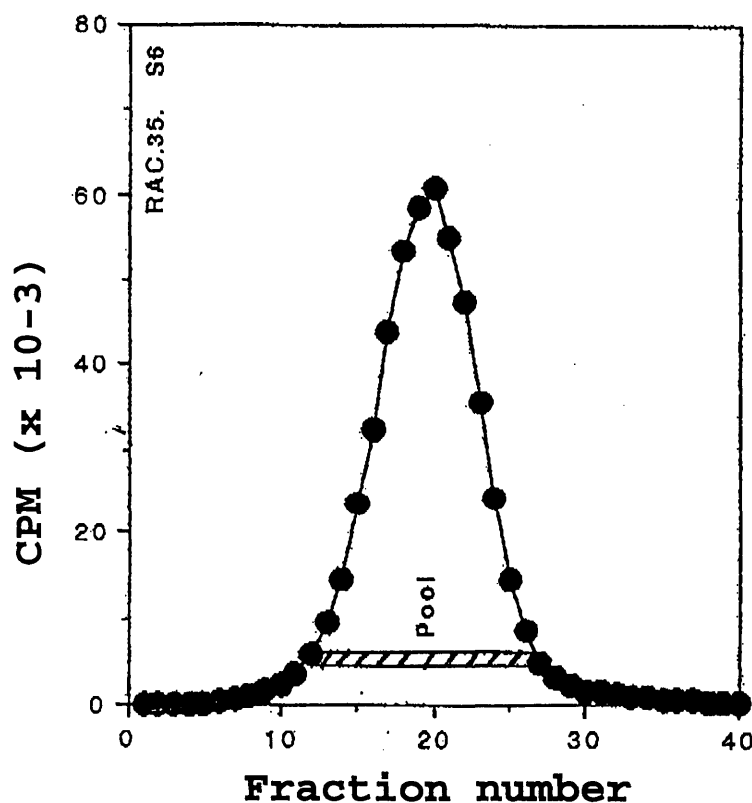


Figure 2-1: Superose 6 chromatograph of GAG chains

2.1.2 “Cold” Chondroitin Sulfate Preparation

“Cold” chondroitin sulfate obtained from Sigma was prepared for use in the transport experiments. The chondroitin sulfate was processed according to a procedure recommended by Dr. Jim Kimura to remove salts and low molecular weight fragments prior to use in the transport experiments. The following are the steps used in the preparation:

1. Papain digest the chondroitin sulfate C (Sigma cat#C4384) overnight using an enzyme-substrate ratio of 1/1000.
2. Boil the digested solution for 15 minutes. The solution should look cloudy.
3. Centrifuge at 5000G for about half an hour. The solution should look clear.
4. Carefully transfer the supernatant into another vial.
5. Add 2 volumes of 0.1 M Sodium Acetate dissolved in 100% ethanol to the supernatant. Shake well.
6. Spin at 5000G for 10 to 15 minutes.
7. Carefully remove and discard the supernatant leaving the pellet.
8. Lyophilize the pellet overnight.

The molecular weight distribution of the “cold” chondroitin sulfate preparation was determined using CL-6B column chromatography. The results are described in section 4.3.2. Prior to each experiment, the prepped chondroitin sulfate was reconstituted in a solution of 0.15 M PBS containing 2 mM EDTA.

2.2 Cartilage Dissection

All the tissue used in this study were freshly harvested from the femoropatellar groove of joints purchased from Bertolino's of South Boston. Several cores were drilled out from the joint, paying special attention to obtaining as flat a surface of cartilage as possible. The cores were individually mounted on a microtome and secured at their bone base with screws. The orientation of each core was adjusted so that its top surface was parallel to the blade. Several top slices of cartilage were removed until a nearly circular shape could be obtained. Finally, several 200 μ m thick disks of cartilage were removed from the core. As much as possible throughout the dissection process, the tissue was maintained over ice and submersed in Triple Punch solution (2 mM EDTA in PBS plus 1ml/100ml dilution of Antibiotic Antimycotic solution from Sigma

(cat#A7292; contains Penicillin 100 U/ml, Streptomycin 0.1 mg/ml, Amphotericin B 0.25 $\mu\text{g/ml}$)

2.3 Transport Experiments

2.3.1 Equipment

Transport Chamber Figure 2-2 provides a simplified schematic of the transport chamber. The plexiglass transport chamber consists of two compartments, an up-

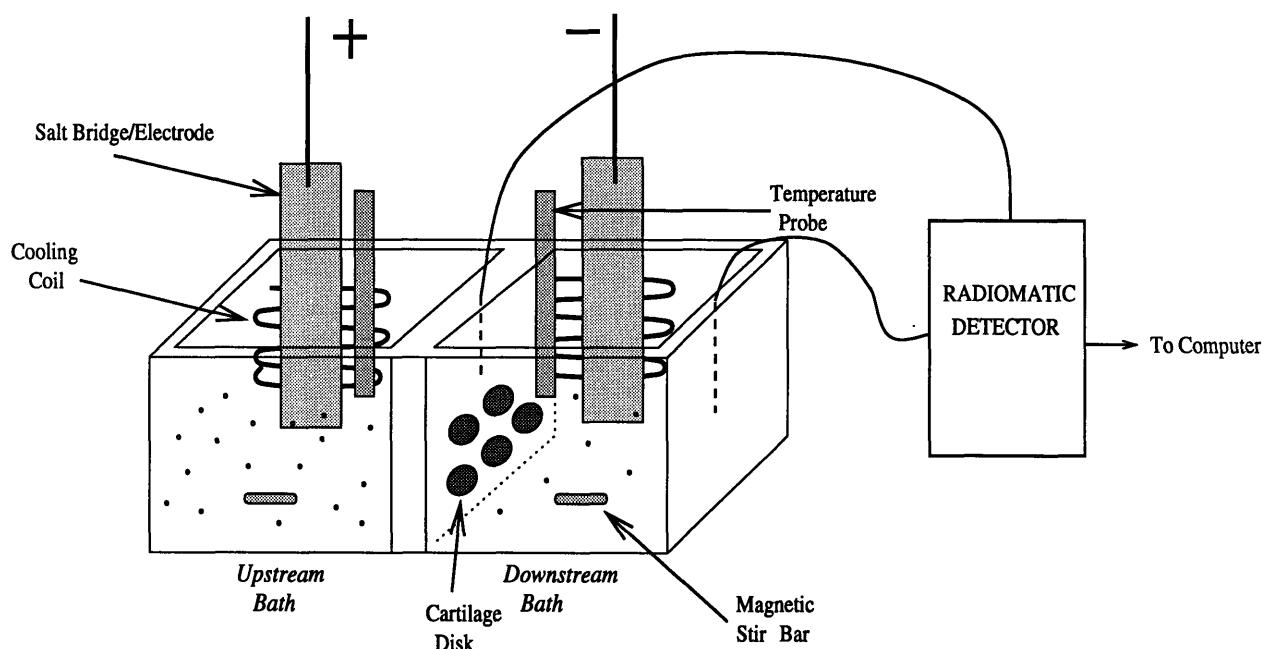


Figure 2-2: Transport Chamber

stream compartment and a downstream compartment, separated by a wall. In the wall are five circular apertures in which five cartilage disks 9 mm in diameter can be mounted. After mounting, the free surface area per cartilage disk exposed to diffusing solutes has a diameter of 7 mm. The thickness of the cartilage disk can be varied; compensation is achieved by using o-rings and gaskets as needed when mounting the cartilage disks in the apertures. The o-rings and gaskets establish a seal so that

the only path of transport between the upstream and downstream compartments is through the cartilage tissue.

Cooling coils consisting of teflon tubing through which refrigerated potassium dichromate circulates were used to keep the upstream and downstream baths below room temperature. The cooling bath was maintained at a relatively steady temperature using a HAAKE A81 refrigerated circulator. Temperature probes were immersed in both the upstream and downstream baths so that temperature could be checked at intervals.

Magnetic stir bars were used to keep the baths well stirred and to minimize formation of a stagnant liquid film at the cartilage surface, a phenomena that increases the effective thickness of the cartilage and reduces solute diffusivity[7].

Salt Bridges and Electrodes Silver electrodes buffered by polyacrylamide salt bridges were used to apply a current density through the cartilage. The recipe for the salt bridges consisted of the following:

- 0.04 g N,N'-methylene-bis-acrylamide crosslinker
- 1.46 g acrylamide
- 15 ml deionized water
- 10 μ l 40% (w/v) ammonium persulfate solution
- 10 μ l TEMED

The salt bridges were cast in plexiglass tubes with two layers of dialysis membrane (molecular weight cutoff: 12,000-14,000) secured to the bottom end. The salt bridges were immersed in the upstream and downstream baths only during current application. 25 mM HEPES buffer diluted in 0.15 M PBS was used in the salt bridges to minimize pH changes.

Radioactive Detector A Radiomatic FLO-ONE detector made by Packard Instrument Company was used to monitor the radioactivity of the downstream bath. The energy window used for the ^{35}S isotope was 18-167. The downstream bath was continuously recirculated through the detector, passing through a solid scintillant cell packed with CaF_2 crystals. The FLO-ONE detector monitors the radioactivity in the solid scintillant cell and interfaces with a computer that samples and records the data every six seconds.

2.3.2 Protease Inhibitors

The following protease inhibitors were prepared for use in the transport experiments:

- 0.1 M PMSF in isopropanol (this is a 100x stock solution)
- 0.5 M Benzamidine-HCl in ethanol (this is a 100x stock solution)
- 2 mM EDTA in PBS, titrated to pH 7 with 1 N NaOH and sterile filtered.

2.3.3 General Protocol

After harvesting the cartilage slices as described in Section 2.2, a more precisely circular disk was punched out of each slice using a $\frac{3}{8}$ " steel punch. The following is the general procedure used for every transport experiment:

1. Equilibrate the freshly harvested cartilage overnight in 0.15 M PBS with "cold" chondroitin sulfate (at $+4^\circ\text{C}$ with protease inhibitors).
2. Measure the wet weight of each cartilage disk, making sure to wipe away any surface moisture prior to weighing.
3. Measure the thickness of each cartilage disk at three different locations using a micrometer.
4. Assemble the chamber with the tissue.

5. Check the chamber for leaks by placing 50 ml of 2 mM EDTA in PBS in each compartment of the chamber. Wait 15 minutes after addition of fluid to each side. Check for leakage from one compartment to another and for leakage from inside the chamber out.
6. Assuming no leaks, replace solutions in both compartments with fresh 2 mM EDTA in PBS (50 mL) containing dissolved “cold” chondroitin sulfate. Add appropriate amount of stock PMSF and Benzamidine-HCl to each bath.
7. Set up cooling system to maintain the temperature of the baths at approximately $20 \pm 2^\circ\text{C}$. Begin stirring. Allow system to equilibrate for at least an hour.
8. Set up tubing to circulate downstream bath through the detector.
9. Obtain baseline slope for a couple of hours. Add radioactive solute to upstream compartment.
10. Allow system to run as long as necessary to get a decent slope. Do a calibration to determine the upstream bath concentration by transferring a small, known volume of fluid from the upstream to the downstream bath. Continue recording downstream radioactivity.
11. Apply current densities of $+26\text{mA}/\text{cm}^2$ and $-26\text{mA}/\text{cm}^2$ for as long an interval as possible until pH of baths begins to move out of an acceptable range. Monitor pH using litmus paper. Make sure pH stays relatively close to 7 ± 1 .
12. Throughout the entire run, keep the chamber sealed as much as possible to minimize evaporation.
13. At the end of the transport experiment, remove the cartilage slices and rinse them with PBS. Lyophilize the tissue and save them for subsequent analysis. Also save the upstream and downstream baths.

2.3.4 GAG Chains Transport

The radiolabelled rat chondrosarcoma GAG chains were initially in powdered form. The entire 4 mg of radiolabelled GAG chains was reconstituted for use in the first transport experiment. After the experiment, the upstream bath was saved in the freezer for reuse in subsequent repeat experiments.

2.3.5 Aggrecan Transport

The aggrecan transport experiments were almost identical to the GAG chain transport experiments. One problem encountered in dealing with the aggrecan, however, was that it tended to stick to the CaF crystals in the solid scintillant cell. To minimize sticking, the detector system was preconditioned with 0.1% BSA prior to starting the experiment. BSA was also added to the downstream bath at a final concentration of 0.01%. In addition, aliquots were taken manually from the upstream and downstream baths; the data collected from the aliquots was compared to the data recorded using the detector.

2.4 Partition Experiments

Cartilage was freshly harvested as described in section 2.2. Subsequently, 3 mm or 9 mm plugs were punched out of the slices. Wet weights and thickness measurements were taken for each plug. The plugs were divided into various conditions and placed into either orange-capped vials or plastic scintillation vials to prevent evaporation. Conditions varied with “cold” CS concentration and/or amount of time allowed for equilibration. In addition to prepped “cold” CS, media for each condition contained radiolabelled aggrecan or GAG chains. Appropriate concentrations of protease inhibitors were also used. All samples were equilibrated on a shaker in a incubator at 37°C.

At the end of the equilibration period, the tissue slices were removed from the media

and washed *briefly* in 0.15 M PBS. The plugs were lyophilized, weighed, and digested with protease K. Samples of both the digested plugs and the media were counted in the scintillation counter. GAG assays were also performed on the digested plugs.

2.5 Auxiliary Procedures

Several standard laboratory protocols were used to gather data as part of the experiments. The following sections provide a brief description of these procedures.

2.5.1 GAG assay

Assaying for GAG content was a standard part of post-experimental analysis for every experiment performed. Typically, the cartilage plugs used in the transport or partition experiments were digested with either papain or protease K and then assayed for GAG content. GAG assays were also performed on fractions collected during column chromatography as described in section 2.5.5. The GAG assay used was a metachromatic, absorbance assay. In the assay, 2 ml of 1,9-dimethylmethylene blue chloride dye solution is added to 20 μ l of sample[3]. The absorbance of the mixture is immediately measured on a Perkin Elmer spectrophotometer with wavelength set at 525 nm. All samples were measured in duplicates. The optical density measurement is compared to measurements obtained from a set of standards made from shark fin chondroitin sulfate (Sigma).

2.5.2 DNA assay

DNA was used as a molecular weight marker during column chromatography as described in section 2.5.5. Consequently, fractions collected during chromatography had to be assessed for DNA content. 100 μ l of the sample was added to 2 ml of Hoechst dye solution; fluorescence measurements were taken using a SPF-500C Spectrofluorometer (SLM Instruments)[8]. Duplicate aliquots were measured for each sample.

2.5.3 Scintillation Counting

Scintillation counting was a necessary part of every experiment. Aliquots from transport experiments, media and digested plugs from partition experiments, and fractions from column chromatography runs all had to be counted. Typically, 50 or 100 μl of sample was added to 2 ml of Ecolume scintillation fluid. A Rackbeta liquid scintillation counter (LKB Wallac) was used to measure radioactivity of samples in CPM. When dual labels were present in the same sample, the raw data was processed to correct for overflow from one channel to the next.

2.5.4 Weights

Both wet weights and dry weights were measured for every cartilage slice used in the transport and partition experiments. Wet weights were taken prior to the experiments. After equilibration in solution, the hydrated cartilage slices were patted on a paper towel to remove surface moisture and then weighed. Dry weights were measured after the experiments. The cartilage slices were lyophilized after the experiments and then weighed. The difference between the wet weight and dry weight of a particular cartilage slice was then used as an estimate of equilibrium water content.

2.5.5 Chromatography

Column chromatography was used to determine molecular weight distribution of the prepped "cold" CS. It was also used to try to detect the presence of free ^{35}S in the upstream bath.

CL-6B Sepharose CL-6B columns from Pharmacia were used for the radiolabelled rat chondrosarcoma GAG chains and the prepped "cold" CS. The following procedure was used to run the columns:

1. Mix 400 μl of 10 $\mu\text{l}/\text{mg}$ stock DNA, 10 μl of 10 $\mu\text{Ci}/\text{ml}$ $^3\text{H}_2\text{O}$, and 500 μl of upstream bath solution. (The DNA and $^3\text{H}_2\text{O}$ serve as molecular weight markers.)

2. Turn on the pump to the column. Allow the fluid level in the column to drop to just above the surface of the bead bed. Turn off pump.
3. Apply the mixture of item 1. Turn on the pump long enough to allow the solution to just sink into the bed.
4. Fill the column to the top with 4 M Guanidine-HCl running buffer (4 M Guanidine-HCl, 1 mM Na₂SO₄·10H₂O, 0.05 M Sodium Acetate, 0.025 M Na₂EDTA, pH 7, degassed) and then connect the column to a reservoir containing the running buffer.
5. Turn on the pump. Using a fraction collector, collect fractions of ~0.65 ml volume at a rate of 18 minutes per fraction. Collect approximately 60 fractions.

The fractions were assayed for DNA, GAG, ³⁵S, or ³H content.

CL-2B Sepharose CL-2B columns from Pharmacia were used for the radiolabelled rat chondrosarcoma aggrecan. The procedure used is exactly the same as that used to run the CL-6B columns as described above.

PD-10 PD-10 fractionation was performed on the last several fractions from the CL-2B columns in hopes of achieving better resolution for determining presence of free ³⁵S. The following steps are used for PD-10 fractionation:

1. Precondition the column to minimize sticking of the aggrecan to the column:
 - (a) Run 20 ml of 4 M Guanidine-HCl buffer through the column.
 - (b) Apply 1 ml of 4% BSA/100 μg CS/10 μl 0.5% phenol red.
 - (c) Elute the column with buffer until all the phenol red is eluted.
2. Pool fractions of interest from the CL-2B run. Mix 0.5 ml of the pooled fractions, 400 μl of 10 μg/ml DNA, 10 μl of 10μCi/ml ³H₂O. Apply to column.

3. Manually add buffer in 0.5 ml volumes. Collect entire 0.5 ml volume in one fraction. Collect approximately 30 to 35 fractions.

All fractions were assayed for DNA, ^{35}S , and ^3H content.

Chapter 3

Theory and Calculations

3.1 Flux Equation

Three mechanisms contribute to solute transport through cartilage: diffusion, migration, and convection. These are incorporated into the Nernst-Planck equation for solute flux, shown below in one dimension[4]:

$$\Gamma = \left(-\bar{D} \frac{\partial \bar{c}(x, t)}{\partial x} + \frac{z}{|z|} u \bar{c} E \right) + \bar{c} v, \quad (3.1)$$

where Γ is the flux of the solute, \bar{D} is the diffusion coefficient of the solute in cartilage, \bar{c} is the intratissue concentration of the solute, u is solute mobility, z is the valence of the solute, v is the intratissue fluid velocity, and E is electric field.

3.1.1 Donnan Equilibrium Partitioning

For ionic solutes like the proteoglycan monomers and GAG chains investigated in this study, the Donnan equilibrium partition relationship must be satisfied. Specifically, the intratissue concentration of the solute is related to its outside bath concentration c by some constant[4]:

$$\left(\frac{\bar{c}_{i+}}{c_{i+}} \right)^{1/|z_{i+}|} = \left(\frac{c_{j-}}{\bar{c}_{j-}} \right)^{1/|z_{j-}|} = \text{constant}, \quad (3.2)$$

where the + refers to positively charged solutes and the - refers to negatively charged solutes. The partitioning of ionic solutes into cartilage is highly sensitive to the tissue GAG content; the concentration of tissue fixed charge groups in combination with the concentrations of the all mobile ionic species present in the tissue must satisfy electroneutrality:

$$\bar{\rho}_m/F + \sum z_i \bar{c}_i = 0, \quad (3.3)$$

where $\bar{\rho}_m$ is tissue fixed charge density and F is Faraday's constant. Experimentally, a partition coefficient k can be measured; this partition coefficient can then be used to relate the intratissue solute concentration to the bath concentrations at the boundaries of the tissue and baths:

$$\bar{c}(x = 0) = kc^u \quad (3.4)$$

$$\bar{c}(x = \delta) = kc^d, \quad (3.5)$$

where c^u and c^d are the upstream and downstream bath concentrations.

3.1.2 Calculation of Diffusion Coefficient

With no applied fields and no hydraulic pressure, the contributions to solute flux due to migration and convection are eliminated from equation 3.1, leaving only the term due to diffusion. In steady state, a linear profile of the solute is assumed. Furthermore, if we assume that the concentration of radiolabelled solute upstream is much greater than the downstream concentration and that the upstream concentration remains constant, the solute flux can be expressed as

$$\Gamma = \bar{D} \frac{kc^u}{\delta}, \quad (3.6)$$

where δ is the cartilage thickness. Conservation of solute particles in the downstream bath requires that the net increase of particles in the downstream bath in a time interval Δt must equal the net influx of particles, thus the following continuity relation

must hold:

$$\bar{D} \frac{k c^u}{\delta} A = V^d \frac{\Delta c^d}{\Delta t}, \quad (3.7)$$

where A is the cross-sectional area of cartilage¹ and V^d is the volume of the downstream bath.

With an unknown upstream solute concentration, the following relation is used to relate the information obtained through the calibration to the values needed to calculate solute diffusivity:

$$\frac{c^u V_{cal}}{V^d} = \Delta c_{cal}^d, \quad (3.8)$$

where V_{cal} is the volume of the aliquot used in the calibration and Δc_{cal}^d is the increase in downstream solute concentration as a result of the calibration. Rearranging the terms in equation 3.8, we can substitute for $\frac{c^u}{V^d}$ in equation 3.7. Furthermore, with the use of radiolabelled solutes, the downstream bath solute concentration is related to the bath radioactivity level as measured by the detector in CPM. Thus the following equation, derived by rearranging equation 3.7 and making appropriate substitutions, can be used directly to calculate the solute diffusivity based upon known or measurable parameters:

$$\bar{D} = \frac{1}{k} \frac{\delta V_{cal}}{A \Delta CPM_{cal}^d} \left(\frac{\Delta CPM^d}{\Delta t} \right). \quad (3.9)$$

3.2 Electroosmotic Convection

The application of a current density across the cartilage tissue during the transport experiments gives rise to an electroosmotic fluid flow, which contributes the convective component to solute flux as described by the Nernst-Planck equation. Figure 3-1 provides a schematic of this phenomenon. The fixed negative charge density of the cartilage tissue attracts an excess of positive counterions into the interstitial tissue space to satisfy electroneutrality requirements. An applied electric field across the

¹This does not account for the porosity of the cartilage tissue.

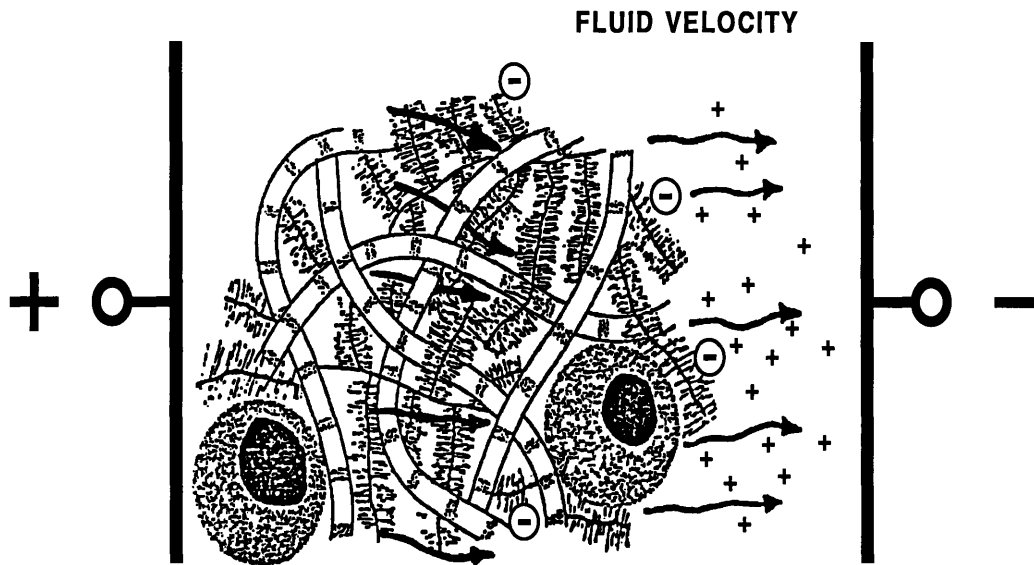


Figure 3-1: Electroosmotic Convection in Cartilage

cartilage causes migration of the mobile ions, which transfer their momentum to the surrounding fluid. Because of the excess of positive counterions, there is an induced net fluid flow in the direction of positive ion migration. The velocity of induced flow depends on the magnitude of the applied field and the fixed negative charge density (GAG content) of the tissue. With aggrecan monomers or GAG chains as the solute of interest, the electroosmotic convective force is always in opposition to the migration force, thus making the net effect of the applied field on solute flux difficult to predict.

Chapter 4

Results

4.1 Partition Coefficients

4.1.1 GAG Chains

Experiment #1

In this experiment, combinations of three different “cold” CS concentrations (20 mg/ml, 30 mg/ml, and 40 mg/ml) and three different time points (44 hours, 99 hours, and 164 hours) gave a total of 9 different conditions. Four 3 mm, 200 μ m thick cartilage plugs were equilibrated in each condition. The partition coefficient K was calculated as

$$K = \frac{CPM_t}{CPM_m},$$

where CPM_t =total CPM in the tissue normalized by (wet weight - dry weight) and CPM_m =total CPM in media normalized by media volume. Table 4.1 contains the calculated partition coefficients. Figure 4-1 shows a plot of the values. The dots, stars, and triangles represent a ‘cold” CS concentration of 20 mg/ml, 30 mg/ml, and 40 mg/ml, respectively. Notice that the partition coefficients for 30 mg/ml CS concentrations were consistently higher than those for 20 mg/ml concentrations at all time points. The same is true for the partition coefficients for 40 mg/ml concentrations relative to those for 30 mg/ml concentrations.

Concentration [$\frac{mg}{ml}$]	Time [hours]	K
20	44	0.07
	99	0.10
	164	0.07
30	44	0.10
	99	0.13
	164	0.09
40	44	0.32
	99	0.17
	164	0.12

Table 4.1: GAG Partition Measurements: Experiment #1

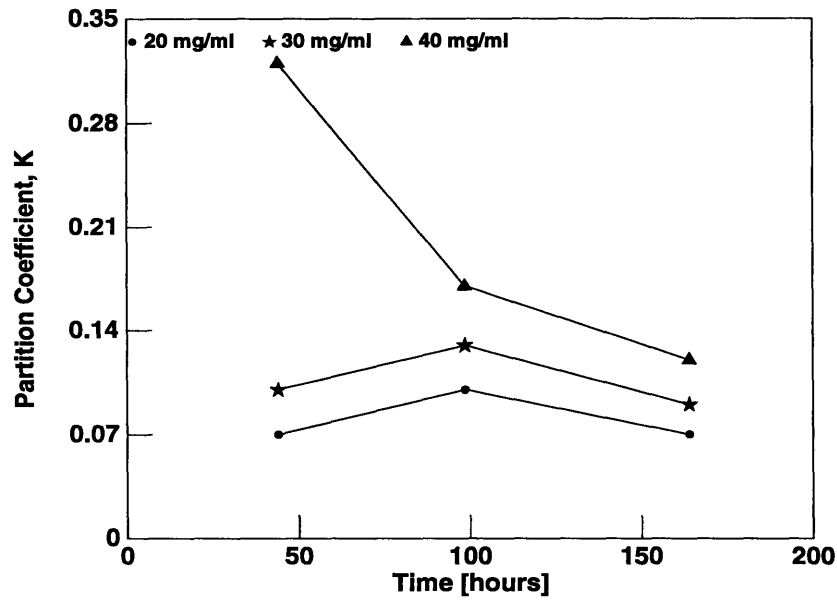


Figure 4-1: GAG Partition Measurements: Experiment #1

Experiment #2

In this experiment, combinations of two different “cold” CS concentrations (20 mg/ml, 30 mg/ml) and three different time points (64 hours, 120 hours, and 205 hours) gave a total of six different conditions. Two 9 mm, 200 μ m thick cartilage plugs were equilibrated in each condition. Table 4.2 contains the calculated partition coefficients. Figure 4-2 is a plot of the values. The values obtained in this experiment were higher

Concentration [$\frac{mg}{ml}$]	Time [hours]	$K \pm \sigma$ (n=2)
20	65	0.44 ± 0.071
	120	0.64 ± 0.636
	216	0.38 ± 0.403
30	65	0.38 ± 0.233
	120	0.24 ± 0.021
	216	0.39 ± 0.410

Table 4.2: GAG Partition Measurements: Experiment #2

than those obtained in the previous experiment; one explanation for this may be that the GAG content of the cartilage used in this experiment was lower than the cartilage GAG content in the previous experiment.¹

4.1.2 Aggrecan Monomers

Three different “cold” CS concentrations (20 mg/ml, 30 mg/ml, 40 mg/ml) and three different time points (48 hours, 96 hours, and 192 hours) gave a total of nine different conditions. Four 3 mm, 200 μ m thick cartilage plugs were equilibrated in each condition. Table 4.3 contains the calculated partition coefficients; the values range from 0.05 to 0.15. Figure 4-3 provides a plot of the partition coefficients. There were no clear trends in the data; partition coefficients did not necessarily increase for higher CS concentrations. One caveat to consider when interpreting the data from this experiment is the stickiness of the monomers; i.e. the monomers may have adhered to

¹GAG content values are provided in Appendix A. Water content values are also provided in Appendix A.

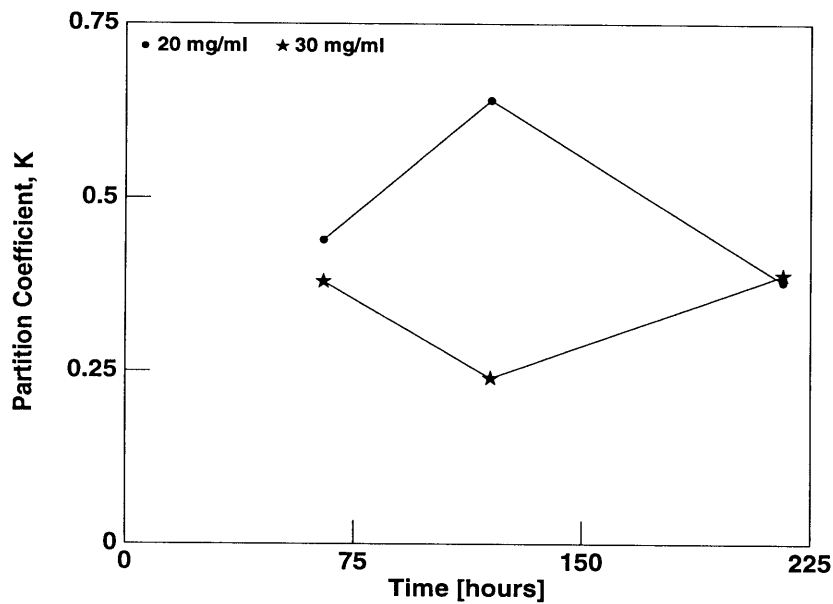


Figure 4-2: GAG Partition Measurements: Experiment #2

Concentration $\left[\frac{mg}{ml}\right]$	Time [hours]	$K \pm \sigma$ (n=2)
20	48	0.15 ± 0.119
	96	0.09 ± 0.056
	192	0.09 ± 0.012
30	48	0.11 ± 0.015
	96	0.08 ± 0.040
	192	0.08 ± 0.039
40	48	0.06 ± 0.009
	96	0.10 ± 0.095
	192	0.05 ± 0.005

Table 4.3: PG Partition Measurements

the walls of the vials, or some of the radioactivity assumed to have penetrated the cartilage tissue plugs may actually just have adhered to the tissue surface.

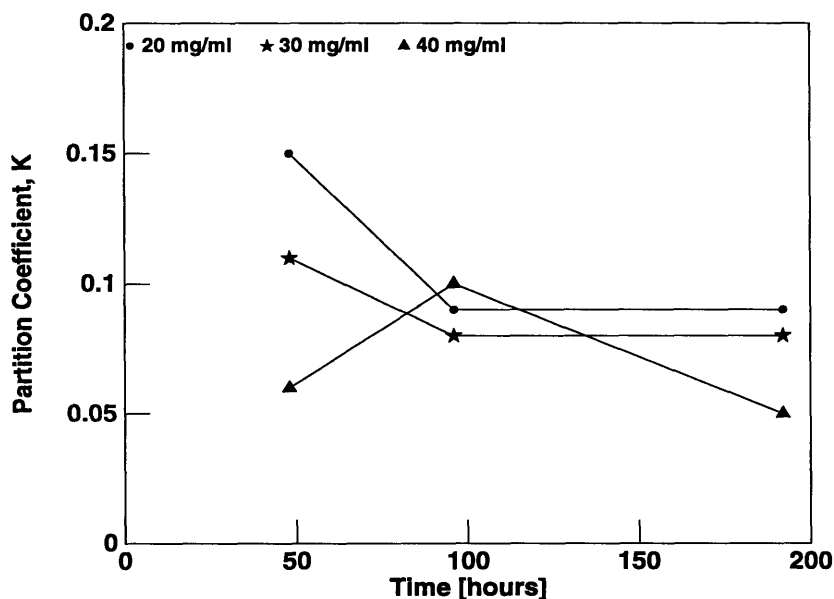


Figure 4-3: Aggrecan Partitioning

4.2 Transport

4.2.1 GAG Chains

Experiment #1

Five 9 mm, 200 μm^2 were used in this experiment. A 20 mg/ml “cold” CS concentration was used in the upstream and downstream baths. Figure 4-4 is a graph of the radioactive counts recorded by the detector system over the entire course of the transport experiment. The ^{35}S -labelled GAG chains were added to the upstream compartment at time $t=91$. The solute was allowed to passively diffuse for 23 hours until time $t=1473$ when a calibration was performed. Subsequent to the calibration, the solute was allowed to passively diffuse for another 2 hours. At time $t=1700$, a -26 mA/cm^2 current was applied across the tissue for approximately 50 minutes. Notice

²This is an estimated thickness. The actual thickness value used for calculating diffusion coefficients was the average of the measured cartilage thickness (provided in Appendix A).

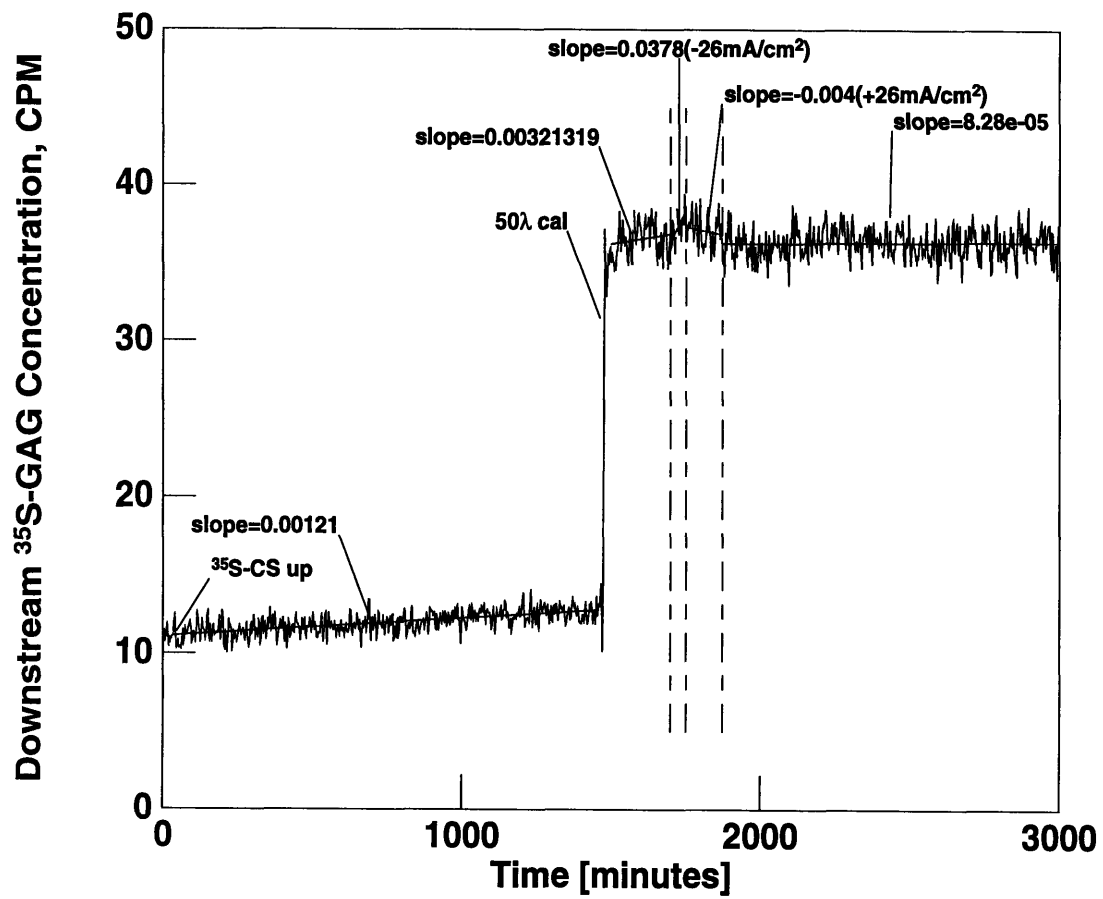


Figure 4-4: CS Transport Experiment #1

that the effect of this negative field was to enhance the flux of the GAG chains. At time $t=1755$, a $+26 \text{ mA/cm}^2$ current was applied across the tissue for approximately 2 hours. The effect of the positive field was to decrease the flux of the GAG chains. In fact, the apparent negative flux recorded during positive field application suggests that electrophoresis dominates the combination of diffusion and electroosmosis. After field application, the solute was again allowed to passively diffuse for another 19 hours before terminating the experiment.

Based upon the slope for the first interval that the solute was allowed to passively diffuse and using a partition coefficient of 0.1, the calculated diffusion coefficient is $\bar{D} = 4.96 \times 10^{-9} \text{ cm}^2/\text{s}$. The negative field *enhanced* the flux over diffusion alone by a factor of ~ 31 . The positive field *reversed* the direction of the flux and increased its magnitude by more than threefold.

Experiment #2

Five 9 mm, 200 μm thick cartilage slices were used in this experiment. The “cold” chondroitin sulfate concentration used in the bath was increased from the 20 mg/ml used in experiment #1 to 30 mg/ml.³ Figure 4-5 shows the radioactive readings recorded during the experiment. At time $t=101$, the ^{35}S -labelled GAG chains were added to the upstream compartment. For the next 24 hours, the solute was allowed to passively diffuse. A 40 μl calibration was performed at time $t=1556$. An 80 μl calibration was performed at time $t=1588$. Subsequently, the solute was allowed to passively diffuse for approximately 11 hours. At $t=2241$, a -26 mA/cm^2 current was applied across the tissue for 70 minutes. At time $t=2425$, a $+26 \text{ mA/cm}^2$ current was applied for 70 minutes. The field applications were repeated to test for reproducibility. At time $t=2559$, a -26 mA/cm^2 current was applied for almost 100 minutes. At time $t=2670$, a $+26 \text{ mA/cm}^2$ current was applied for approximately 50 minutes. The experiment was ended 80 minutes after the last field application.

³Higher concentrations resulted in such a high fluid viscosity that excessive bubbling began to occur and flow was almost nonexistent.

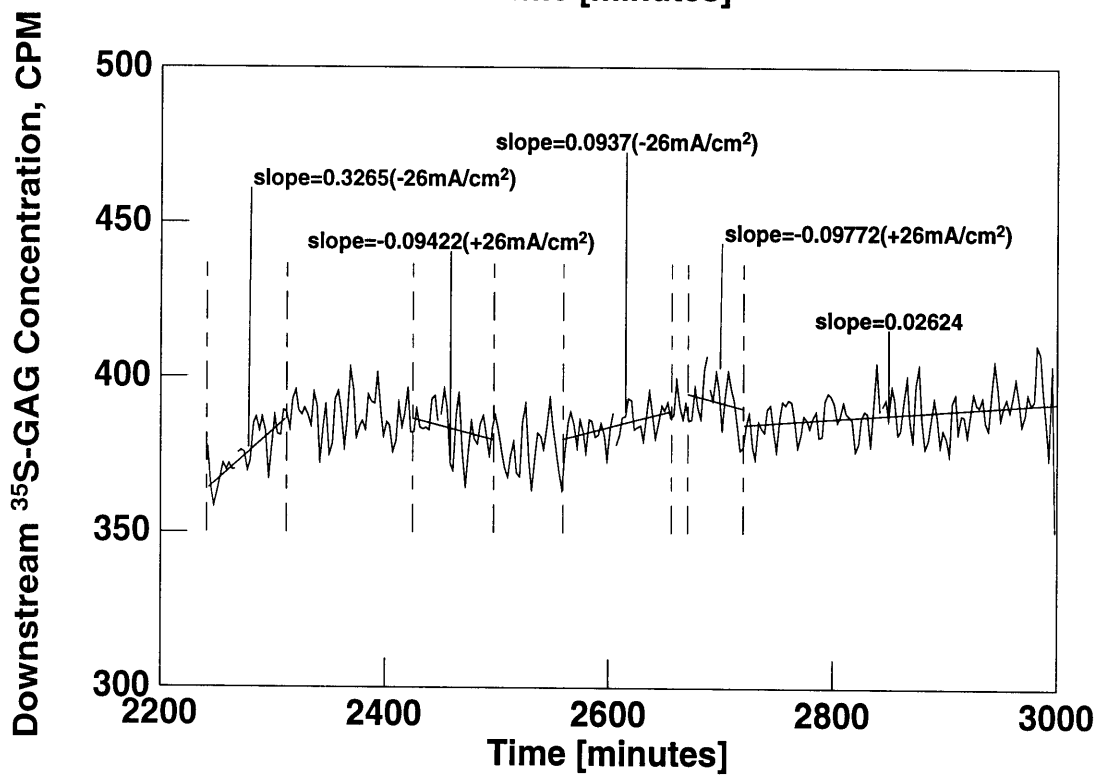
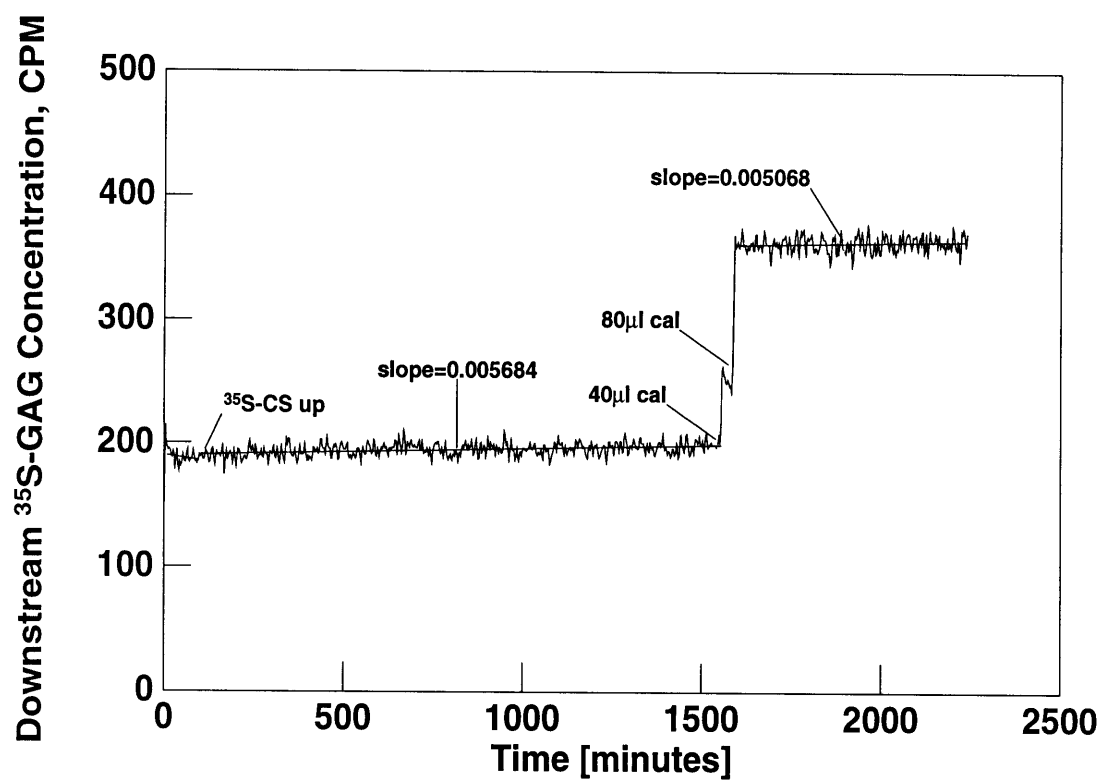


Figure 4-5: CS Transport Experiment #2

The results of this transport experiment were similar to those obtained in the first experiment. Taking into account a partition coefficient of 0.1, the diffusion coefficient calculated from the first diffusive slope was $\bar{D} = 7.79 \times 10^{-9} \text{ cm}^2/\text{s}$. Consistent with what was observed in the first experiment, both negative field applications enhanced the solute flux. The first negative field application increased the flux by a factor of ~ 57 . The second negative field application increased the flux by a factor of ~ 16 . Another result consistent with what was observed in the first experiment is that both positive field applications reversed the direction of flux, in both cases with a 16 to 17-fold increase in magnitude.

Experiment #3

The third transport experiment was basically a repeat of the second transport experiment. Figure 4-6 shows the time course of increase in downstream radioactivity during the experiment. The radiolabelled GAG chains were added upstream at $t=52$. A $-26 \text{ mA}/\text{cm}^2$ current was applied at $t=2462$ for about 70 minutes and then again at $t=2768$ for about 70 minutes. A $+26 \text{ mA}/\text{cm}^2$ current was applied at $t=2633$ for about 70 minutes and then again at $t=2890$ for about 60 minutes. Finally, a $200 \mu\text{l}$ calibration was performed near the end of the experiment at $t=2970$ (not shown on the graph).

The diffusion coefficient calculated based upon the best fit slope spanning the time from $t=400$ to $t=2456$ is $\bar{D} = 1.23 \times 10^{-8} \text{ cm}^2/\text{s}$ (using a 0.1 partition coefficient). Consistent with what was observed in the previous experiments, both negative field applications enhanced the solute flux, the first one by a factor of 20 and the second one by a factor of 25. The effect of the first positive field was consistent with the first two experiments; it reversed the direction of solute flux, increasing the magnitude of flux by 25-fold. The second positive field failed to conform with what had been previously observed in that it did not reverse the direction of solute flux. A closer inspection of the data recorded during the second positive field application, however, reveals that the radioactivity seems to increase initially during the first 1/3 of the

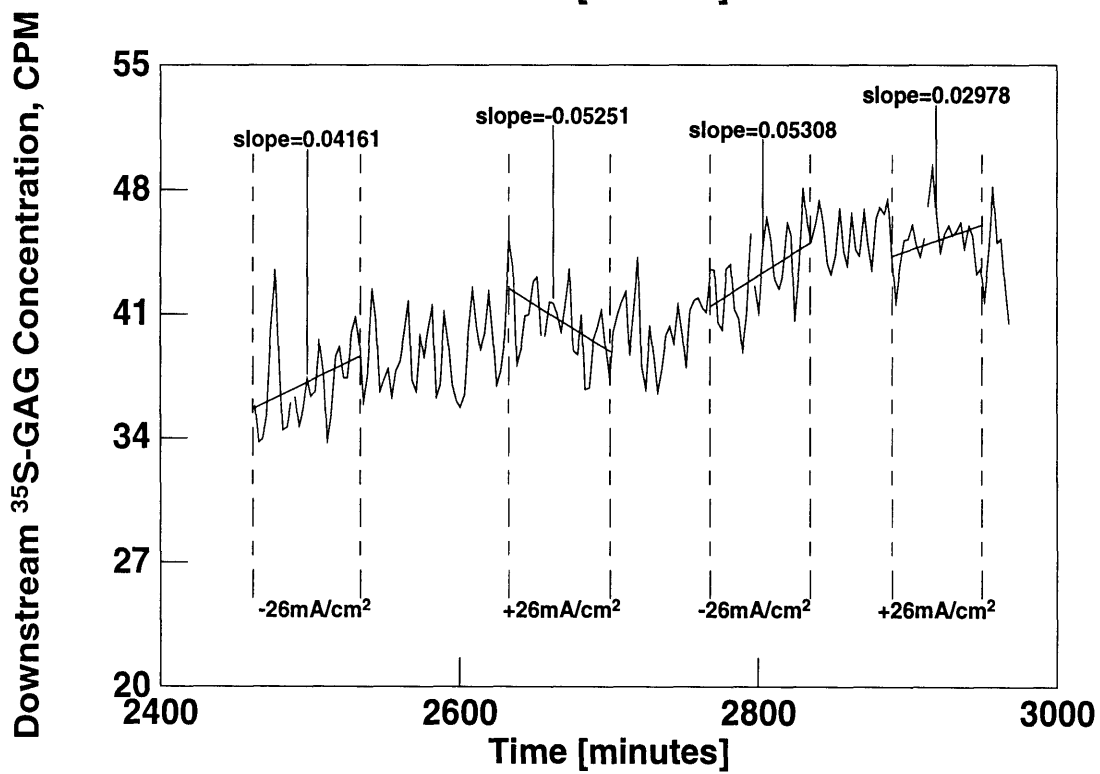
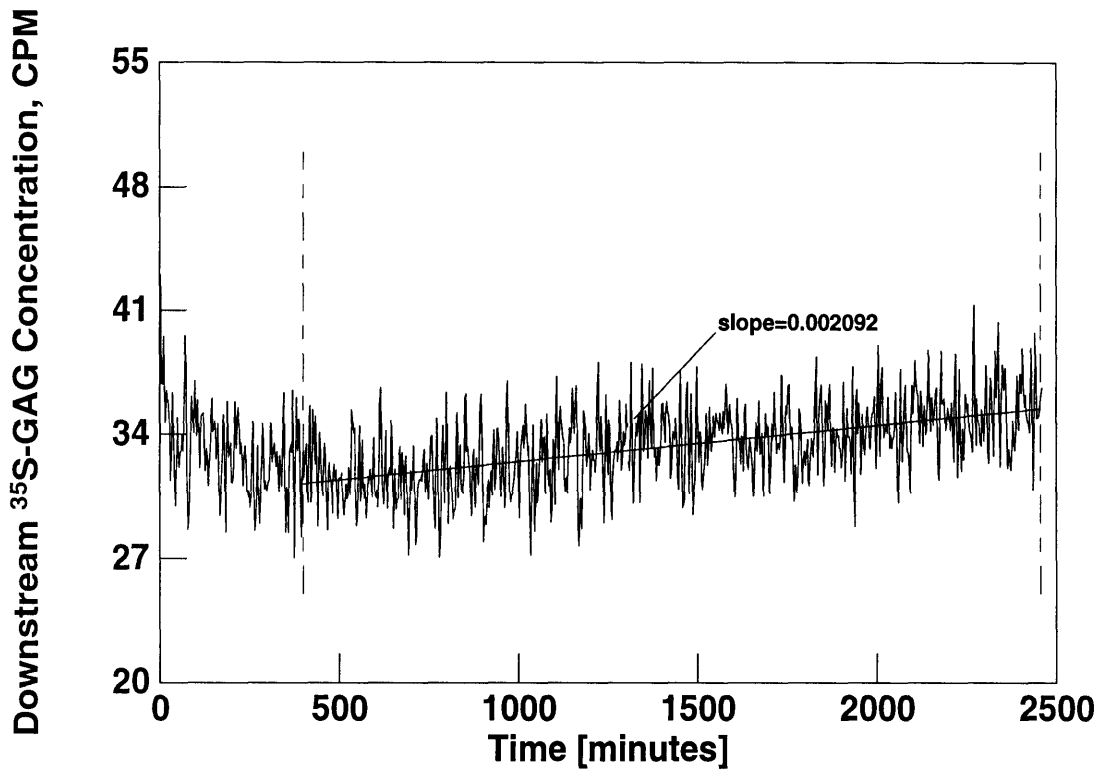


Figure 4-6: CS Transport Experiment #3

interval but that a negatively sloped line could be fit to the data recorded in the subsequent 2/3 of the interval.

4.2.2 Aggrecan Monomers

Experiment #1

Four 9 mm, 200 μm thick cartilage slices were used in this experiment. A 25 mg/ml “cold” CS concentration was used in the upstream and downstream baths. Figure 4-7 shows the increase in downstream radioactivity measured by the detector over the course of the experiment. The radiolabelled PG monomers were added at $t=122$ and allowed to diffuse through the cartilage for 32 hours. Subsequently, a 200 μl calibration was performed at $t=2060$. Two field applications of each polarity were applied during the experiment. A -26 mA/cm^2 current was applied at $t=2600$ for 60 minutes and then again at $t=2830$ for another 60 minutes. A $+26 \text{ mA/cm}^2$ current was applied at $t=2720$ for 60 minutes and at $t=2920$ for 45 minutes.

Using a partition coefficient of 0.1, the diffusion coefficient calculated based upon the best fit slope for the first interval of diffusion is $\bar{D} = 1.06 \times 10^{-7} \text{ cm}^2/\text{s}$. Notice that the slope for the second interval of diffusion (after the calibration) is much larger than the slope during the first interval (before the calibration). Part of this may be attributed to monomers sticking to the scintillation cell; this explanation is supported by the fact that the slope of the trace immediately after the calibration is initially pretty large but clearly decreases over time. Both negative field applications increase the flux of the monomer, the first time by a factor of 20 and the second time by a factor of 10 relative to the observed pre-calibration diffusive flux. The positive field applications reverse the direction of the flux, increasing the magnitude by 8-fold once and 18-fold the second time.

50 μl aliquots were removed from the downstream bath over the course of the experiment and subsequently counted in the scintillation counter. The data collected from

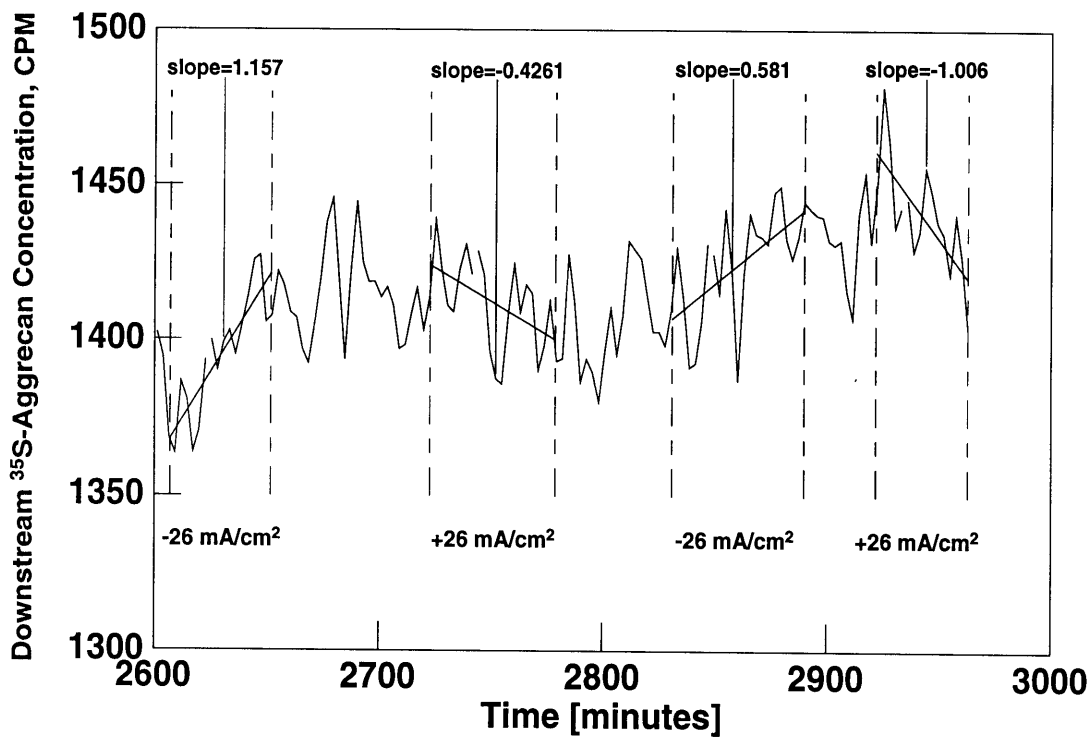
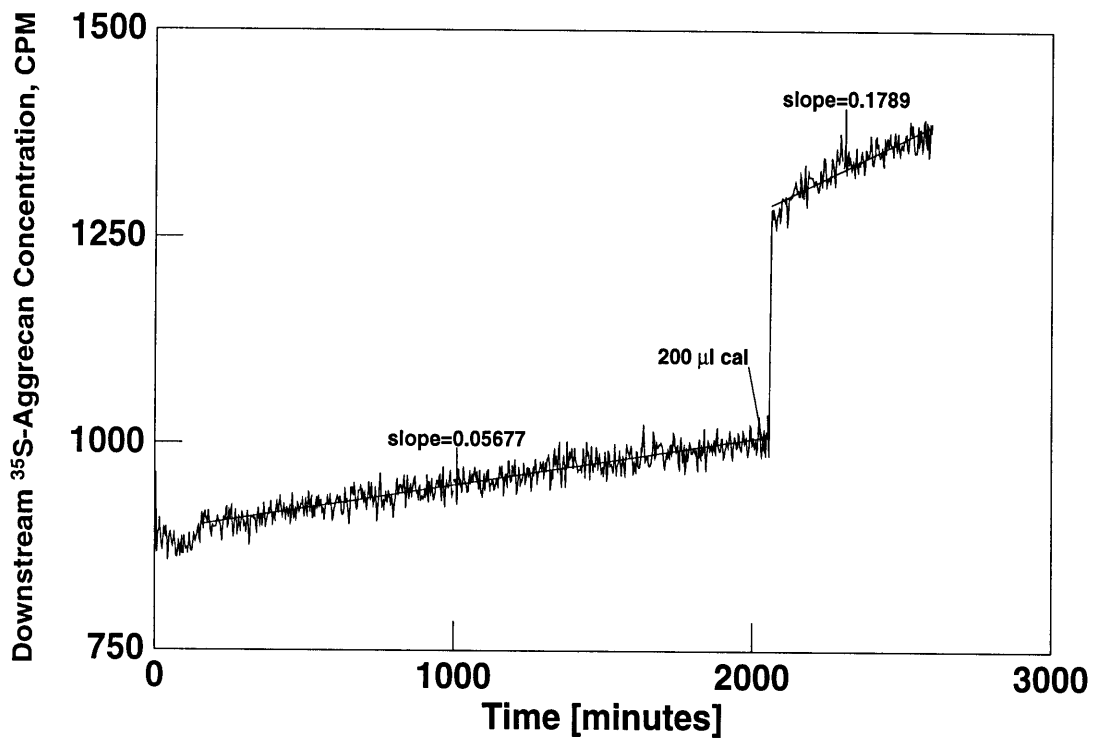


Figure 4-7: PG Transport Experiment #1

these aliquots are plotted in figure 4-8. The dots represent the actual data points; the lines are the best fit curves to the data points. Using a partition coefficient of 0.1, the

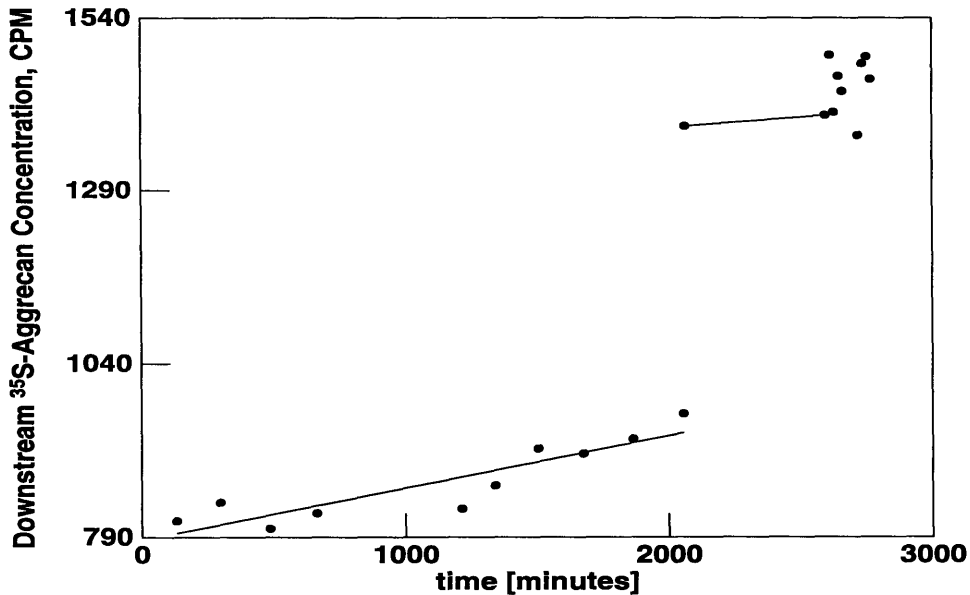


Figure 4-8: PG Transport Experiment #1: Aliquots

diffusion coefficient calculated based on data collected from the aliquots for the first interval of diffusion is $\bar{D} = 8.95 \times 10^{-8} \text{ cm}^2/\text{s}$. This compares well with the diffusion coefficient calculated based on data recorded by the detector system. Unfortunately, the data from the aliquots removed during the field applications were too scattered to afford any meaningful interpretation.

One disturbing observation is that the diffusion coefficient derived based upon the PG monomer transport data is larger than the diffusion coefficients calculated based on data from all three of the GAG chain transport experiments. A possible explanation is that the PG monomer preparation contained a fraction of free ³⁵S. This possibility is discussed later in this chapter in section 4.3.3.

Experiment #2

Five 9 mm cartilage slices of average thickness $240 \pm 12 \mu\text{m}$ were used in this experiment. As with the first experiment, a 25 mg/ml “cold” CS concentration was used in the upstream and downstream baths. A plot of the downstream CPM measured over

time by the detector is shown in figure 4-9. The radiolabelled PG monomers were added to the upstream bath at $t=125$ and allowed to diffuse through the cartilage for almost 34 hours until $t=2155$ at which time a $200 \mu\text{l}$ calibration was performed. Unfortunately, despite the presence of BSA in the bath, there was a lot of sticking of the PG monomers to the scintillation cell as evidenced by the large post-calibration slope that tapers off over time. Over the next 29 hours after the calibration, the system began to saturate in terms of the amount of sticking; the best fit slope for the last four hours of this interval was 0.09 (compared to the pre-calibration slope of 0.023). A -26 mA/cm^2 current was applied across the cartilage at $t=3899$ for 80 minutes and at $t=4246$ for 70 minutes. A $+26 \text{ mA/cm}^2$ current was applied at $t=4093$ for 70 minutes and at $t=4384$ for 50 minutes.

The diffusion coefficient calculated based upon the best fit slope during the pre-calibration diffusion interval is $\bar{D} = 8.58 \times 10^{-8} \text{ cm}^2/\text{s}$ (using a partition coefficient of 0.1). The best fit slopes for the data collected during the field applications are shown in figure 4-9; however, they seem inconsistent with what was observed in the first experiment. The data collected in this experiment seemed particularly noisy after the first field application; notice that CPM recorded changed drastically even during intervals in which no fields were applied. As a side note, unexpected changes in back pressure level occurred during the time after the first field application suggesting that some unidentified phenomena may have contaminated the data collected during the field applications.

As with the previous experiment, $50 \mu\text{l}$ aliquots were collected from the downstream bath periodically over the course of the run. The data collected from these aliquots are plotted in figure 4-10. The diffusion coefficient calculated based upon the slope fit to the data collected during the first diffusive interval is $\bar{D} = 5.06 \times 10^{-8} \text{ cm}^2/\text{s}$ (using a partition coefficient of 0.1), a value consistent with that calculated based on data collected using the detector system. This diffusion coefficient is also consistent with the diffusion coefficients calculated based on the previous experiment.

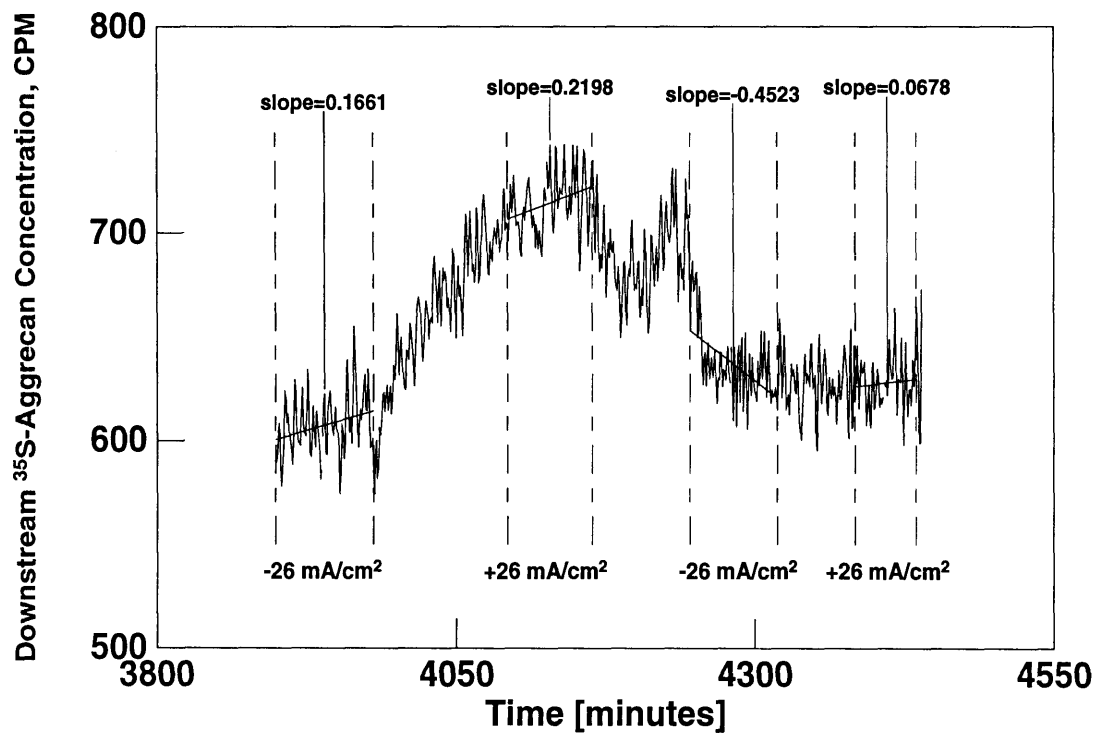
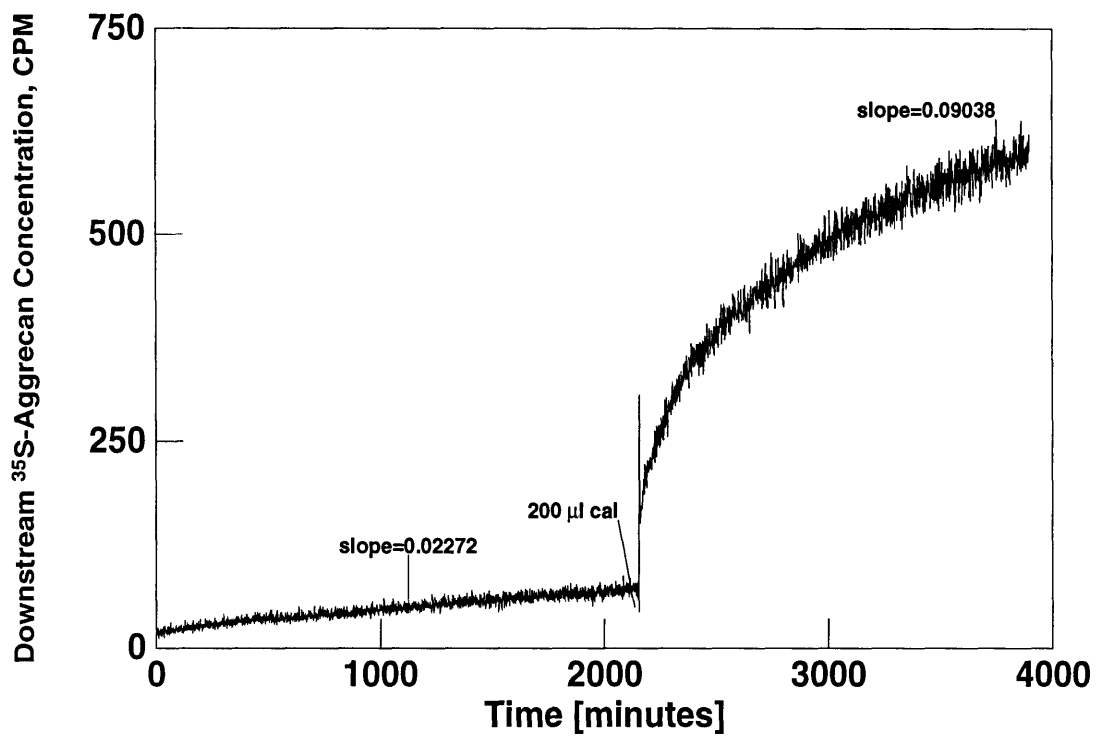


Figure 4-9: PG Transport Experiment #2

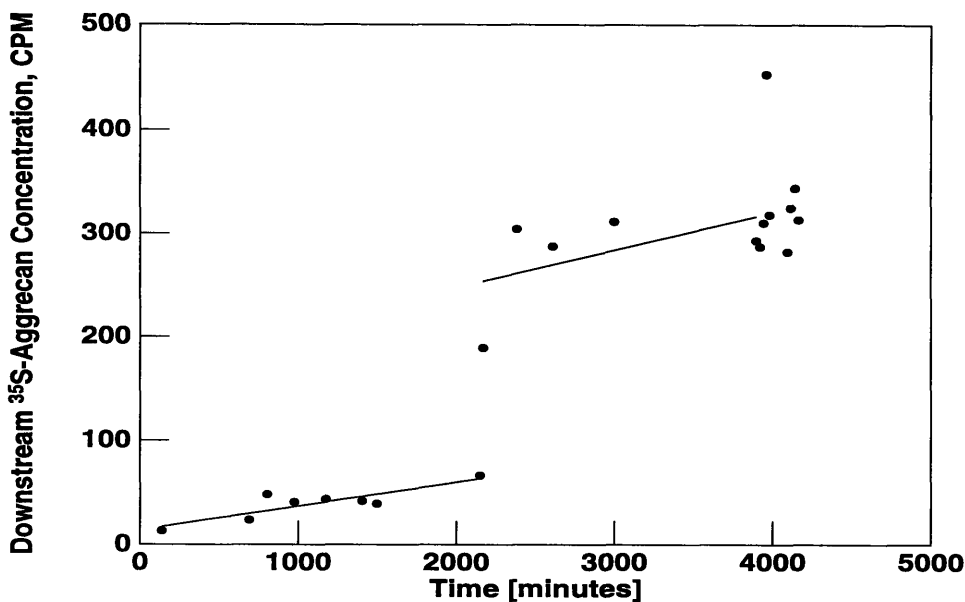


Figure 4-10: PG Transport Experiment #2: Aliquots

4.3 Chromatography

CL-6B, CL-2B, and PD-10 column chromatography were all used to analyze the solute preparations. For the CL-2B and CL-6B columns, DNA and tritium were used as markers for the void volume, V_o , and total volume, V_t , of the column, respectively. In particular the *peak* of the DNA curve marked the void volume and the *peak* of the tritium curve marked the total volume. The elution profiles shown in the next couple of sections are plotted as a function of a normalized variable K_{av} , which is defined as

$$K_{av} = \frac{V - V_o}{V_t - V_o},$$

where all the V 's have units of fraction number.

4.3.1 Free ³⁵S in GAG Chain Preparation

An aliquot of the upstream bath containing the rat chondrosarcoma GAG chains was run on a Sepharose CL-6B column according to the method described in section 2.5.5. Fifty seven fraction were collected. The peak of the DNA marker occurred at fraction 18, and the peak of the tritium marker occurred at fraction 46. Figure 4-11 shows

the CL-6B elution profile of the ^{35}S radiolabelled GAG chain preparation. The x-axis is normalized to be K_{av} and the y-axis is CPM displayed on a logarithmic scale. The amount of radiolabelled molecules eluted after the tritium peak seems insignificant. In fact, assuming that everything coming out of the column after the tritium peak is free label, the free label accounts for only 0.81% of the total distribution.

The absence of free label in the GAG chain preparation is not surprising since it was pooled from the peak fractions of a Superose-6 run as mentioned in section 2.1.1. A possible concern is that the ^{35}S label could be lost over time; however, the results shown in figure 4-11 dismiss any suspicions about the stability of the label.

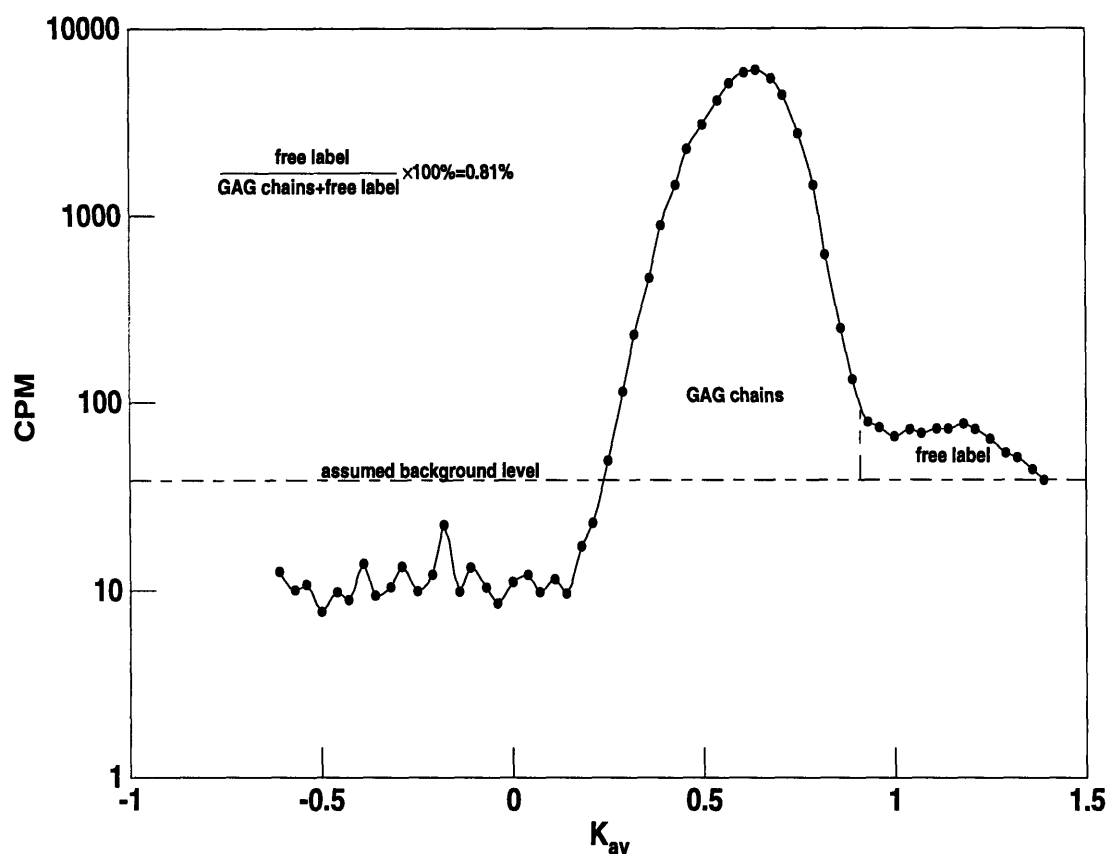


Figure 4-11: CL6B Chromatography of Rat Chondrosarcoma GAG chains

4.3.2 Characterization of “Cold” Chondroitin Sulfate

The shark fin chondroitin sulfate from Sigma that was prepared as described in section 2.1.2 was run on a Sepharose CL-6B column to determine its molecular weight distribution. The purpose of this step was to check for any obvious anomalies in the distribution that might skew the results of the transport experiments. Figure 4-12 shows the elution profile of the shark fin chondroitin sulfate as denoted by the triangles. Superimposed on the graph is the elution profile for the radiolabelled rat chondrosarcoma GAG chains (denoted by the dots) run simultaneously on the same column. Again, the x-axis is normalized to be K_{av} . The two curves look very similar, although the “cold” chondroitin sulfate preparation appears to be a bit smaller and more polydisperse.

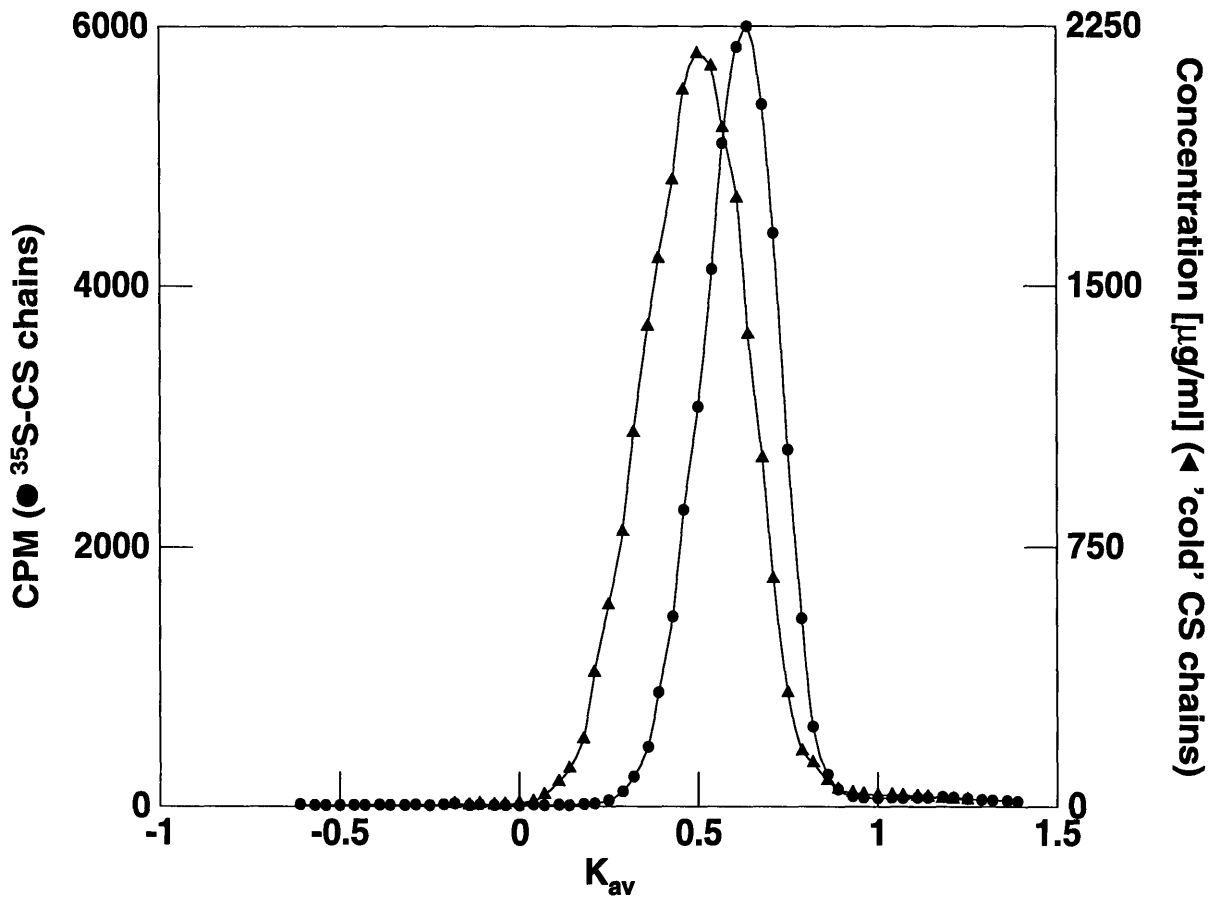


Figure 4-12: CL6B Chromatography: “Cold” CS vs. Rat Chondrosarcoma CS

4.3.3 Free ^{35}S in Aggrecan Preparation

In an attempt to determine the presence of any free label (or degradation fragments) in the rat chondrosarcoma aggrecan preparation, an aliquot of the upstream bath was run on a Sepharose CL-2B column according to the method described in section 2.5.5. Fifty nine fractions were collected. The peak of the DNA marker occurred at fraction 19, and the peak of the tritium marker occurred at fraction 49. Figure 4-13 shows the CL-2B elution profile of the aggrecan bath with the x-axis normalized to be K_{av} . The absence of a second peak after the tritium marker ($K_{av}=1$) suggests that there was no significant amount of free label; however, because the ^{35}S counts were still above background by the time the tritium had eluted, more resolution was desired.

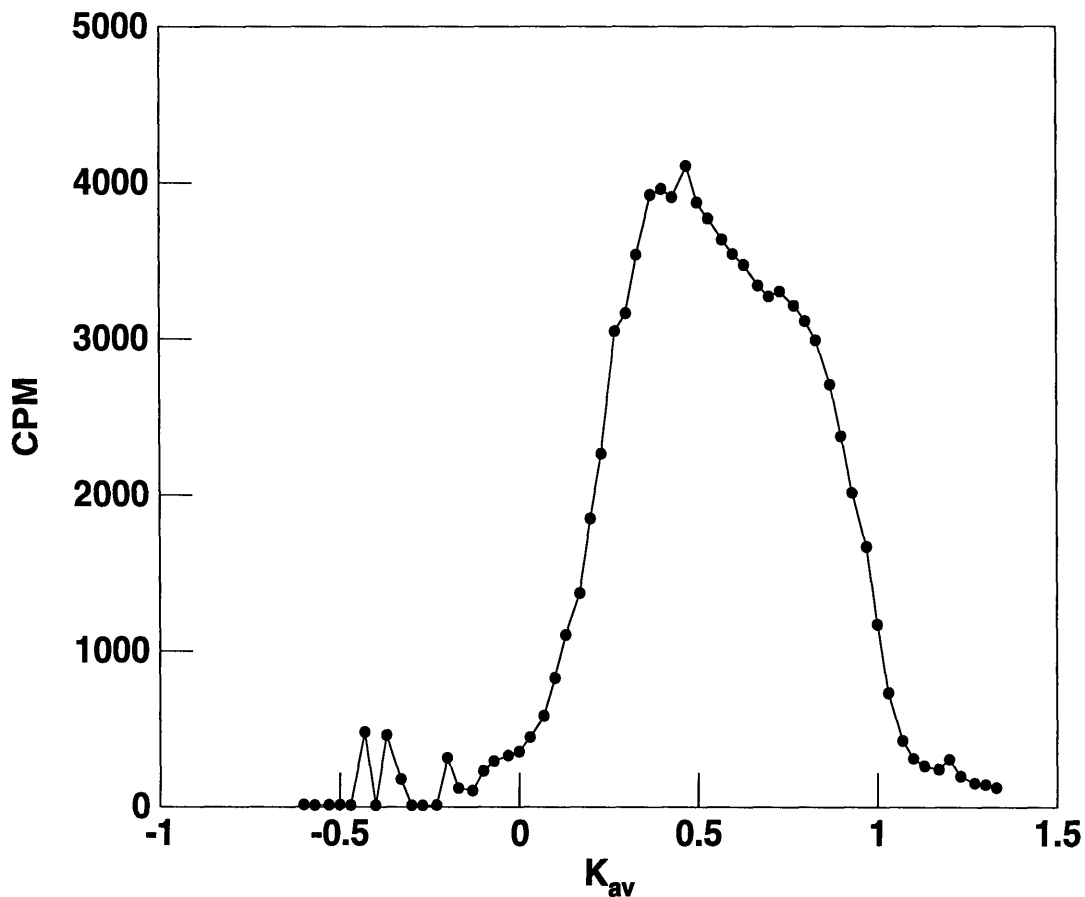


Figure 4-13: CL2B Chromatography of Rat Chondrosarcoma aggrecan

In an attempt to look more closely at the fractions coming out of the CL-2B column after the tritium, fractions 49 through 59 were pooled together and run on a PD-10 column according to the method described in section 2.5.5. The elution profile for the run is shown figure 4-14. The ^{35}S curve is denoted by the triangles; notice that there is only one peak in the curve. For reference, the figure also shows the tritium elution profile (denoted by the dots).

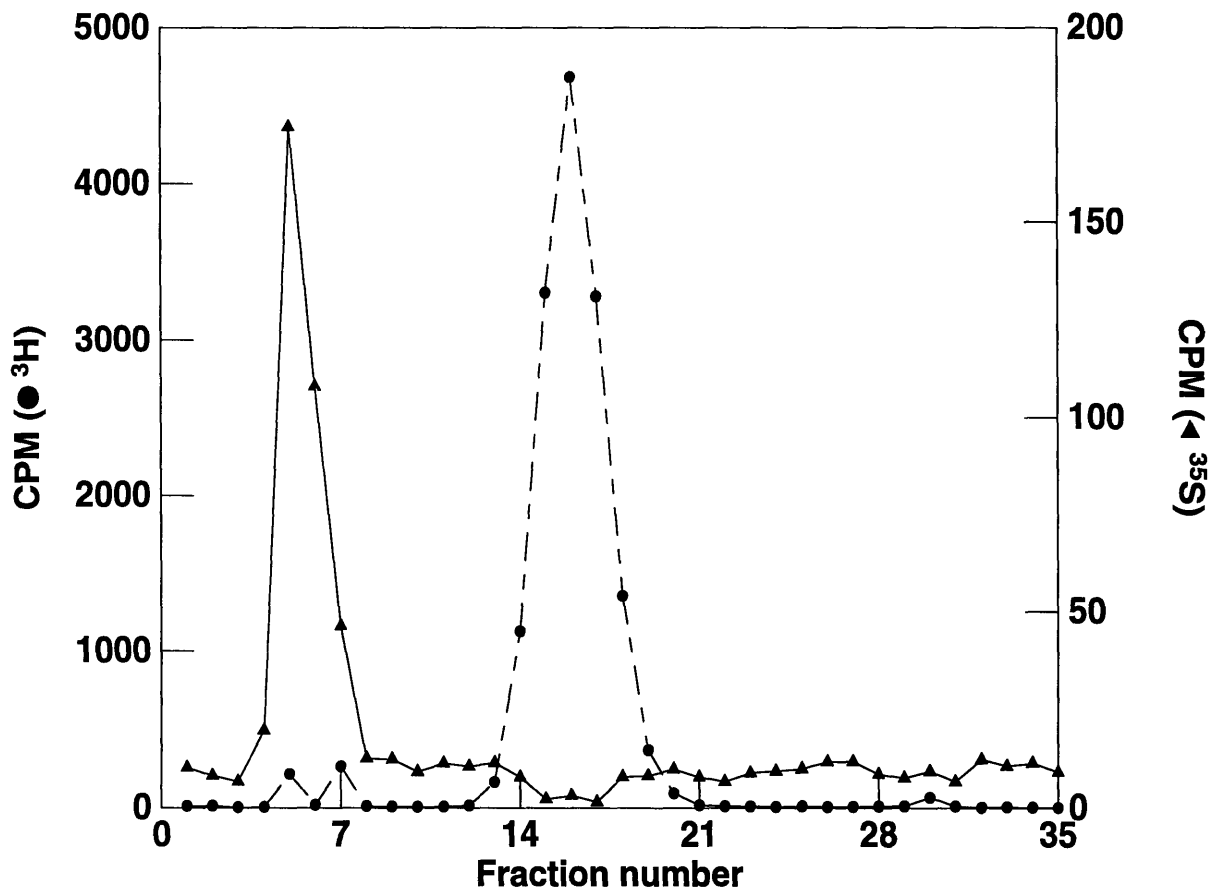


Figure 4-14: PD10 Fractionation of Fractions 49-59 from CL2B

Based upon the data from Figures 4-13 and 4-14, there appears to be no free label in the preparation used for the transport experiments. On the other hand, the elution profile obtained from the CL-2B clearly indicates the polydispersity of the aggrecan preparation and suggests the presence of small aggrecan fragments.

Chapter 5

Discussion

5.1 Experimental Difficulties

Some of the difficulties that arose in carrying out the experiments in this study include issues related to evaporation of bath fluid, changes in bath pH, presence of free ^{35}S in the solute preparations, and sticking of the aggrecan monomer to the scintillation cell.

Because of the prolonged nature of the experiments that were carried out, evaporation of the bath fluid was a serious concern, as excessive evaporation could skew the measurements. To minimize evaporation, the transport chamber was sealed as much as possible with plexiglass lids covering the upstream and downstream compartments, rubber stoppers inserted in the openings for the salt bridges when fields weren't being applied, and parafilm essentially wrapped around the entire assembly. In addition, the baths were kept below room temperature. Despite the precautions taken to keep the chamber sealed, evaporation still occurred, typically on a magnitude of a few milliliters but, in some cases, up to 20% of the total bath volume.

Changes in bath pH during field applications were closely monitored. The need to maintain the bath pH around physiologic levels limited the amount of time during which a field could be applied. While the imposed time limitation is not a concern

for smaller ionic solutes, it introduces some uncertainty about the reliability of the slopes obtained during field applications for the large aggrecan monomers and GAG chains because of the naturally small flux rate of these molecules.

As mentioned earlier, the presence of free ^{35}S in the solute preparations was a key concern. Based upon the chromatography results for the GAG chains and the protocol used for obtaining the GAG chains, there is no reason to believe that any significant amount of free-label was present in the GAG chain preparation. On the other hand, the chromatography results for the aggrecan monomers were ambiguous. The fact that the calculated diffusivity for the aggrecan monomer was larger than the calculated diffusivity for the GAG chains further suggests that there may have been some small degradation fragments present in the aggrecan monomer preparation.

A serious problem with no clear remedy was the sticking of the aggrecan monomers to the solid scintillant cell. The use of BSA in the baths improved the situation; however, considerable sticking still occurred, especially in the second aggrecan transport experiment.

Despite the difficulties described above, the experiments yielded valuable results. This is evidenced by the consistency in measurements obtained from repeat experiments. In cases where the precise magnitude of measurements obtained may not be completely accurate, there were at least definite trends in the data.

5.2 Interpretation Within Context of Current Literature

The diffusion coefficients calculated for the rat chondrosarcoma GAG chains based upon the three transport experiments performed are $4.96 \times 10^{-9} \text{ cm}^2/\text{s}$, $7.79 \times 10^{-9} \text{ cm}^2/\text{s}$, and $1.23 \times 10^{-8} \text{ cm}^2/\text{s}$. Maroudas reported diffusion coefficients for Dextran40

(MW 40K) ranging from $1.03 \times 10^{-8} \text{ cm}^2/\text{s}$ to $2.8 \times 10^{-8} \text{ cm}^2/\text{s}$ [10]. The fact that GAG chains are slightly smaller in molecular weight than Dextran40 would lead one to predict a larger diffusion coefficient for the GAG chains compared to Dextran40; on the other hand, the negative charges of the GAG chains might be expected to slow their movement through the cartilage matrix. Consequently, the calculated diffusion coefficients for the GAG chains seem reasonable.

The diffusion coefficients calculated based on the PG monomer transport experiments range from $5.06 \times 10^{-8} \text{ cm}^2/\text{s}$ to $1.06 \times 10^{-7} \text{ cm}^2/\text{s}$. This value is slightly larger than the diffusion coefficients found for the GAG chains. As mentioned in Chapter 1, Comper and coworkers found the diffusion coefficients of rat chondrosarcoma proteoglycan monomers and CS chains in solution to be very similar for concentrations above 10 mg/ml[2]. Depending upon how much, if any, free-label was present in the monomer preparation used, Comper's theory that the movement of monomers is dominated largely by its CS chains may hold for transport through cartilage as well.

5.3 Summary

In summary, diffusion coefficients were measured for PG monomers and GAG chains that seemed consistent with data available from literature. Furthermore, the data obtained during current applications suggests that the electrophoretic component of flux for PG monomers and GAG chains dominates the combination of diffusion and convection.

5.4 Future Research

An interesting next step will be to perform transport studies using PG disaccharide components, prepared from the ^{35}S -labelled GAG chains. In addition, it would be desirable to perform repeats of the experiments carried out in this study, especially the partition experiments. Finally, separation of the electroosmotic convective and

electrophoretic effects is another challenge still left to tackle.

An interesting topic originally included in the intended scope of this study is the effect of the presence of an intact articular surface (topmost 200 μ m layer) on the transport of PG component molecules. Previous studies by Setton et al[12] and Torzilli[13] have focused on the influence of an intact articular surface on fluid transport and solute diffusion, respectively. A decrease in fluid permeability was observed with the presence of an intact surface[12]. Torzilli reported that removal of the articular surface decreased solute concentration levels inside the cartilage tissue and the diffusion rates decreased for a variety of solutes with the exception of dextran-77[13]. This topic seems particularly relevant to transport of PG components because degraded PG fragments must pass through the articular surface layer during transport out of cartilage. Therefore, it would be important to repeat our experiments using specimens that contain an intact articular surface, and to compare the resulting effects on partition and transport of aggrecan, CS-GAG chains, and disaccharide constituents.

Appendix A

Tables

The following equations define the hydration and GAG content for a slice of cartilage:

$$\text{Water Content} = \frac{\text{Wet Weight} - \text{Dry Weight}}{\text{Wet Weight}} \times 100\%$$

$$\text{GAG Content} = \frac{\text{GAG Weight}}{\text{Dry Weight}} \times 100\%$$

Slice #	Water Content	GAG Content	Thickness [μm]
1	71.2	16.2	
2	81.0	18.0	226
3	73.8	16.0	233
4	78.7	16.9	200
5	75.6	15.0	

Table A.1: Auxiliary Data for GAG Chain Transport: Experiment #1

Slice #	Water Content	GAG Content	Thickness [μm]
1	77.4		
2	80.0		
3	77.0		231
4	78.7		
5	80.0		223

Table A.2: Auxiliary Data for GAG Chain Transport: Experiment #2

Slice #	Water Content	GAG Content	Thickness [μm]
1	74.8	14.5	265
2	78.0	15.1	267
3	75.0	13.8	277
4	74.9	15.7	225
5	77.1	16.2	260

Table A.3: Auxiliary Data for GAG Chain Transport: Experiment #3

Slice #	Water Content	GAG Content	Thickness [μm]
1	76.6	13.0	263
2	72.5	10.3	234
3	73.4	13.7	239
4	77.0	17.6	234

Table A.4: Auxiliary Data for Aggrecan Transport: Experiment #1

Slice #	Water Content	GAG Content	Thickness [μm]
1			237
2			222
3			245
4			245
5			253

Table A.5: Auxiliary Data for Aggrecan Transport: Experiment #2

Concentration [$\frac{mg}{ml}$]	Time [hours]	Water Content	GAG Content	Thickness [μm]
20	44	75.8	17.81	224
	99	76.5	15.89	232
	164	77.6	18.37	215
30	44	77.5	17.57	217
	99	77.4	18.04	219
	164	77.3	17.12	220
40	44	73.1	17.36	228
	99	71.7	13.62	216
	164	77.2	15.88	225

Table A.6: Auxiliary Data for GAG Chain Partition: Experiment #1

Concentration [$\frac{mg}{ml}$]	Time [hours]	Water Content	GAG Content	Thickness [μm]
20	65	77.1	13.4	249
	120	82.6	12.1	255
	216	80.3	8.6	259
30	65	83.1	15.6	251
	120	73.8	10.4	247
	216	75.5	8.1	242

Table A.7: Auxiliary Data for GAG Chain Partition: Experiment #2

Concentration [$\frac{mg}{ml}$]	Time [hours]	Water Content	GAG Content	Thickness [μm]
20	48	79.0		234
	96	81.1		227
	192	81.1		234
30	48	79.1		236
	96	74.5		234
	192	75.0		220
40	48	76.4		222
	96	74.8		232
	192	75.3		214

Table A.8: Auxiliary Data for Aggrecan Partition

Bibliography

- [1] J Buckwalter, E Hunziker, L Rosenberg, R Coutts, M Adams, and D Eyre. Articular cartilage: Composition and structure. In S L-Y Woo and J A Buckwalter, editors, *Injury and Repair of the Musculoskeletal Soft Tissues*, pages 405–425. American Academy of Orthopaedic Surgeons, Park Ridge, IL, 1988.
- [2] W D Comper. Physicochemical aspects of cartilage extracellular matrix. In B Hall and S Newman, editors, *Cartilage: Molecular Aspects*, pages 59–96. CRC, Boca Raton, Fl., 1991.
- [3] R W Farndale, D J Buttle, and A J Barrett. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochimica et Biophysica Acta*, 883:173–177, 1986.
- [4] A J Grodzinsky. *Fields, Forces and Flows in Biological Tissues and Membranes*. MIT, 1992.
- [5] G S Harper and B N Preston. Molecular shrinkage of proteoglycans. *Journal of Biological Chemistry*, 262:8088–8095, 1987.
- [6] D Heinegard and A Oldberg. Structure and biology of cartilage and bone matrix noncollagenous macromolecules. *The FASEB Journal*, 3:2042–2051, 1989. cartilage, collagen fiber, proteoglycan, hyaluronate binding region, matrix protein, bone sialoprotein.
- [7] F Helfferich. *Ion Exchange*, pages 346–349. McGraw-Hill, New York, NY, 1962.

- [8] Y-J Kim, R L Y Sah, J Y H Doong, and A J Grodzinsky. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Analytical Biochemistry*, 174:168–176, 1988.
- [9] A Maroudas. Transport of solutes through cartilage: Permeability to large molecules. *Journal of Anatomy*, 122:335–347, 1976.
- [10] A Maroudas. Physicochemical properties of articular cartilage. In M A R Freeman, editor, *Adult Articular Cartilage, 2nd ed.*, pages 215–290. Pitman, Tunbridge Wells, England, 1979.
- [11] R L Sah. *Biophysical Regulation of Matrix Synthesis, Assembly, and Degradation in Dynamically Compressed Calf Cartilage*. PhD thesis, Massachusetts Institute of Technology, Cambridge, MA, 1990.
- [12] L A Setton, W Zhu, and V C Mow. The biphasic poroviscoelastic behavior of articular cartilage: role of the surface zone in governing the compressive behavior. *Journal of Biomechanics*, 26:581–592, 1993.
- [13] P A Torzilli. Effects of temperature, concentration and articular surface removal on transient solute diffusion in articular cartilage. *Med & Biol. Eng. & Comput.*, 31:s93–s98, 1993.