





This is to certify that the  
 dissertation entitled  
 Turnover of Cell Surface Proteoglycans  
 In Cultured Fibroblasts

presented by

James H. Brauker

has been accepted towards fulfillment  
 of the requirements for

Ph.D. degree in Biochemistry

  
 Major professor

Date Aug. 12, 1986

TURNOVER OF CELL SURFACE PROTEOGLYCAN  
IN CULTURED FIBROBLASTS

By

James H. Brauker

A DISSERTATION

Submitted to  
Michigan State University  
in partial fulfillment of the requirements  
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1986

4276413

ABSTRACT  
TURNOVER OF CELL SURFACE PROTEOGLYCANS  
IN CULTURED FIBROBLASTS

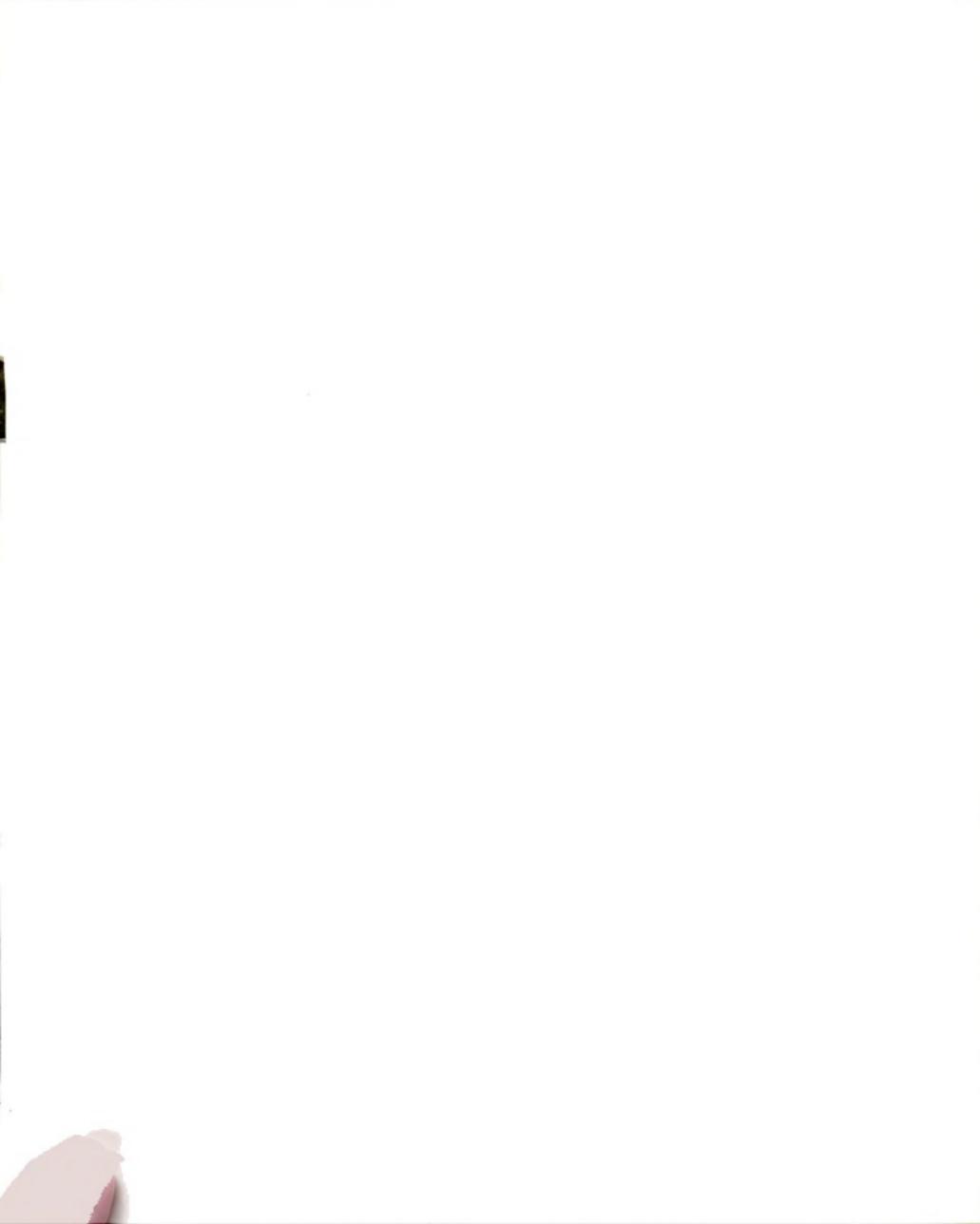
By  
James H. Brauker

Human fibroblasts were cultured in  $^{35}\text{SO}_4^{-2}$  to label the glycosaminoglycans (GAGs); the extracellular matrix was derived from these labeled cells by removal of cells with the chelating agent ethylene glycol-bis-( $\beta$ -amino ethyl ether)N,N'-tetra acetic acid (EGTA). When unlabeled cells were plated onto these radiolabeled extracellular matrices, two distinct events were observed: a) the cells actively released [ $^{35}\text{S}$ ]PG from the matrix and; b) the cells degraded a large fraction (20-40% in 24 hours) of the matrix PG, releasing free sulfate. The latter degradation event could be inhibited in a specific dose-dependent manner by addition of mannose 6-phosphate ( $K_i = 60 \mu\text{M}$ ) to the culture medium. Analyses of this effect in terms of the saccharide specificity,  $\text{NH}_4\text{Cl}$  sensitivity, and the requirement for cells suggest that both an intracellular compartment and the mannose 6-phosphate receptor that binds lysosomal enzymes at the cell surface may play important roles in the turnover of PG of the extracellular matrix.

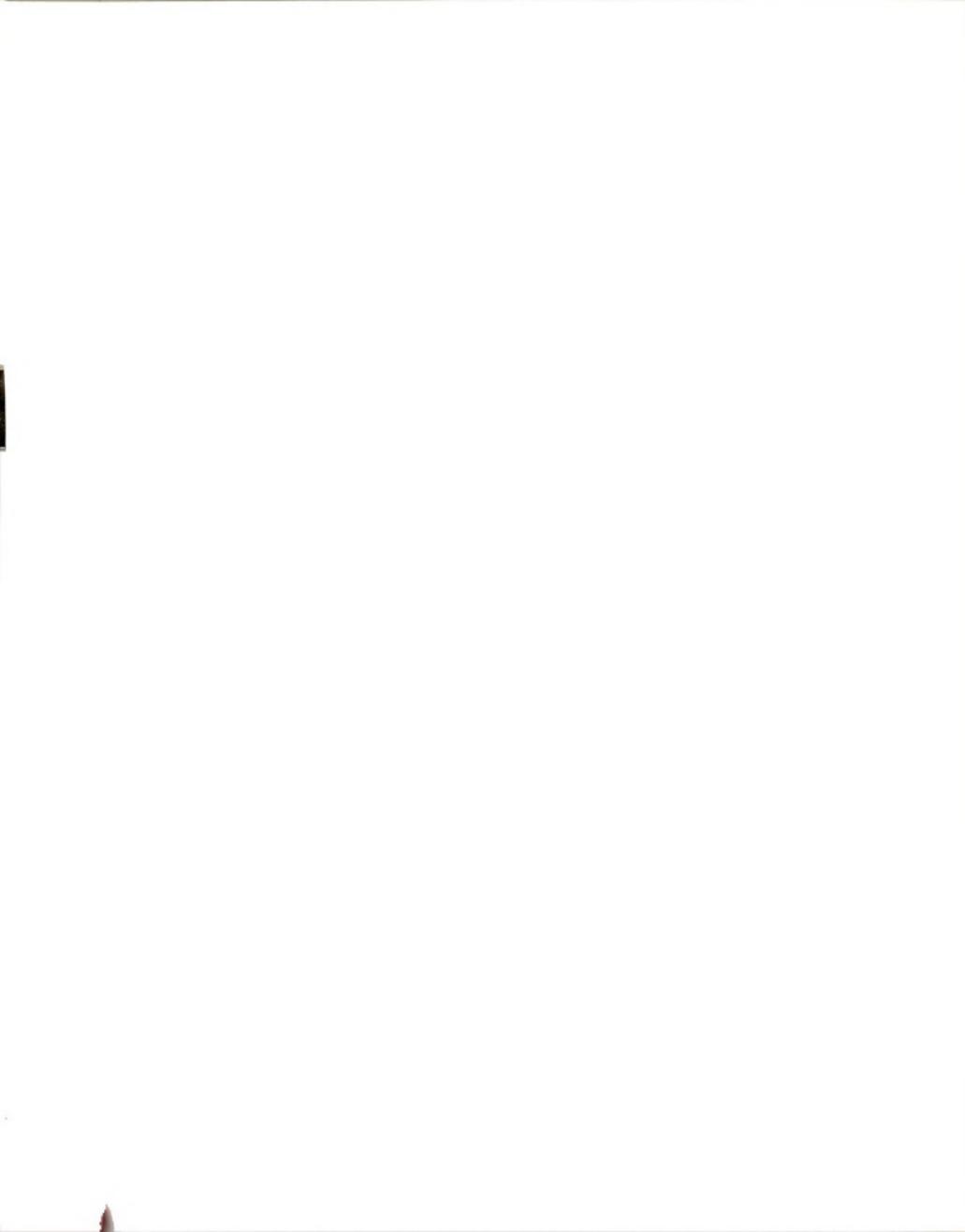
The turnover of PG of the cell surface was also studied in I-cell fibroblasts. These cells have a deficiency of lysosomal enzymes both at the cell surface and within the cell, resulting in the intracellular storage of partially degraded GAGs. Our analysis of these cells indicated no apparent perturbation of the turnover of the PG of the cell surface. The cell surface heparan sulfate (HS) PG is internalized by the cells at a rate comparable to that of normal cells. Once internalized, the HS is acted on by an endoglycosidase. This HS endoglycosidase activity appears to be unaffected by the I-cell defect, suggesting that it is a non-lysosomal enzyme.

Treatment of normal cells with  $\text{NH}_4\text{Cl}$ , which inhibits intracellular lysosomal enzyme activity, resulted in defective GAG degradation, mimicking that observed in I-cells. However, the initial steps in the turnover of cell surface PGs, including internalization, separation from core protein, and endoglycosidase activity was unaffected by treatment with  $\text{NH}_4\text{Cl}$ .

These data support the hypothesis that partial (HS endoglycosidase) and complete (mannose 6-phosphate inhibitable) degradation of GAGs may occur in compartments other than lysosomes.



For Muggy



#### ACKNOWLEDGEMENTS

I owe thanks to John Wang for supporting me and giving me the freedom to "noodle around", while at the same time teaching me to set realistic goals. I would also like to thank Dr. Richard Anderson for teaching me the importance of attending to details (although I don't like doing it any more than previously), and for his listening ear.

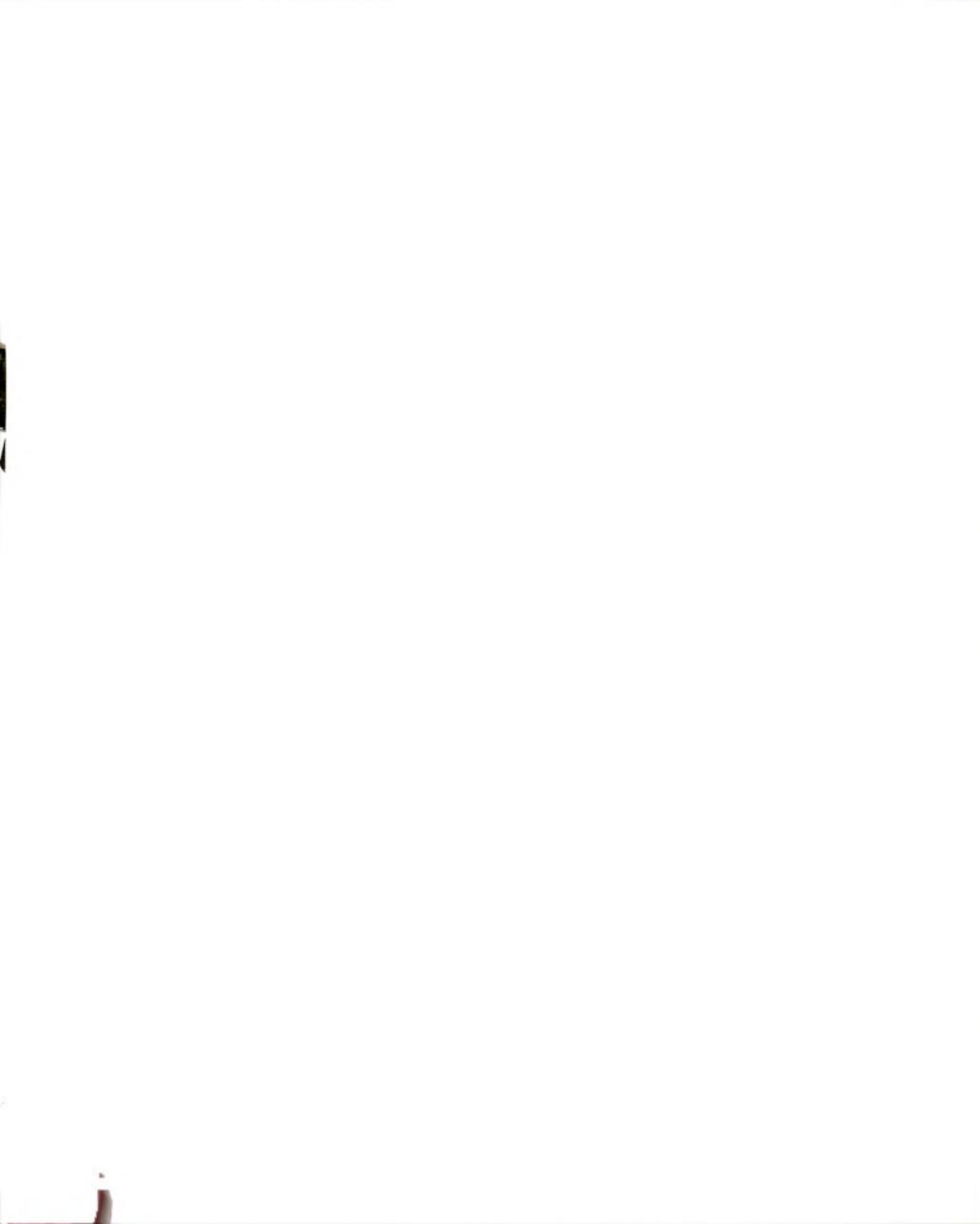
I wish to thank my wife, Diane, for always knowing where my head was at, understanding why it was there, and waiting for it to cycle around to ground zero occasionally. She is also a good fishing partner.

To those lab mates who worked with me, whether they were my teachers, students, or "worthy opponents", I owe a debt which I hope I can repay over the coming years as we all venture forth to various grant review committees. I owe special thanks to Cal Roff and Liz Cowles. They worked with me, had faith in me, and will be remembered warmly as coauthors of my first two papers.

To Craig Thomas, Lee Morehouse, and others who shared time with me around pool tables I can only say; "I'm just happy to be here".

## TABLE OF CONTENTS

	page
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
ABBREVIATIONS.....	xi
INTRODUCTION.....	1
 CHAPTER 1 LITERATURE REVIEW	
Structure and synthesis of proteoglycans.....	3
Secreted proteoglycans.....	6
Cell surface proteoglycans.....	8
Intracellular proteoglycans.....	11
Degradation of GAG chains.....	12
Transport and distribution of lysosomal enzymes.....	17
References.....	21
 CHAPTER 2 THE EFFECT OF MANNOSE 6-PHOSPHATE ON THE TURNOVER OF THE PROTEOGLYCANS IN THE EXTRACELLULAR MATRIX OF HUMAN FIBROBLASTS...	
Summary.....	31
Introduction.....	32
 Materials and Methods	
Cell lines and culture conditions.....	35
Preparation of radiolabeled SAM.....	36
Digestion of SAM by unlabeled cells or conditioned medium.....	36
Harvest and bulk quantitation of radioactivity.....	39
Quantitation of degraded material.....	40
Analysis of SAM fraction by column chromatography.....	41



## Results

Characterization of $^{35}\text{SO}_4$ -labeled SAM by column chromatography.....	42
Solubilization of SAM components into the medium.....	45
Column chromatography of solubilized SAM components.....	45
Kinetics, dose response curve and saccharide specificity of the M6P effect.....	51
Evidence that M6P affects a cell-associated compartment.....	58

Discussion.....	60
-----------------	----

Acknowledgements.....	64
-----------------------	----

References.....	65
-----------------	----

## CHAPTER 3 TURNOVER OF CELL SURFACE PROTEOGLYCANS IN CULTURED FIBROBLASTS: COMPARISON BETWEEN NORMAL AND I-CELLS.....

67

Summary.....	68
--------------	----

Introduction.....	70
-------------------	----

### Materials and Methods

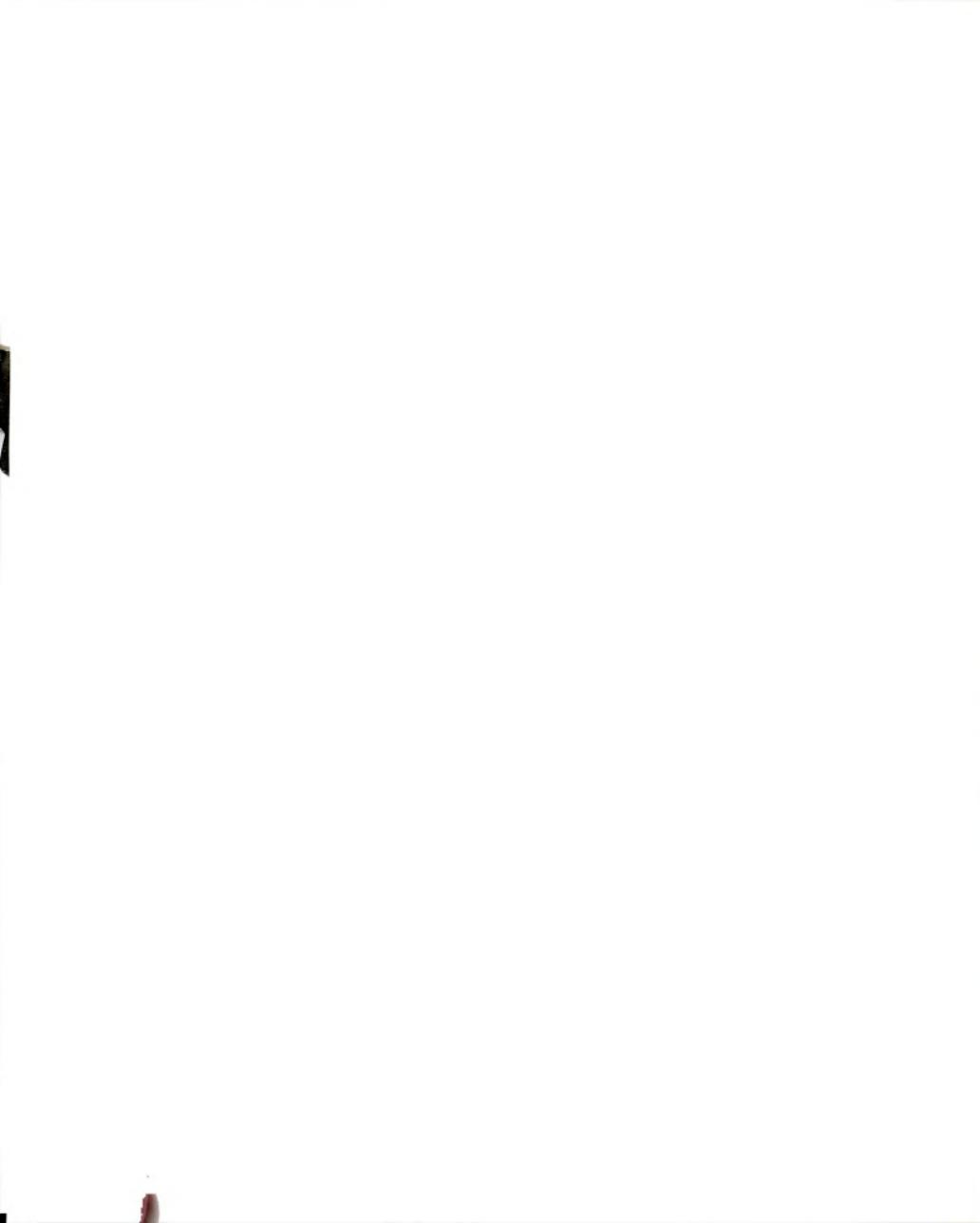
Cell culture and radiolabeling procedures....	73
Harvest of cell fractions during label-chase experiments.....	74
Chemical and enzymatic degradation of GAGs...	76
Gel filtration.....	77

### Results

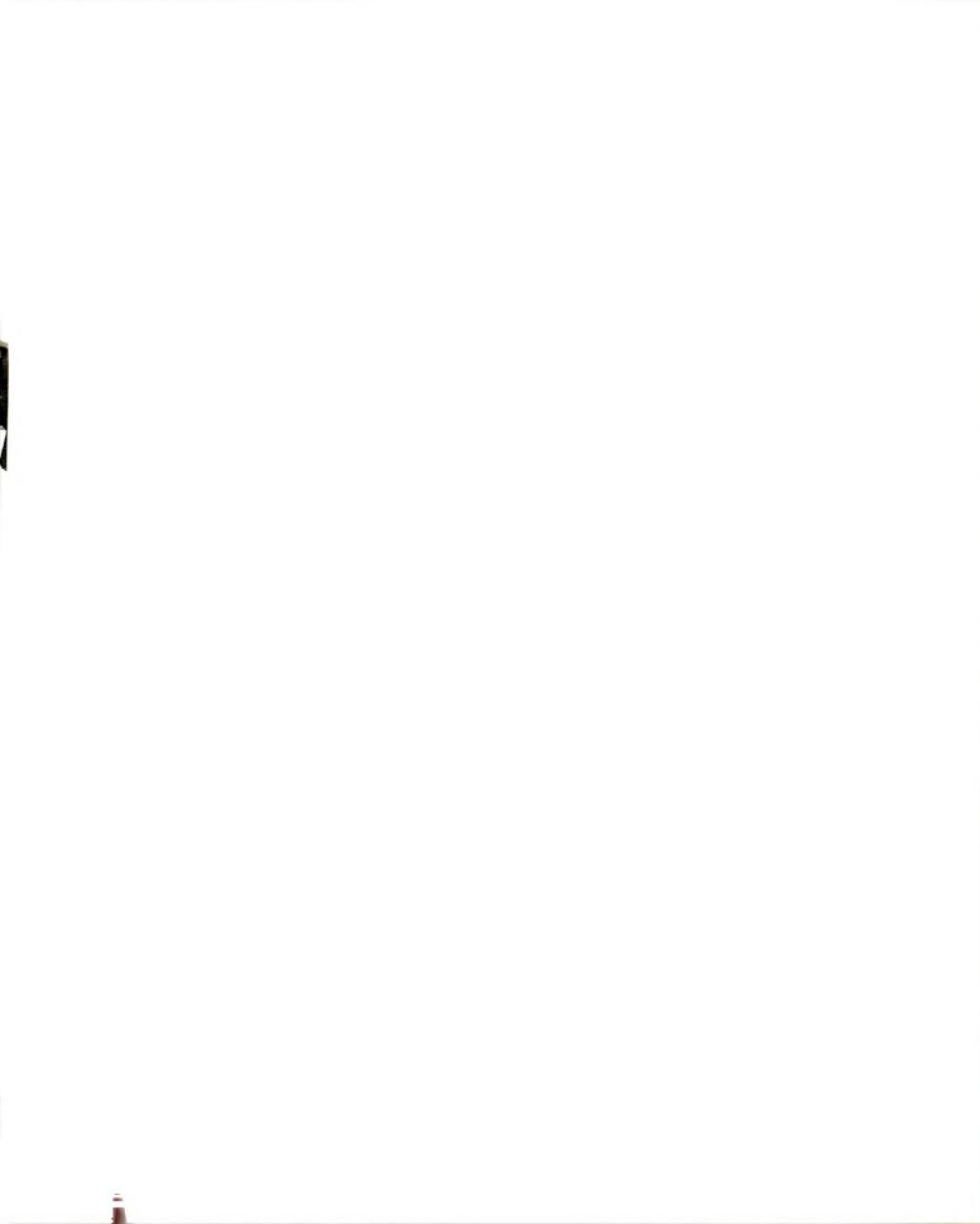
Turnover of $^{35}\text{SO}_4$ -labeled components in cells.....	78
Molecular weight distribution of $^{35}\text{SO}_4$ -labeled components in the cell monolayer...	81
Characterization of the cell surface PG.....	85
Effect of trypsin of the turnover of pericellular PG.....	88
Shedding and uptake of pericellular PG.....	92
Characterization of intracellular GAGs.....	101
Turnover of intracellular GAGs.....	109

Discussion.....	113
-----------------	-----

References.....	120
-----------------	-----

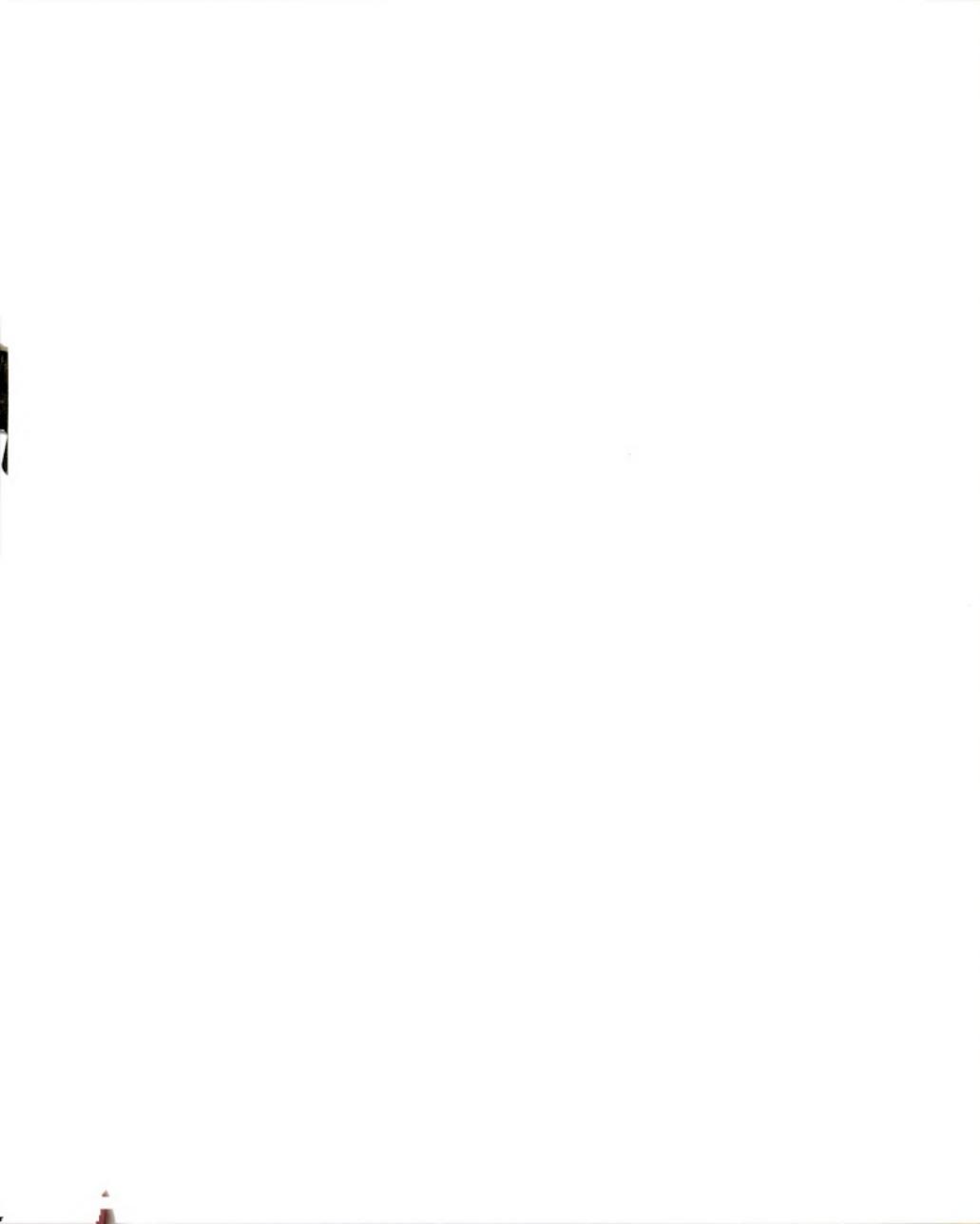


CHAPTER 4	TURNOVER OF CELL SURFACE PROTEOGLYCANS IN CULTURED FIBROBLASTS: INTERNALIZATION AND EARLY STEPS IN DEGRADATION.....	123
	Summary.....	124
	Introduction.....	126
	Materials and Methods	
	Cell culture and radiolabeling procedures....	128
	Harvest of cell fractions during label-chase experiments.....	128
	Gel filtration.....	130
	Chemical and enzymatic degradation of GAGs...	131
	Cell fractionization.....	131
	Results	
	The effect of $\text{NH}_4\text{Cl}$ on the turnover of $^{35}\text{SO}_4$ -labeled components.....	134
	Characterization of intracellular GAGs in $\text{NH}_4\text{Cl}$ treated cultures.....	137
	The effect of $\text{NH}_4\text{Cl}$ on the turnover of the intracellular GAGs.....	142
	Analysis of the intracellular GAGs in subcellular fractions.....	146
	Discussion.....	150
	References.....	157



LIST OF TABLES

Table	Page
Chapter 2	
1. Quantitation of degraded [ $^{35}\text{S}$ ]PG after various incubations.....	52
2. Quantitation of degraded [ $^{35}\text{S}$ ]PG produced by SL66 cells in the presence of various saccharides.....	58



LIST OF FIGURES

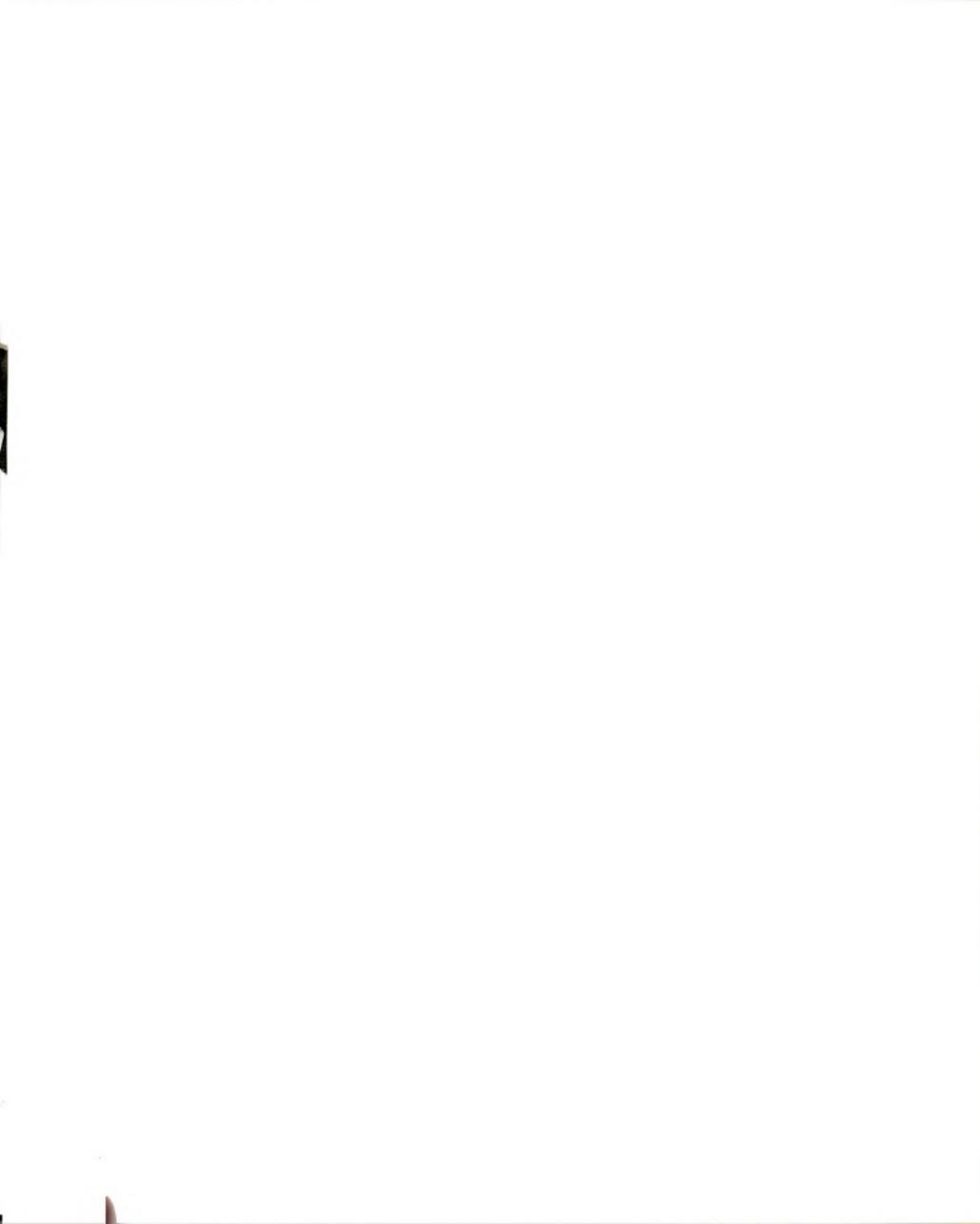
Figure	Page
Chapter 1	
1. Subunit structures of the glycosaminoglycans of cultured fibroblasts.....	4
2. Schematic representation of the steps in the degradation of heparan sulfate.....	14
Chapter 2	
1. Protocol for the preparation of radiolabeled SAM and subsequent digestion of SL66 cells.....	37
2. Chromatographic profile of SAM components before and after digestion with pronase.....	43
3. Time course of release of radioactivity from SAM..	46
4. Sepharose CL6B chromatography of soluble radio-labeled material released from SAM by SL66 cells..	49
5. Time course of the generation of degraded [ $^{35}\text{S}$ ]PG by SL66 cells from SAM.....	53
6. Dose response of M6P effect on the generation of degraded [ $^{35}\text{S}$ ]PG from SAM by SL66 cells.....	55
Chapter 3	
1. Kinetics of the turnover of $^{35}\text{SO}_4$ -labeled components in the intracellular and pericellular compartments of SL66 and I-cells.....	79
2. Gel filtration of $^{35}\text{SO}_4^{-2}$ -labeled components derived from the whole cell monolayer and the intracellular fraction of SL66 cells and I-cells during a label and chase experiment.....	82
3. Gel filtration of $^{35}\text{SO}_4^{-2}$ -labeled components derived from the pericellular fraction of I-cells labeled for 24 hours with $^{35}\text{SO}_4^{-2}$ .....	86



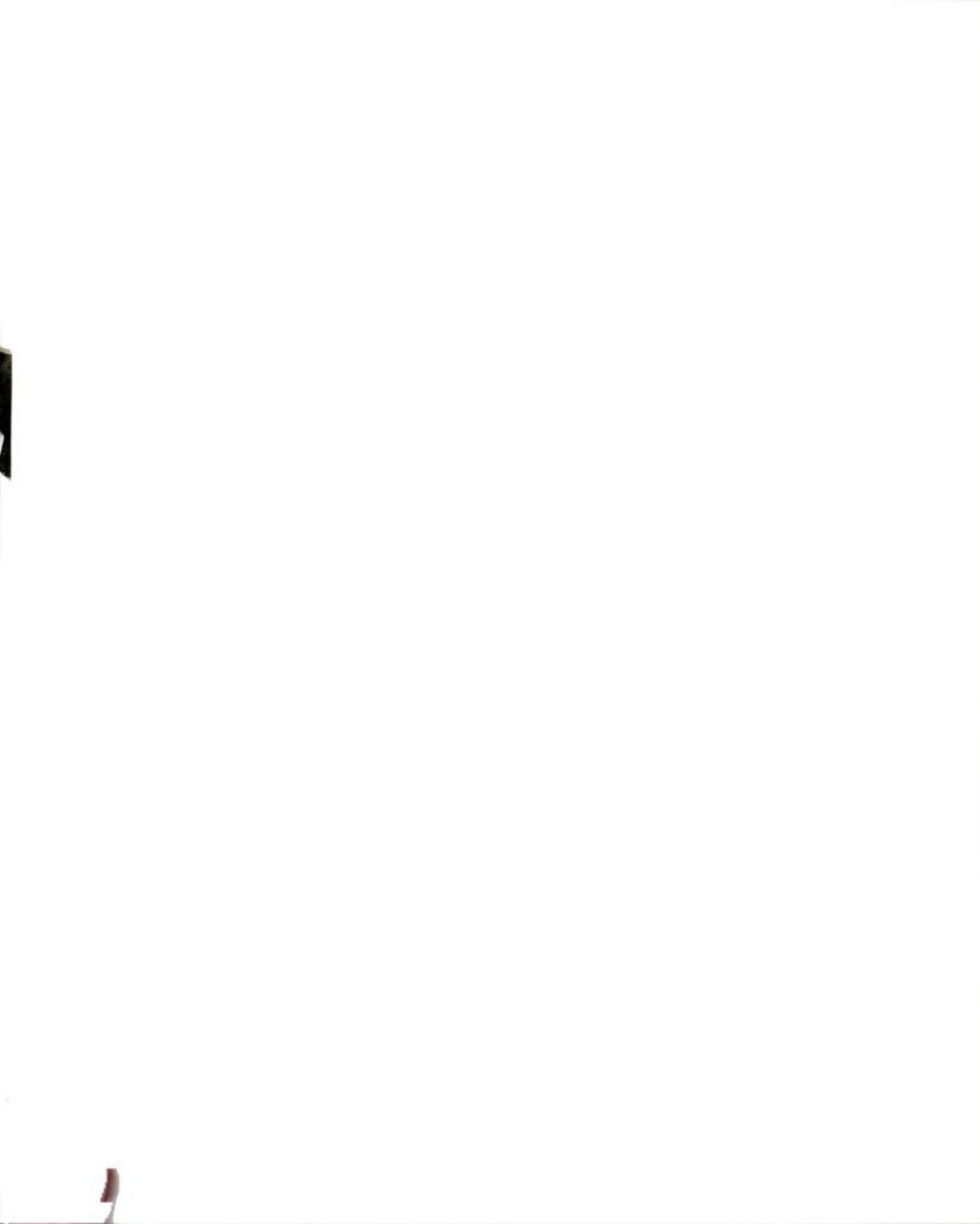
4.	Kinetics of the turnover of $^{35}\text{SO}_4^{-2}$ -labeled components derived from the intracellular and pericellular compartments of SL66 and I-cells.....	89
5.	Kinetics of the appearance of LMW radioactivity and HMW radioactivity in the medium of SL66 and I-cells labeled with $^{35}\text{SO}_4^{-2}$ .....	93
6.	Gel filtration of $^{35}\text{SO}_4^{-2}$ -labeled components in the medium of SL66 cells and I-cells.....	96
7.	Gel filtration of $^{35}\text{SO}_4^{-2}$ -labeled components in the medium of SL66 and I-cells.....	99
8.	Quantitation of internalized and shed $^{35}\text{SO}_4^{-2}$ -labeled macromolecules from the pericellular fraction of SL66 and I-cells.....	102
9.	Gel filtration of $^{35}\text{SO}_4^{-2}$ -labeled components of the intracellular fraction of I-cells, Sanfilippo cells, and heterozygous I-cells.....	105
10.	Gel filtration of $^{35}\text{SO}_4^{-2}$ -labeled components corresponding to region B and C (figure 9) of the intracellular fraction of I-cells after chemical treatments.....	107
11.	Kinetics of the turnover of $^{35}\text{SO}_4^{-2}$ -labeled components corresponding to regions B and C of the intracellular fraction of I-cells.....	110

#### Chapter 4

1.	Kinetics of the turnover of $^{35}\text{SO}_4^{-2}$ -labeled components in the intracellular and pericellular compartments of untreated and $\text{NH}_4\text{Cl}$ treated cells.....	135
2.	Gel filtration of $^{35}\text{SO}_4^{-2}$ -labeled components derived from the intracellular fraction of untreated and $\text{NH}_4\text{Cl}$ treated cells during a label and chase experiment.....	138
3.	Gel filtration of $^{35}\text{SO}_4^{-2}$ -labeled components corresponding to region B and C (figure 2) of the intracellular fraction of $\text{NH}_4\text{Cl}$ treated cells after chemical treatments.....	140
4.	Kinetics of the turnover of $^{35}\text{SO}_4^{-2}$ -labeled components corresponding to regions B and C (fig. 2) of the intracellular fraction of $\text{NH}_4\text{Cl}$ treated cells.....	143



5. Percoll density gradient fractionation of  
NH<sub>4</sub>Cl treated cells.....147
6. Schematic diagram of proposed pathways for the  
degradation of GAGs in fibroblasts.....153

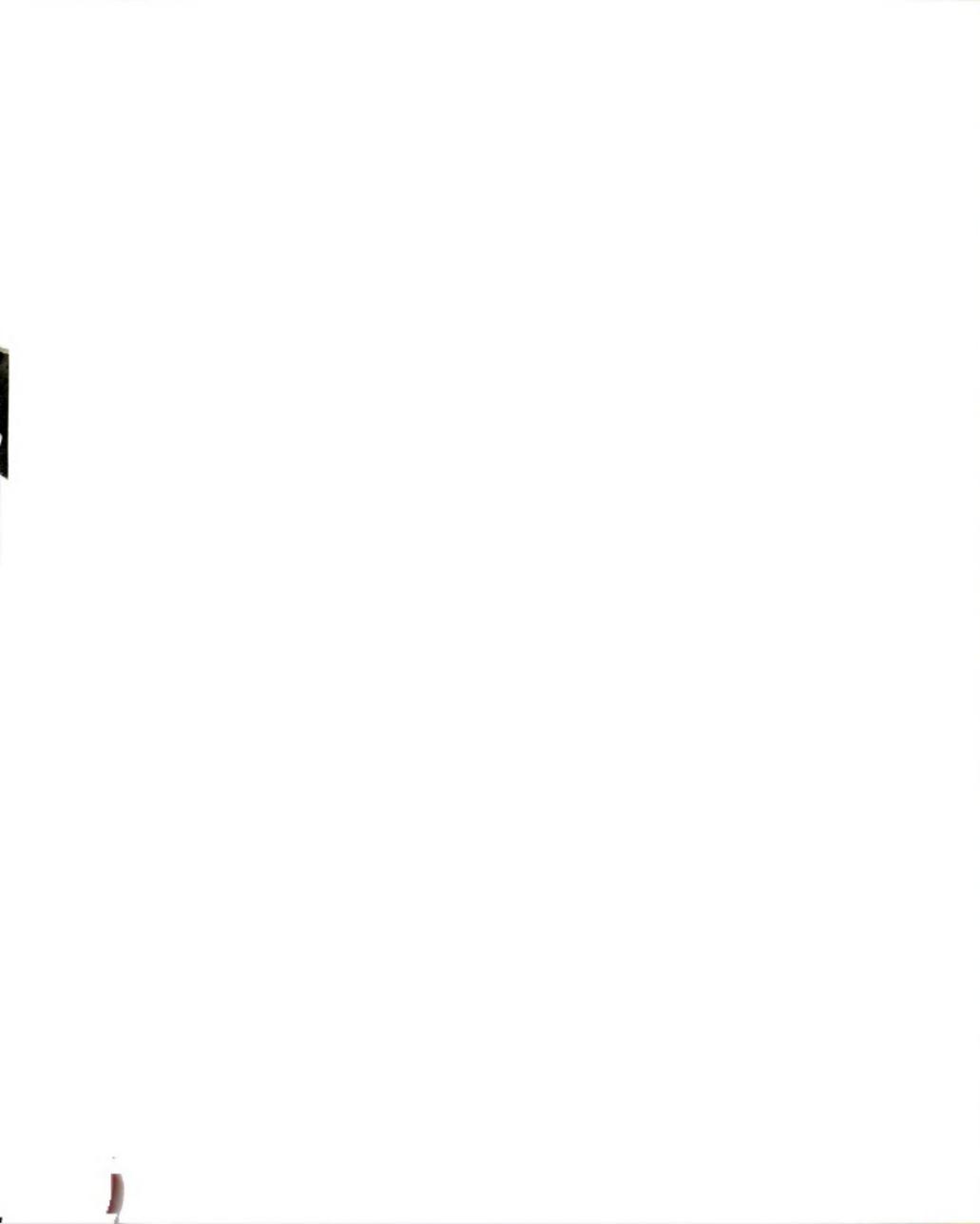


## ABBREVIATIONS

C'ase	chondroitin ABC lyase
CM	conditioned medium
cpm	counts per minute
CS	chondroitin sulfate
DL	dense lysosomes
dpm	disintegrations per minute
DS	dermatan sulfate
DSPG	dermatan sulfate proteoglycan
EGTA	ethyleneglycol-bis-( $\beta$ -aminoethyl ether)N,N' tetraacetic acid
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GAG	glycosaminoglycan
GalN	galactosamine
GlcN	glucosamine
GlcUA	glucuronic acid
HMW	high molecular weight
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
IdUA	iduronic acid

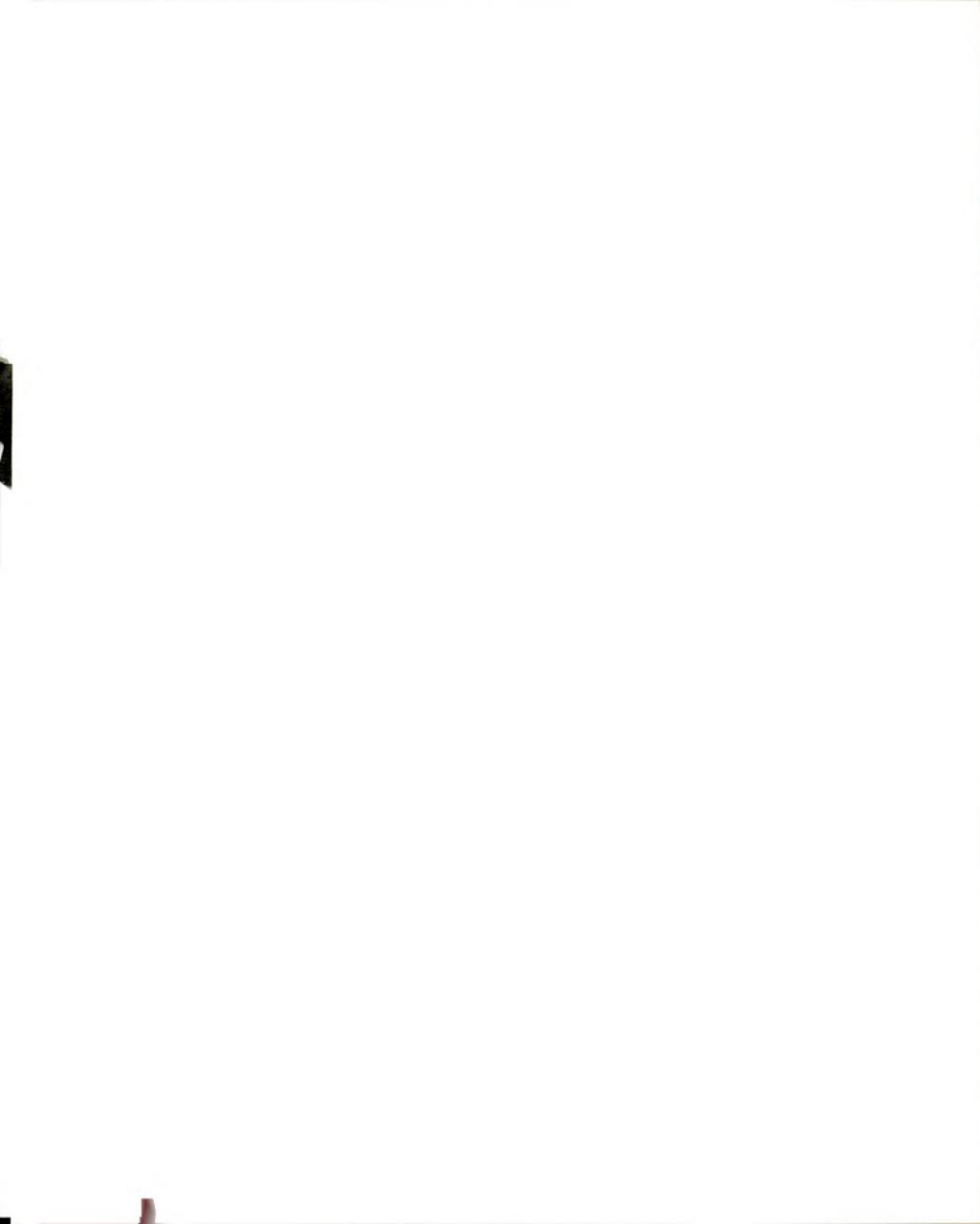


LL	light lysosomes
LMW	low molecular weight
MEM	modified Eagle's medium
M6P	mannose 6-phosphate
MPS	mucopolysaccharidosis
PBS	phosphate buffered saline
PG	proteoglycan
SAM	substratum attached material
SDS	sodium lauryl sulfate



## INTRODUCTION

This thesis describes the turnover of proteoglycans (PG) at the cell surface and in the extracellular matrix of human fibroblasts. The literature review should serve as an introduction to the distribution and turnover of the types of PG studied in cultured cells, and the transport of the enzymes involved in their degradation. We are mainly interested in the enzymes involved in the catabolism of pericellular PG, and whether some of the steps in their degradation occur in extralysosomal compartments. We have chosen two main approaches to address these issues. One approach is to analyze the metabolism of the PG of normal cells cultured in the presence and absence of mannose 6-phosphate (M6P). M6P competes with lysosomal enzymes for binding sites at the cell surface, but does not inhibit the intracellular transport of the enzymes to lysosomes. Therefore, if M6P perturbs the degradation or turnover of extracellular matrix PG, it would most likely be affecting the lysosomal enzyme action in a non-lysosomal compartment. The other approach is to study cells that have dysfunctional lysosomes (I-cells). Identification of the characteristics of the undegraded fragments "stored" in the lysosomes of I-cells, in comparison to the precursors



for degradation internalized from the cell surface/extracellular matrix, would allow us to deduce the non-lysosomal steps of PG degradation.

The experimental data of this thesis provide evidence that non-lysosomal compartments, and at least one non-lysosomal enzyme (heparan sulfate endoglycosidase) are involved in the degradation of the PG internalized from the cell surface and extracellular matrix.

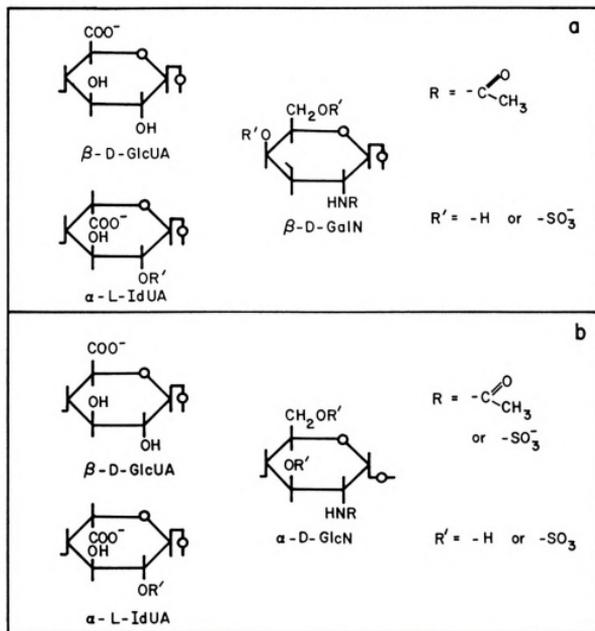
Chapter 1  
LITERATURE REVIEW

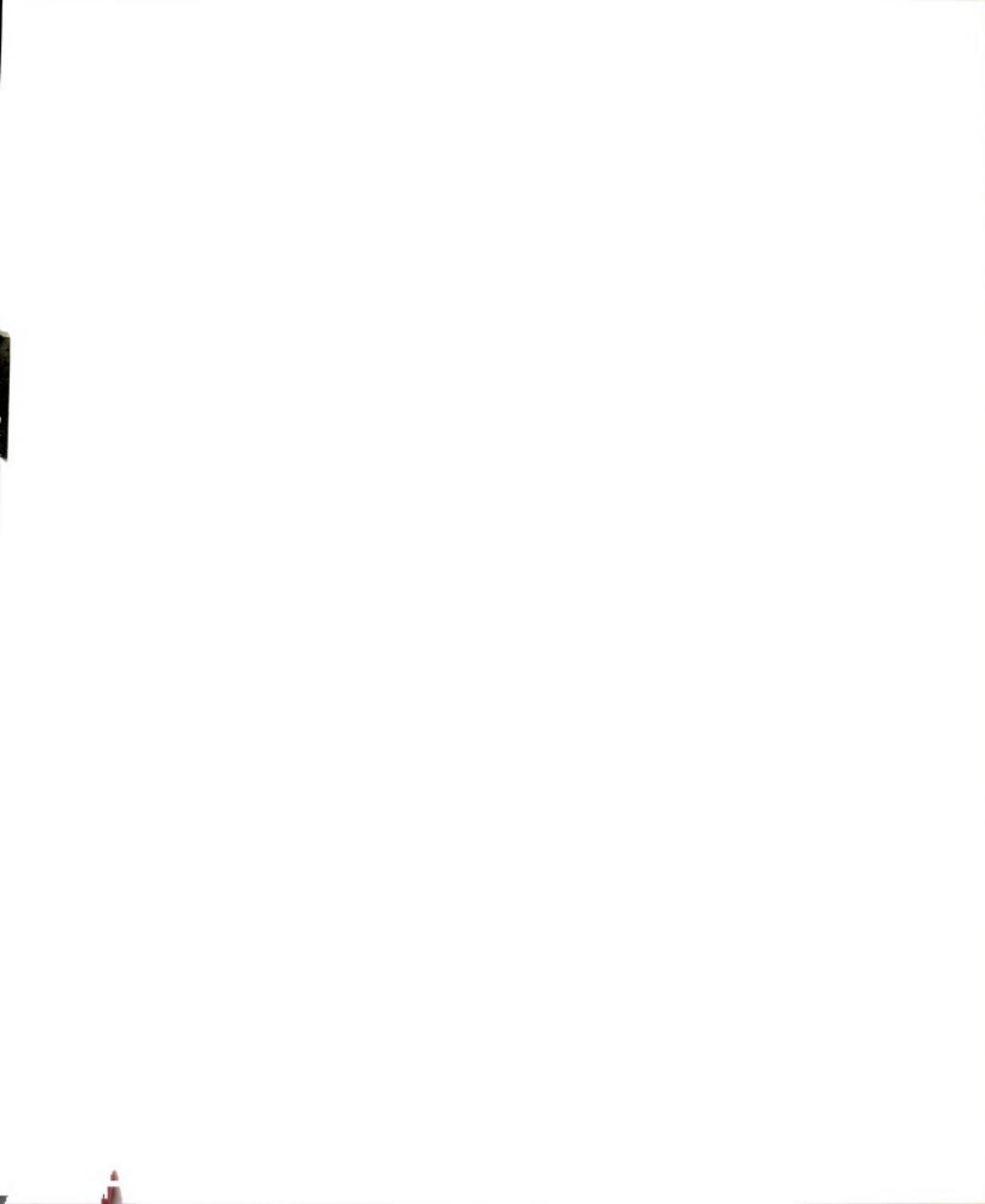
Structure and Synthesis of Proteoglycans

Proteoglycans (PG) are molecules composed of linear polysaccharide chains covalently linked to glycoproteins called core proteins (1-4). The polysaccharide chains, called glycosaminoglycans (GAGs) are composed of repeating disaccharide units consisting of a hexosamine (D-glucosamine or D-galactosamine) and an uronic acid (D-glucuronic acid or its 5-epimer, iduronic acid). Ester sulfate groups are present at various locations on the disaccharide units, and N-sulfate groups are found on heparan sulfate (HS) molecules. Because little is known about the various core proteins of proteoglycans, the molecules are usually classified according to the chemical nature of the GAG chain. The human fibroblast synthesizes three classes of GAG chains, called dermatan sulfates, (DS), chondroitin sulfates (CS) (DS and CS will be referred to as galactosaminoglycans), and heparan sulfates (HS). The subunit structures of these polysaccharides are illustrated in figure 1. Each of these GAG chains is linked to serine residues of the core protein by a trisaccharide linkage region (1-4).



Figure 1. Subunit structures of the glycosaminoglycans of cultured fibroblasts: a) Subunit structures of the galactosaminoglycans. Chondroitin sulfate is composed of galactosamine (GalN) and glucuronic acid (glcUA) dimers, whereas dermatan sulfate dimers contain galactosamine linked to either glucuronic acid or iduronic acid (IdUA); b) The subunit structure of heparan sulfate is a dimer of glucosamine (GlcN) and either glucuronic acid or iduronic acid. All three types of glycosaminoglycans may be sulfated and/or acylated as indicated by the R groups. Many combinations of dimers are observed based on the carbohydrate components, linkage between carbohydrates, and the degree of sulfation and acylation.





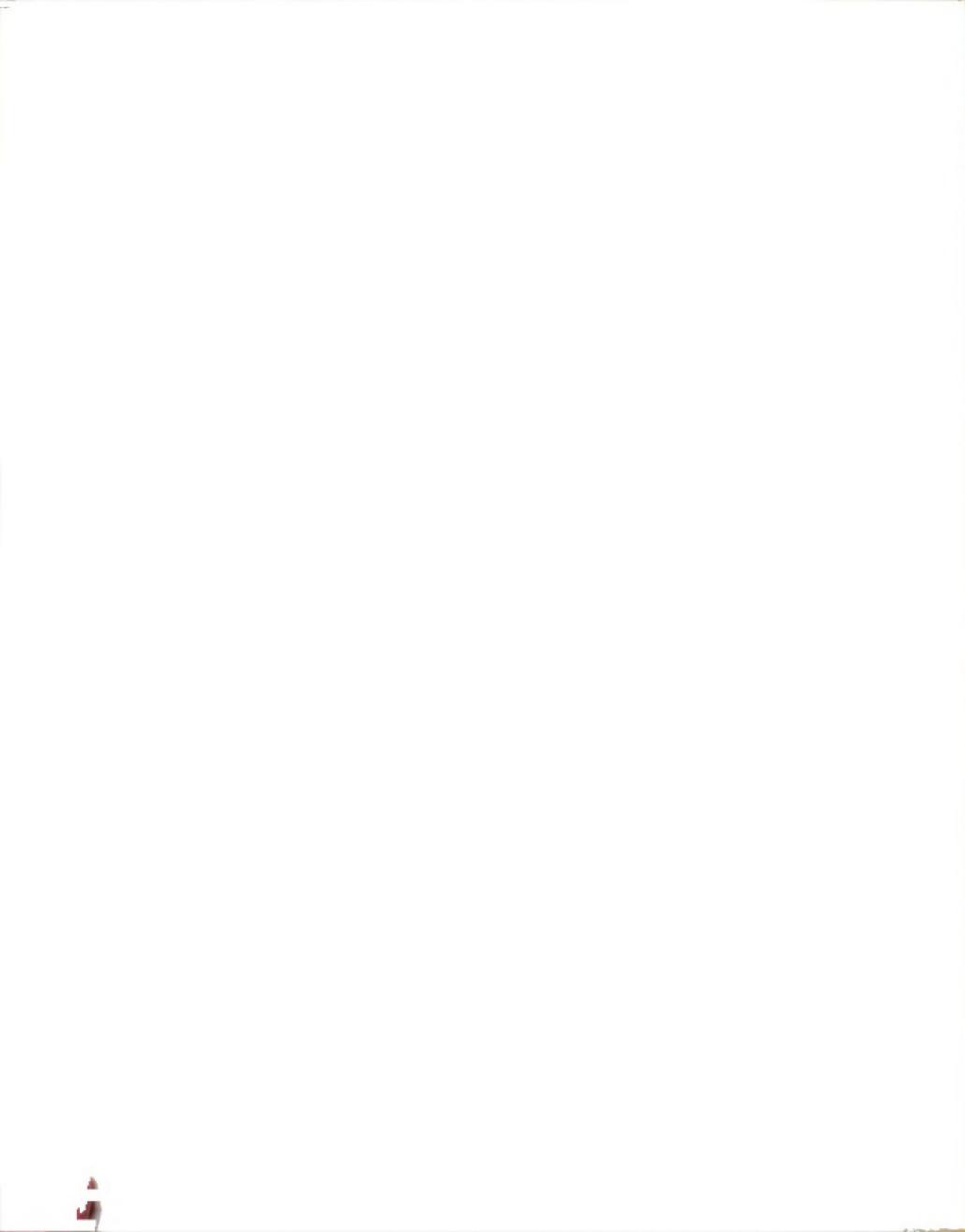
Proteoglycans are assembled in the Golgi apparatus by addition of monosaccharides to specific sites on core protein glycoproteins. The glycoproteins, containing O & N-linked oligosaccharides, are synthesized in the rough endoplasmic reticulum and then transported to the Golgi apparatus (1,3,5,6). Either in the Golgi or just prior to reaching the Golgi (6,7), they are recognized by a specific enzyme, xylose-transferase, which is responsible for initiating the synthesis of the linkage region. The xylose-transferase links a xylose residue to the protein at the hydroxyl group of serine. Two galactose molecules are added to the xylose by galactosyl transferase to make the complete linkage region gal-gal-xyl-ser (1-4). The disaccharide units are then added by enzymatic transfer of hexosamines & uronic acids from the appropriate UDP-sugar onto the non-reducing end of the chain. Chain elongation proceeds to a specified number of carbohydrate dimers, when termination of chain synthesis occurs by an as yet unspecified mechanism. Completed PG molecules may be secreted from the cell, transported to the cell surface, or transported directly to intracellular degradative compartments (8). These three pathways are discussed in detail below.

#### Secreted Proteoglycans

The dominant PG secreted into the medium of fibroblasts (80% of secreted PG) is a low molecular weight

(LMW) iduronate rich dermatan sulfate (9-13). These dermatan sulfate proteoglycan (DSPG) molecules are exported rapidly from the cell with a half life of only a few minutes (14) and apparently have a low affinity for the cell surface or for the extracellular matrix of fibroblastic cells grown on plastic. Although the small DSPG does not become associated with the matrix in cultures of fibroblasts, it is an important component of the extracellular matrix of many tissues. It has a core protein Mr of 38,000 (9), and an overall Mr of 70,000 - 100,000, depending on the number and size of DS GAG chains (16-19), and the number of oligosaccharide chains (18,20). Immunological studies revealed a relationship between the fibroblast DSPG and the DSPG of sclera and tendon (21). Histochemical and immunohistochemical studies indicate that it binds to type I collagen from rat tail tendon (22). Abnormal biosynthesis of the DSPG by fibroblasts may be the underlying cause of Coffin-Lowry syndrome, a disorder marked by mental retardation, skin disorders, and skeletal abnormalities (23).

The reports cited above indicate that there is secretion of the DSPG in fibroblast cultures, and that this product is important in vivo in connective tissue structure, but not important in maintenance of extracellular matrix for adhesion of fibroblasts in culture. This observation bears similarity to the case of procollagen synthesis and secretion by cultured cells.



Although both procollagen and procollagen processing enzymes are secreted by cultured fibroblasts (26, 27), very little collagen is produced (25,26), nor is it an important component of fibroblast adhesion sites (24). Interestingly, when fibroblastic cells are grown on collagen substrata, the secreted DSPG binds strongly to collagen fibrils in the matrix (15). Apparently, in the artificial conditions of the culture dish, both procollagen and DSPG are secreted, but do not associate as they would in vivo to produce an extracellular matrix.

Two other classes of PG are also found in the medium of fibroblasts. One is a high molecular weight (HMW) glucuronic acid rich DSPG and the other is a HMW HSPG. Both of these molecules are proposed to be cell surface PG which have been shed into the medium by a process which is slower than secretion of the LMW DSPG, thus they represent only a minor fraction of the total extracellular PG (10,13). Because these molecules are found at the cell surface, they may play important roles as matrix components or as cell surface receptors of fibroblasts (30-33). It may be that they are released from the cell surface by enzymes during such processes as cell migration and mitosis (33,40).

#### Cell Surface Proteoglycans

Heparan sulfate is the predominant cell surface PG of most cultured cells (34). Evidence is accumulating which suggests that the majority of the cell surface PG of rat



hepatocytes (36,37), mammary epithelial cells (35), glial cells and endothelial cells (38) are intercalated into the plasma membrane by hydrophobic regions of the core protein. The structures and sizes of these HSPG vary from one cell type to the next, but the general pattern is the same. The cell surface HS are membrane intercalated, but can be released into the medium by protease activity. Protease activity releases a soluble PG into the medium, leaving behind the small, unglycosylated, membrane intercalated fragment of the protein core. Treatment of cells with trypsin releases a soluble fragment of similar size to that which is "shed" into the medium during normal in vitro cell processes. For example, when cells round up in mitosis (40), or when stimulated to round up by treatment with EGTA (33) HS molecules are released into the medium, apparently by an enzymatic activity (33).

While at the cell surface, the HSPG may act as adhesive molecules, mediating binding of cells to other extracellular components such as fibronectin (41-43), laminin (44,45), and collagens (46). While interacting with extracellular matrix components, the PG may serve as a linkage molecule between the matrix and the cytoskeleton. Some evidence indicates co-distribution patterns between cell surface PG and intracellular actin (47). Moreover, the core protein of the cell surface PG of mammary epithelial cells may bind actin (48,49). Interestingly, this particular core protein is a hybrid, carrying both HS

and CS chains (50). As yet, there is no evidence as to whether the small percentage of CS on the cell surface of fibroblasts is covalently linked to the cell surface HSPG.

Some of the components of the pericellular matrix are only loosely associated with the cell surface. These molecules may be separated from the cells by treatment with heparin, which competes for GAG binding sites on the cell surface (51,52). Evidence from several laboratories indicates that the cell can actively bind and endocytose soluble and matrix associated molecules (53-59), via receptors that recognize the GAG chains (55,57), or protein cores (59).

The cell surface PGs of fibroblasts have a half-life of between 10 and 20 hours. They leave the cell surface by being either shed into the medium or internalized. From 45% (60) to 75% (61) of the PG leaving the cell surface of skin fibroblasts do so by internalization. This makes them likely candidates as cell surface receptors which might bind ligands and transport them to lysosomes (31). Indirect evidence, i.e. the competitive inhibition of binding by GAG chains, indicates that lipoprotein lipase (62-65) and platelet factor 4 (66) may be bound to cell surface GAGs on endothelial cells. More conclusive evidence suggests that the core protein of the cell surface HSPG of human fibroblasts may be similar to or identical to transferrin receptors. Fransson et al (67,68) have demonstrated transferrin binding activity in the HSPG, and



that the core protein can bind monoclonal antibodies that recognize transferrin receptors (67). Moreover, the core protein, like the transferrin receptor, occurs as a disulfide linked dimer having a subunit Mr of 80,000 - 100,000. When cleaved by trypsin, the HSPG becomes soluble, and the core protein is no longer a disulfide bonded dimer, but is a monomer having an Mr of about 70,000. Similarly, trypsin treatment of transferrin receptor releases soluble monomers with Mr of 70,000 (69).

#### Intracellular Proteoglycans

Those cell surface PG which are internalized by fibroblasts become associated mainly with a degradative compartment (8). Some evidence exists, however, for a small pool of intracellular GAG chains which have a long half-life. Fedarko and Conrad (70) have identified a nuclear fraction of HS, (representing about 6% of the total cell associated HS) which has an unusual disaccharide structure present in high amounts. They suggested that, because of the long transit time to the nucleus (2 hours), the nuclear HS may be a unique catabolite of the HSPG which is endocytosed from the cell surface. HS may play a role in the regulation of nuclear activity, based on the observation that heparin, when added to isolated nuclei, stimulates both DNA replication and transcription (71-75). Heparin also affects the activities of a number of other enzymes found in the nucleus, including cAMP dependent

protein kinase N II (76), and topoisomerase I (77).

The remainder of the intracellular GAG appears to be in a degradative compartment. No function has been assigned to these intermediate sized fragments of GAG chains found in the cells. These molecules will be discussed below in terms of the enzyme systems used by the cells to degrade them.

#### Degradation of GAG chains

The degradation of the GAG chains of internalized proteoglycans has been studied extensively. However, little information is available as to whether, or how, the protein core of internalized PG is degraded, although there is evidence for extracellular cleavage of core proteins of PGs (78-81). In cartilage, release of PG monomers from the matrix results from cleavage near the hyaluronate binding region of the core protein (82-84). Once endocytosed, soluble or cell surface core proteins are rapidly separated from GAG chains. This leads to difficulty in further analyzing the degradation of core proteins, since the probes used to study PG usually involve labeling the GAG chains and/or purification schemes based on properties of GAGs (1-4).

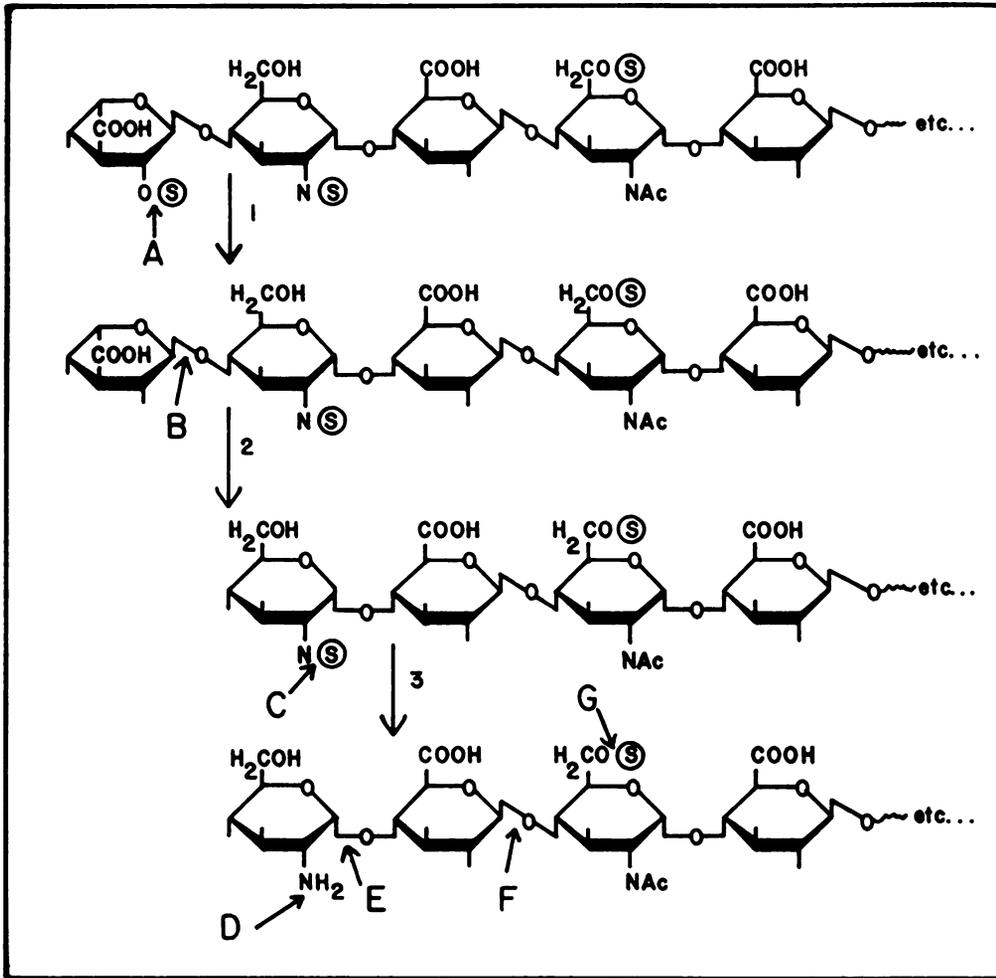
The steps in the degradation of the internalized GAGs are thought to occur predominantly in lysosomes, although indirect evidence indicates the possibility of non-lysosomal endoglycosidase activity against HS (85). Klein et al (86) have reported that there is an HS endoglucuronidase

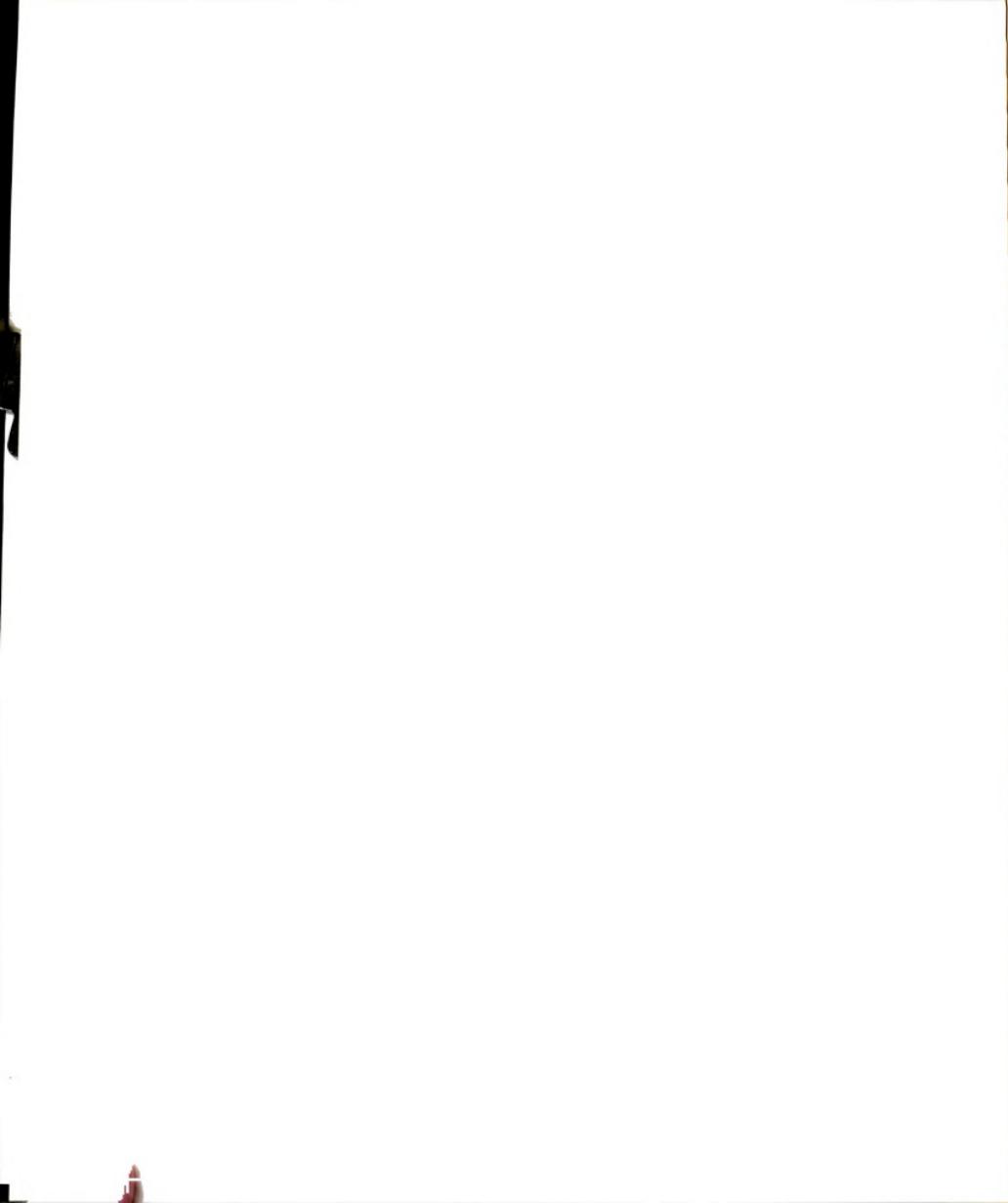
activity in human fibroblasts, but presented no evidence as to its cellular localization. Data from other studies suggest that HS endoglucuronidases are common features of several cell types and tissues including platelets (87-89), activated T-lymphocytes (90), rat liver (91), and human placenta (92,93). The pH optimum for the activity of the HS endoglycosidase is around 6, which is high for lysosomal enzymes but somewhat low for neutral enzymes.

Endoglycosidase activity also occurs against CS chains in chick chondrocytes (94) and liver (95). Moreover, CS is a substrate of lysosomal hyaluronidase (96,97). However, as yet, no hyaluronidase activity has been observed in human fibroblasts (98,99).

The GAG chains which are found in the lysosomes, whether or not they have been previously acted on by endoglycosidases, are substrates for the action of several glycosidases and sulfatases which act sequentially as exoenzymes at the non-reducing end of the GAG chains (100-102). The scheme for the degradation of HS is shown in figure 2. Each enzyme in the pathway changes the structure of the GAG chain so that it becomes a substrate for the next enzyme in the pathway. Thus, for HS degradation, three glycosidases, two sulfatases, a sulfamatase, and an N-acetyl transferase act in sequence to free monomers from the polysaccharide (101). Degradation of the galactosaminoglycans occurs in a similar manner, differing only in the specificity of the participating enzymes.

Figure 2. Schematic representation of the steps in the degradation of heparan sulfate. The heparan sulfate chain consists of repeating disaccharides of glucosamine and either glucuronic acid or iduronic acid. A series of lysosomal enzymes act at the non-reducing of the chain to modify (usually by hydrolytic cleavage) the structure of the chain, making it a substrate for the next enzyme in the pathway. The diagram illustrates the actions of iduronate sulfatase (A) which removes the O-sulfate at the 2 position of iduronic acid. The next enzyme in the pathway is  $\alpha$ -iduronidase (B), which can then remove the iduronic acid residue. These activities are followed by those of heparan N-sulfatase (C), and a specific lysosomal acetyl coA transferase (D) which links an acetyl group to the nitrogen of glucosamine. This alteration makes the terminal monomer a substrate for  $\alpha$ -N-acetylglucosaminidase (E). Sites of action of  $\beta$ -glucuronidase (F) and N-acetyl glucosamine 6-sulfatase (G) are also shown.





If one of the exoenzymes of the GAG degradation pathway is missing, the GAG chain is not depolymerized because the substrates for the successive enzymes in the pathway are not uncovered. Much of our knowledge about the exoenzymes of GAG catabolism has come from studies of human genetic diseases called mucopolysaccharidoses (MPS). In cells from MPS patients, the gene for production of a lysosomal enzyme is defective, resulting in little or no enzyme activity appearing in the lysosomes (100-104). When GAG chains are transported to lysosomes in these cells they are degraded only until the substrate for the missing enzyme is produced at the reducing end of the GAG chain. Since cells have a low capacity to clear polymers from their lysosomes [some lysosomal enzymes have half-lives in the cell of 30 days (105)], the undegraded GAGs are stored intracellularly, resulting in a myriad of secondary molecular, cellular and clinical defects (100-104). Studies of the MPS diseases over the last 15 years have facilitated the discovery and/or purification of most of the exoenzymes involved in the degradation of GAGs. For example, HS GAG chains require the activity of a number of exoenzymes in the lysosomes. A group of MPS diseases exist (collectively called Sanfilippo disease) which are specific for HS (100,102). The several subtypes of Sanfilippo disease arise from deficiencies of different lysosomal enzymes, each of which are coded for by different genes, but have in common the function of participating in the



degradation of HS. Absence of any one of these gene products results in storage of HS in the cells of patients, resulting in identical clinical phenotypes. Presently, four subtypes of Sanfilippo syndrome have been identified; Sanfilippo type A [heparan N-sulfatase deficiency (106-108)], type B [ $\beta$ -N-acetylglucosidase (109,110)], type C [N-acetyl transferase deficiency (111-,112)], and type D [ $\beta$ -N-acetylglucosamine-6-sulfate sulfatase deficiency (113)] (see figure 2 for details of HS degradation). The identities of these gene products were discovered because it was found that the secretions of normal cells contained corrective factors which could be internalized by diseased cells and correct the phenotype. Thus, the enzymes could be purified based on assays of GAG degradation in cultured fibroblasts (100-104).

#### Transport and Distribution of Lysosomal Enzymes

The enzymes of the lysosome, including those exoenzymes of the pathway of GAG degradation, receive a chemical transport marker in the Golgi which allows them to be segregated from secretory proteins and transported to lysosomes (114,115). Much of our information about the pathway for the transport of lysosomal enzymes comes from studies of fibroblastic cells from patients with a genetic disease known as I-cell disease (mucopolipidosis II) (114-116). Cultured fibroblasts of I-cell patients synthesize precursor lysosomal enzymes, which, instead of being

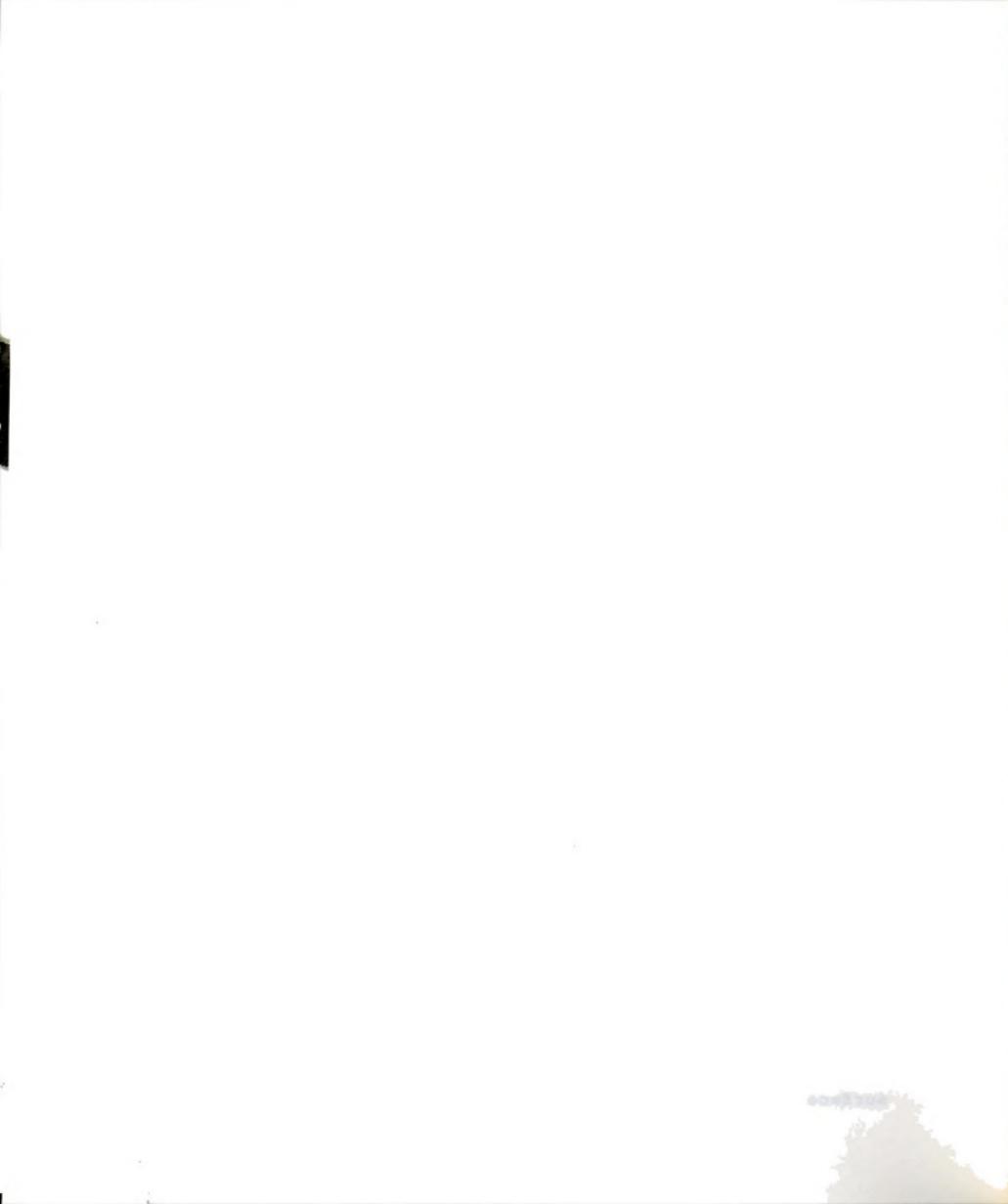
localized into lysosomes, are secreted into the surrounding medium (115,117). Other evidence indicated that lysosomal enzymes from normal cells could be selectively pinocytosed by cells via specific cell surface receptors, and subsequently transported to lysosomes (114,115). When similar experiments were performed using the enzymes secreted by I-cells, they were not taken up by normal cells. In contrast, enzymes from normal cells could be taken up by I-cells. These data indicated that the receptors for transport of lysosomal enzymes were unaffected in I-cell fibroblasts, but the enzymes themselves were affected such that they were not recognized by the receptors. Subsequent studies revealed that the defect of lysosomal enzymes in I-cell patients was an abnormal chemical structure of the oligosaccharide chains. Specifically, the oligosaccharides lacked mannose 6-phosphate (M6P) residues which are necessary components (117-119) for recognition of the lysosomal enzymes by specific membrane receptors called M6P receptors (120-122). It has now been demonstrated that the primary gene defect in I-cell disease is in the phosphotransferase required for the construction of the M6P recognition marker on the oligosaccharides of lysosomal enzymes (123). When the transferase is missing, lysosomal enzymes do not find their way to lysosomes, but are instead secreted from the cells. Therefore, I-cells have a deficiency of the lysosomal enzymes involved in the degradation of GAGs, and they



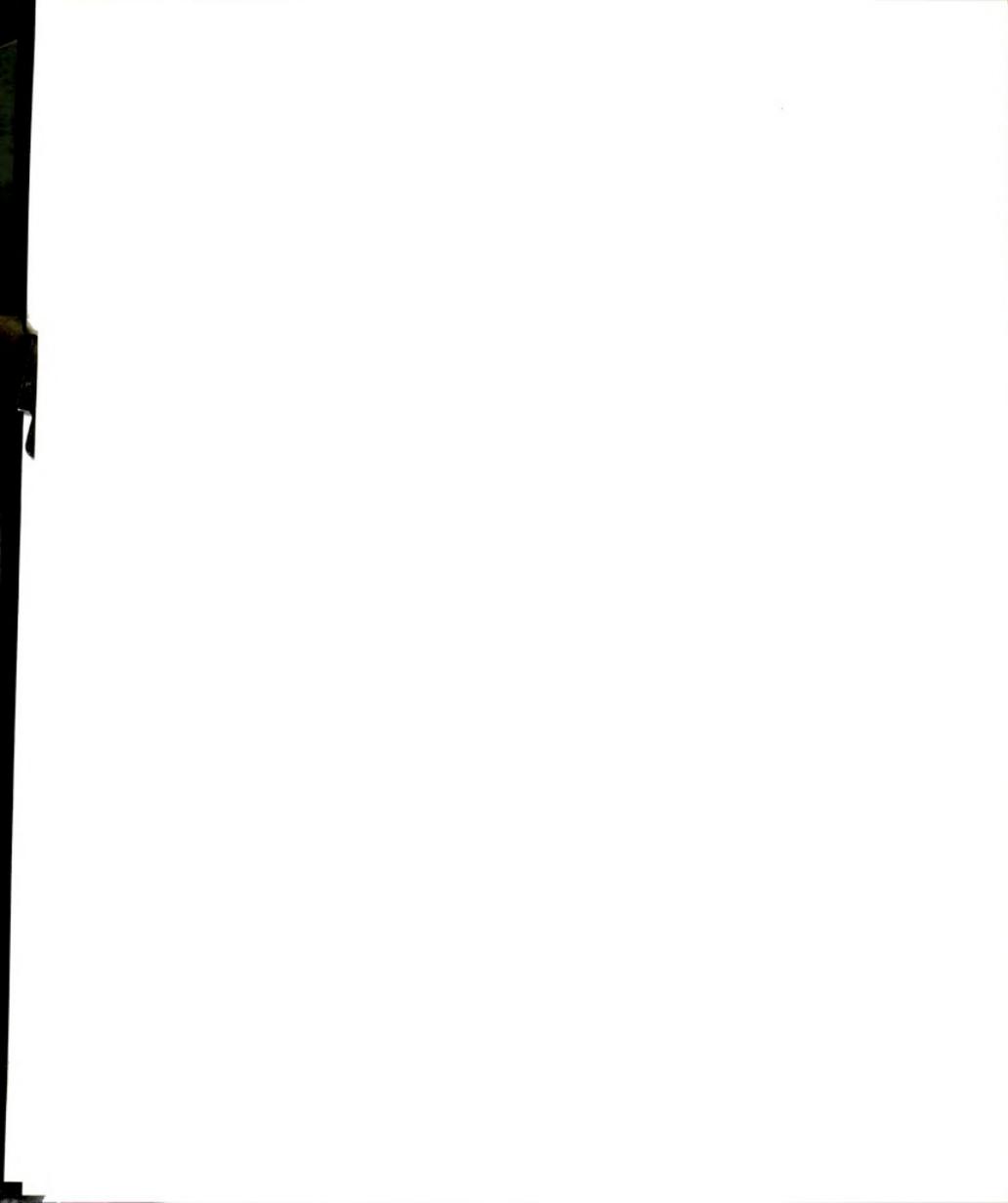
consequently store partially degraded GAG chains in their lysosomes (124).

The observation, cited above, that fibroblasts can bind and endocytose lysosomal enzymes via cell surface receptors, raised the question whether lysosomal enzymes are transported to lysosomes by first being secreted and then recaptured (115,125). Long term treatment of cells with M6P did not inhibit the localization of lysosomal enzymes to lysosomes (126,127) despite the fact that M6P could compete with lysosomal enzymes for binding sites on the cell surface, even displacing previously bound enzymes (128,129). These results argue against the secretion-recapture hypothesis. Recent quantitation of the M6P receptor showed that only about 20% of the M6P receptors were localized to the cell surface (130), whereas the rest were located inside the cell where they play a role in transporting lysosomal enzymes via an intracellular route to the lysosomes.

The localization of lysosomal enzymes to the cell surface is puzzling since the pH optimum of lysosomal enzymes is too low for these enzymes to be active in the neutral conditions of the culture medium (131). Recent evidence suggests that cell surface lysosomal enzymes may play a role in the degradation of the PG of the extracellular matrix of human fibroblasts. Exposure of cells to M6P [which removes lysosomal enzymes from the cell surface (128,129)] disrupted the kinetics of the turnover

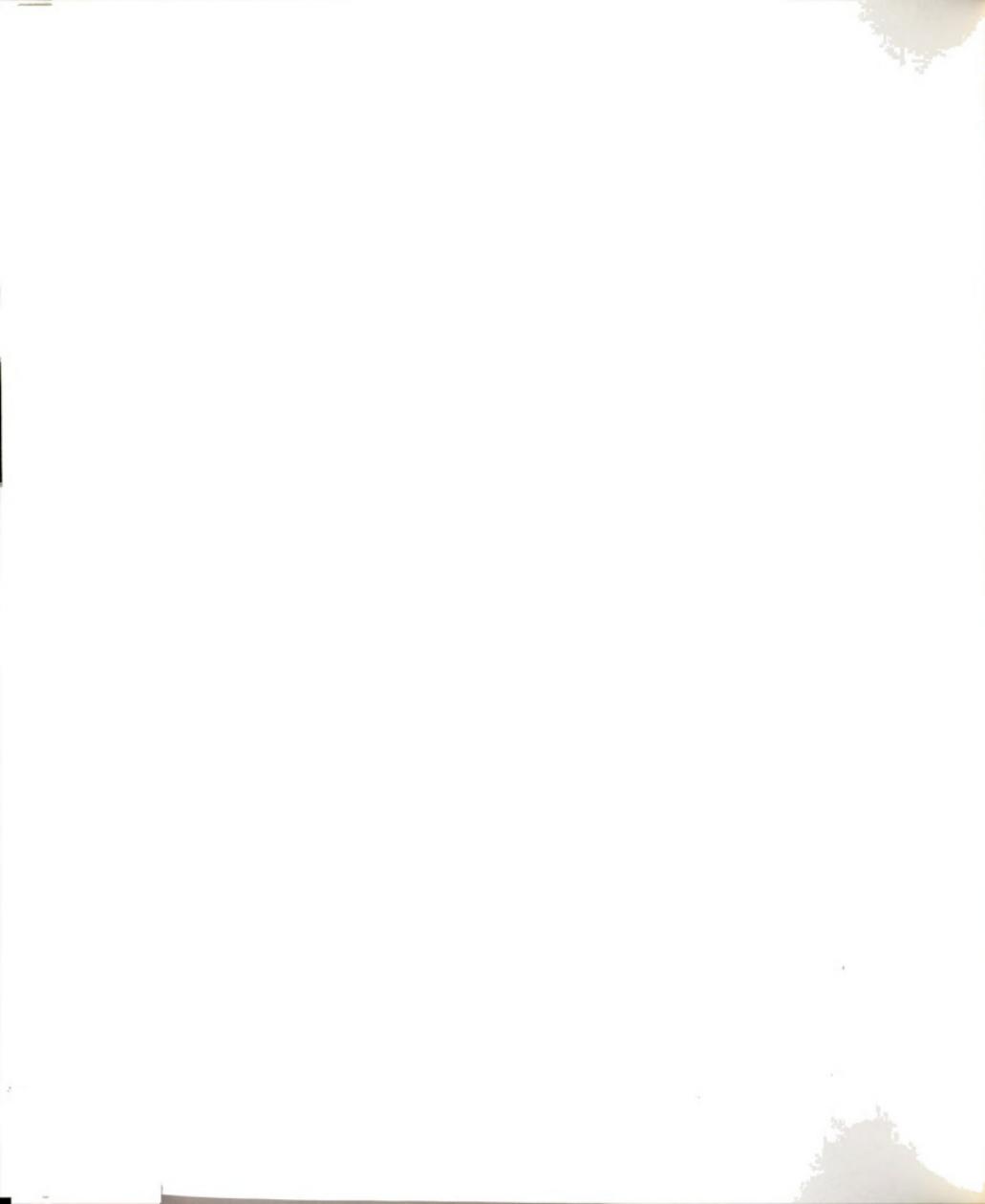


of cell surface GAGs (132,133). Moreover, when cells were plated on an extracellular matrix containing  $^{35}\text{SO}_4^{-2-}$  labeled PGs, the cells internalized and degraded the PGs, and this degradation was inhibited by M6P (134). Thus, it was proposed that some internalized extracellular matrix PGs may be degraded in an acid compartment of the cell which requires a secretion-recapture mechanism for delivery of lysosomal enzymes.



#### REFERENCES

1. Roden, L. (1980) In The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W., ed.), Plenum, New York. pp. 267-371.
2. Dorfman, A. (1981) In Cell Biology of the Extracellular Matrix. (Hay, E.D., ed.) Academic Press, New York. pp. 115-138.
3. Rodin, L., and Horowitz, M.I. (1978) In The Glycoconjugates, Vol. 2 (Horowitz, M.I., and Pigman, W., ed.) Academic Press, New York. pp. 3-71.
4. Hascall, V.C., and Hascall, G.K. (1981) In Cell Biology of the Extracellular Matrix (Hay, E.D., ed.) Plenum, New York. pp. 39-63.
5. Fellini, S.A., Hascall, V.C., and Kimura, J.H. (1984) *J. Biol. Chem.* 259, 4634-4641.
6. Kimura, J.H., Lohmander, L.S., and Hascall, V.C. (1984) *J. Cell. Biochem.* 26, 261-278.
7. Hoffman, H.-P., Schwartz, N.B., Roden, L., and Prockop, D.J. (1984) *Connect. Tissue Res.* 12, 151-163.
8. Fratantoni, J.C., Hall C.W., and Neufeld E.F. (1968) *Proc. Natl Acad. Sci. U.S.A.* 60, 699-706.
9. Glossyl, J. Beck, M., and Kresse, H. (1984) *J. Biol. Chem.* 259, 14144-14150.
10. Vogel, K.G., and Peterson, D.W. (1981) *J. Biol. Chem.* 256, 13235-13242.
11. Coster, L., Carlstedt, I., and Malmstrom, A. (1979) *Biochem. J.* 183, 669-681.
12. Carlstedt, I., Coster, L., and Malmstrom, A. (1981) *Biochem. J.* 197 217-225.
13. Coster, l., Carlstedt, I., Malmstrom, A., and Sarnstrand, B. (1984) *Biochem. J.* 220, 575-582.
14. Vogel, K.G., and Kendall, V.F. (1980) *J. Cell. Physiol.* 103 475-487.



15. Gallagher, J.T., Gasiunas, N., and Schor, S.L. (1983) *Biochem. J.* 215, 107-116.
16. Coster, L., and Fransson, L.-A. (1981) *Biochem. J.* 193, 143-153.
17. Damle, S.P., Coster, L., and Gregory, J.D. (1982) *J. Biol. Chem.* 257, 5223-5527.
18. Nakamura, T., Matsunaga, E., and Shinkai, H. (1983) *Biochem. J.* 213, 289-296.
19. Rosenberg, L.C., Choi, G.U., Tang, L.-H., Johnson, T.L., Pal, S., Webber, C., Reiner, A., and Poole, A.R. (1985) *J. Biol. Chem.* 260, 6304-6313.
20. Shinkai, H., Nakamura, T., and Matsunaga, E. (1983) *Biochem. J.* 213, 297-304.
21. Heinegard, D., Bjerne-Persson, A., Coster, L., Franzen, A., Gardell, S., Malmstrom, A., Paulsson, M., Sandfalk, R., and Vogel, K. (1985) *Biochem. J.* 230, 181-194.
22. Scott, J.E., and Orford, C.R. (1981) *Biochem. J.* 197, 213-216.
23. Beck, M., Glossl, J., Ruter, R., and Kresse, H. (1983) *Pediatr. Res.* 17, 926-929.
24. Culp, L.A., and Bensusan, G. (1978) *Nature (Lond.)* 273, 680-682.
25. Kleinman, H.K., Klebe, R.J. and Martin, G.R. (1981) *J. Cell Biol.* 88, 473-485.
26. Hay, E.D., (1981) In Cell Biology of the Extracellular Matrix (Hay, E.D., ed.) Plenum, New York. pp. 379-409.
27. Lichenstein, J.R., Martin, G.R., Kohn, L.D., Byers, P.H., and McKusick, V.A. (1973) *Science* 182, 298-300.
28. Layman, D.L., and Ross, R. (1973) *Arch. Biochem. Biophys.* 157, 451-456.
29. Goldberg, B., Taubman, M.B., and Radin, B. (1975) *Cell* 4, 45-50.
30. Bernfield, M., Banerjee, S., Koda, J., and Rapraeger, A. (1984) In The Role of the Extracellular Matrix in Development (Trelsted, R.L., ed.) Alan R. Liss, Inc. New York pp. 545-572.



31. Hook, M., Kjellan, L., Johansson, S., and Robinson, J. (1984) *Ann. Rev. Biochem.* 53, 847-869.
32. Rollins, B., Cathcart, M., and Culp, L. (1982) In The Glycoconjugates, Vol. 3 (Horowitz, M., ed.) Academic Press, pp. 289-329.
33. Rapraeger, A., and Bernfield, M. (1985) In Diseases of Connective Tissue: Molecular Pathology of the Extracellular Matrix (Uitto, J. and Perjda, A., ed.) Marcell Dekkar, Inc., New York. pp. 2-32.
34. Gallagher, J., Lyon, M., and Steward, W. (1986) *Biochem. J.* 236, 313-325.
35. Rapraeger, A., and Bernfield, M. (1983) *J. Biol. Chem.* 258, 3632-3637.
36. Kjellen, L., Petterson, I., and Hook, M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 5371-5375.
37. Kjellen, L., Oldberg, A., Hook, M. (1980) *J. Biol. Chem.* 255, 10407-10413.
38. Norling, B., Glimelius, B., Wasteson, A. (1981) *Biochem. Biophys. Res. Comm.* 103, 1265-1272.
39. Rapraeger, A., and Bernfield, M. (1985) *J. Biol. Chem.* 260, 4103-4109.
40. Kraemer, P., and Tobey, R. (1972) *J. Cell Biol.* 55, 713-717.
41. Ruoslahti, E., and Engvall, E. (1980) *Biochem, Biophys. Acta* 631, 350-358.
42. Yamada, K.M., Kennedy, D.W., Kimata, K., and Pratt, R.M. (1980) *J. Biol. Chem.* 255, 6055-6063.
43. Stamatoglou, S., and Keller, J. (1982). *Biochim. Biophys. Acta* 719, 90-97.
44. Sakashita, S., Engvall, E., and Ruoslahti, E. (1980) *FEBS letters* 116, 243-246.
45. Woodley, D.T., Rao, C.N., Hassell, J.R., Liotta, L.A., Martin, G.R., and Kleinman, H.K. (1983) *Biochim. Biophys, Acta* 761, 278-283.
46. Koda, J., Rapraeger, A., and Bernfield, M. (1985) *J. Biol. Chem.* 260, 8147-8162.

47. Woods, A., Hook, M., Kjellen, L., Smith, C.G., and Rees, D.A. (1984) *J. Cell Biol.* 95, 1743-1753.
48. Rapraeger, A., and Bernfield, M. (1982) In The Extracellular Matrix (Hawkes, S.P., and Wang, J.L., ed.) Academic Press, New York. pp265-269.
49. Rapraeger, A., Jalkanen, M., and Bernfield, M. (1986) In Biology of the Extracellular Matrix, Vol. VII: Biology of Proteoglycans (Wight, T.N., and Mecham, R.P., ed.) Academic Press, New York. pp 265-269.
50. Rapraeger, A., Jalkanen, M., Endo, E., Koda J., and Bernfield, M. (1985) *J. Biol. Chem.* 260, 11046-11052.
51. Kjellen, L., Oldberg, A., and Hook, M. (1980) *J. Biol. Chem.* 255, 10407-10413.
52. Kraemer, P.M. (1977) *Biochem. Biophys. Res. Comm.* 78, 1334-1340.
53. Kresse, H., Tekolf, W., von Figura, K., and Buddecke, E. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* 356, 943-952.
54. Truppe, W., and Kresse, H. (1978) *Eur. J. Biochem.* 85, 351-356.
55. Prinz, R., Schwermann, J., Buddecke, E., vonFigura, K. (1978) *Biochem. J.* 176, 671-676.
56. vonFigura, K., Kresse, H., Meinhard, U., and Holtfrerich, D. (1978) *Biochem. J.* 170, 313-320.
57. Glossl, J., Schubert-Prinz, R., Gregory, J.D., Damle, S.P., vonFigura, K., and Kresse, H. (1983) *Biochem. J.* 215, 295-301.
58. Schmidt, A., Buddecke, E. (1985) *Eur. J. Biochem.* 153, 269-273.
59. Smedsrod, B., Kjellen, L. and Pertoft, H. (1985) *Biochem. J.* 229, 63-71.
60. Kresse, H., von Figura, K., Buddecke, E., and Fromme, H.G. (1975) *Hoppe-Seyler's Physiol. Chem.* 356, 929-941.
61. Cowles, E.A., Brauker, J.H., and Anderson, R.L., (1986) *Exp. Cell Res.* (In press).
62. Olivecrona, T., Bengtsson, G., Marklund, S.-E., Lindahl, U., and Hood, M. (1977) *Fed. Proc.* 36, 60-65.



63. Brown, W.V., Wang-Iverson, P., and Paterniti, J.R. (1981) In Chemistry and Biology of Heparin (Lundblad, W.V., Brown, K. G., Mann, H. and Roberts, R., ed.) Elsevier/North Holland, Amsterdam. pp. 175-185.
64. Cheng, C.-F., Oosta, G.M., Bensadoun, A., and Rosenberg, R.D. (1981) *J. Biol. Chem.* 256, 12893-12898.
65. Shimada, K., Gill, P.-J., Silbert, J.E., Douglas, W.H.J., and Fanburg B.L. (1981) *J. Clin. Invest.* 68, 995-1002.
66. Busch, C., Dawes, J., Pepper, D.S., and Wasteson, A. (1980) *Thromb. Res.* 19, 129-135.
67. Fransson, L.-A., Carlstedt, I., Coster, L, and Malmstrom, A. (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81, 5657-5661.
68. Fransson, L.A., Coster, L., Carlstedt, I., and Malmstrom, A. (1985) *Biochem. J.* 231, 683-687.
69. Schnieder, C., Sutherland, R., Newman, R., and Greaves, M. (1982) *J. Biol. Chem.* 257, 8516-8522.
70. Fedarko, N.S., and Conrad, H.E. (1986) *J. Cell Biol.* 102, 587-599.
71. Arnold, E.A., Yawn, D.H., Brown, D.G., Wyllie, R.C., and Coffey, D.S. (1972) *J. Cell Biol.* 53, 737-757.
72. Dynan, W.S., and Burgess, R.R. (1981) *Biochemistry* 18, 4581-4588.
73. Furakawa, K., and Bhavanandan, V.P. (1983) *Biochim. Biophys. Acta* 740, 466-474.
74. Kovacs, J., Frey, A., and Seifert, K.H. (1981) *Biochem Int.* 3, 645-653.
75. Kraemer, R.J., and Coffey, D.S. (1970) *Biochim. Biophys. Acta* 224, 553-567.
76. Hara, T., Takahashi, K., and Endo, H. (1981) *FEBS lett.* 128, 33-36.
77. Ishii, K., Katase, A., Andoh, T., and Seno, M. (1982) *Biochem. Biophys. Res. Comm.* 104, 541-547.
78. Caputo, C.B., MacCallum, D.K., Kimura, J.H., Schrode, J., and Hascall, V.C. (1980) *Arch. Biochem. Biophys.* 204, 220-233.



79. Galloway, W.A., Murphy, G., Sandy, J.D., Gavrilovic, J., Cawston, T.E. and Reynolds, J.J. (1983) *Biochem. J.* 209, 741-752.
80. Heinegard, D., and Hascall, V.C. (1974) *J. Biol. Chem.* 249, 4250-4256.
81. Roughly, P.J. (1977) *Biochem. J.* 167, 639-646.
82. Handley, C.J., and Lowther, D.A. (1977) *Biochim. Biophys. Acta* 500, 132-139.
83. Sandy, J.D., Brown, H.L.G., and Lowther, D.A. (1978) *Biochim. Biophys. Acta* 543, 536-544.
84. Tyler, J.A. (1985) *Biochem. J.* 225, 493-507.
85. Yanagishita, M., and Hascall, V.C. (1984) *J. Biol. Chem.* 259, 10270-10283.
86. Klein, U., Kresse, H., and von Figura, K. (1976) *Biochem. Biophys. Res. Comm.* 69, 158-166.
87. Oosta, G.M., Favreau, L. V., Beeler, D.L., and Rosenberg, R.D. (1982) *J. Biol. Chem.* 257, 11248-11255.
88. Wasteson, A., Hook, M. and Westermark, B. (1976) *FEBS lett.* 64, 218-221.
89. Yahalom, J., Elder, A., Fuks, Z., and Vlodaysky, I. (1984) *J. Clin. Invest.* 74, 1842-1849.
90. Naparstek, Y., Cohen, I.R., Guks, Z., and Vlodaysky, I. (1984) *Nature (London)* 310, 241-244.
91. Hook, M., Wasteson, A., and Oldberg. A. (1975) *Biochem. Biophys. Res. Comm.* 67, 1422-1428.
92. Klein, U., and von Figura, K. (1976) *Biochem. Biophys. Res. Comm.* 73, 569-576.
93. Klein, U., and von Figura, K. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* 360, 1465-1471.
94. Glaser, J.H., and Conrad, H.E. (1979) *J. Biol. Chem.* 254 2316-2325.
95. Takagaki, K., Nakamura, T., Majima, M., and Endo, M. (1985) *FEBS lett.* 181, 271-274.
96. Hoffman, P., Meyer, K., and Linker, A. (1956) *J. Biol. Chem.* 219, 653-663.

97. Meyer, K., and Rapport, M.M. (1950) Arch. Biochem. 27, 287-293.
98. Klein, U., and von Figura, K. (1980) Biochim. et Biophys. Acta 630, 10-14.
99. Arbogast, B., Hopwood, J.J., and Dorfman, A. (1975) Biochem. Biophys. Res. Comm. 67, 376-382.
100. Sly, W.S. (1980) In Metabolic Control and Disease (Brady P.K., and Rosenberg, L.E., ed.) W.B. Saunders, Philadelphia, pp. 545-581.
101. von Figura, K., and Klein, U. (1981) In Lysosomes and Lysosomal Storage Diseases (Callahan J.W., and Lowden, J.A., ed.) Raven Press, New York. pp. 229-248.
102. McKusick, V.A., Neufeld, E.F., and Kelly, T.E. (1983) In The Metabolic Basis of Human Disease (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., and Brown, M.S., ed.) McGraw-Hill pp.751-777.
103. Neufeld, E.F., and Fratantoni, J.C. (1970) Science 169, 141-146.
104. Neufeld, E.F., and Timple, W.L., and Shapiro, L.J. (1975) Ann. Rev. Biochem. 44, 357-376.
105. Skudlarek, M.D., Novak, E.K., and Swank, R.T. (1984) In lysosomes in Biology and Pathology (Dingle, J.T., Dean, R.T., and Sly, W., ed.) Elsevier, New York. pp. 17-43.
106. Kresse, H., and Neufeld, E.F. (1972) J. Biol. Chem. 247, 2164-2170.
107. Kresse, H. (1973) Biochem. Biophys. Res. Comm. 54, 1111-1118.
108. Matalon, R., and Dorfmen, A. (1974) J. Clin. Invest. 54, 907-912.
109. O'Brien, J.S. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1720-1722.
110. von Figura, K., and Kresse, H. (1972) Biochem. Biophys. Res. Comm. 48, 262-269.
111. Kresse, H., von Figura, K., and Klein, U. (1978) Eur. J. Biochem. 92, 333-339.
112. Klein, U., Kresse, H., and von Figura, K. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5185-5188.

113. Kresse, H., Paschke, E., von Figura, K., Gilberf, W., Fuchs, W. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 6822-6826.
114. Hickman, S., and Neufeld, E.F. (1972) Biochem. Biophys. Res. Comm. 49, 992-999.
115. Neufeld, E.F., and Mckusick, V.A., (1983) In The Metabolic Basis of Human Disease (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., and Brown, M.S., ed.) McGraw-Hill. pp 778-787.
116. Miller, A.L., Kress, B.C., Lewis, L., Stein, R., and Kinnon, C. (1980) Biochem. J. 186, 971-975.
117. Kaplan, A., Achord, D.T., and Sly, W.S. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2026-2030.
118. Hasilik, A., Rome, L.H., and Neufeld, E.F. (1979) Fed. Proc. 38, 467.
119. Natowitz, M.R., Chi. M.-M., Lowry, O.H., and Sly, W.S. (1979) Proc. Natl. Acad. Sci. 76, 4322-4326.
120. Sahagian, G.G., Distler, J., and Jourdian, G.W. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 4289-4293.
121. Steiner, A.W., and Rome, L.H. (1982) Arch. Biochem. Biophys. 214, 681-687.
122. Fischer, H.D., Creek, K.E., and Sly, W.S. (1982) J. Biol. Chem. 257, 9938-9943.
123. Reitman, M.L., Varki, A., and Kornfeld, S. (1981) J. Clin. Invest. 67, 1574-1579.
124. Schmickel, R.D., Distler, J.J., and Jourdian, G.W. (1975) J. Lab. Clin. Med. 86, 672-682.
125. Neufeld, E.F., Sando, G.N., Garvin, A.J., and Rome, L.H. (1977) J. Supra. Struct. 6, 95-101.
126. von Figura, K., and Weber, E. (1978) Biochem. J. 176, 943-956.
127. Vladutiu, G.D., and Rattazze, M. (1979) J. Clin. Invest. 63, 595.
128. Gonzalez-Noriega, A., Grubb, J.H., Talkad, V., and Sly, W.S. (1980) J. Cell Biol. 85, 839-852.
129. Rome, L.H., Weissman, B., and Neufeld, E.F. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2331-2334.

130. Fischer, H.D., Gonzalez-Noriega, A., and Sly, W.S. (1980) J. Biol. Chem. 255, 5069.
131. Dean, R.T., and Barrett, A.J. (1976) Essays Biochem. 12, 1-40.
132. Roff, C.F., Wozniak, R.W., Blenis, J., and Wang, J.L. (1983) Exp. Cell Res. 144, 333-344.
133. Roff, C.F., and Wozniak, R.W. (1982) In Extracellular Matrix (Hawkes, S.P., and Wang, J.L., ed.) Academic Press, New York. pp. 329-333.
134. Brauker, J.H., Roff, C.F., and Wang, J.L. (1986) Exp. Cell Res. 164, 115-126.



Chapter 2

The Effect of Mannose 6-phosphate on the Turnover  
of the Proteoglycans in the Extracellular  
Matrix of Human Fibroblasts

James H. Brauker, Calvin F. Roff and John L. Wang

Department of Biochemistry  
Michigan State University  
East Lansing, MI 48824



## SUMMARY

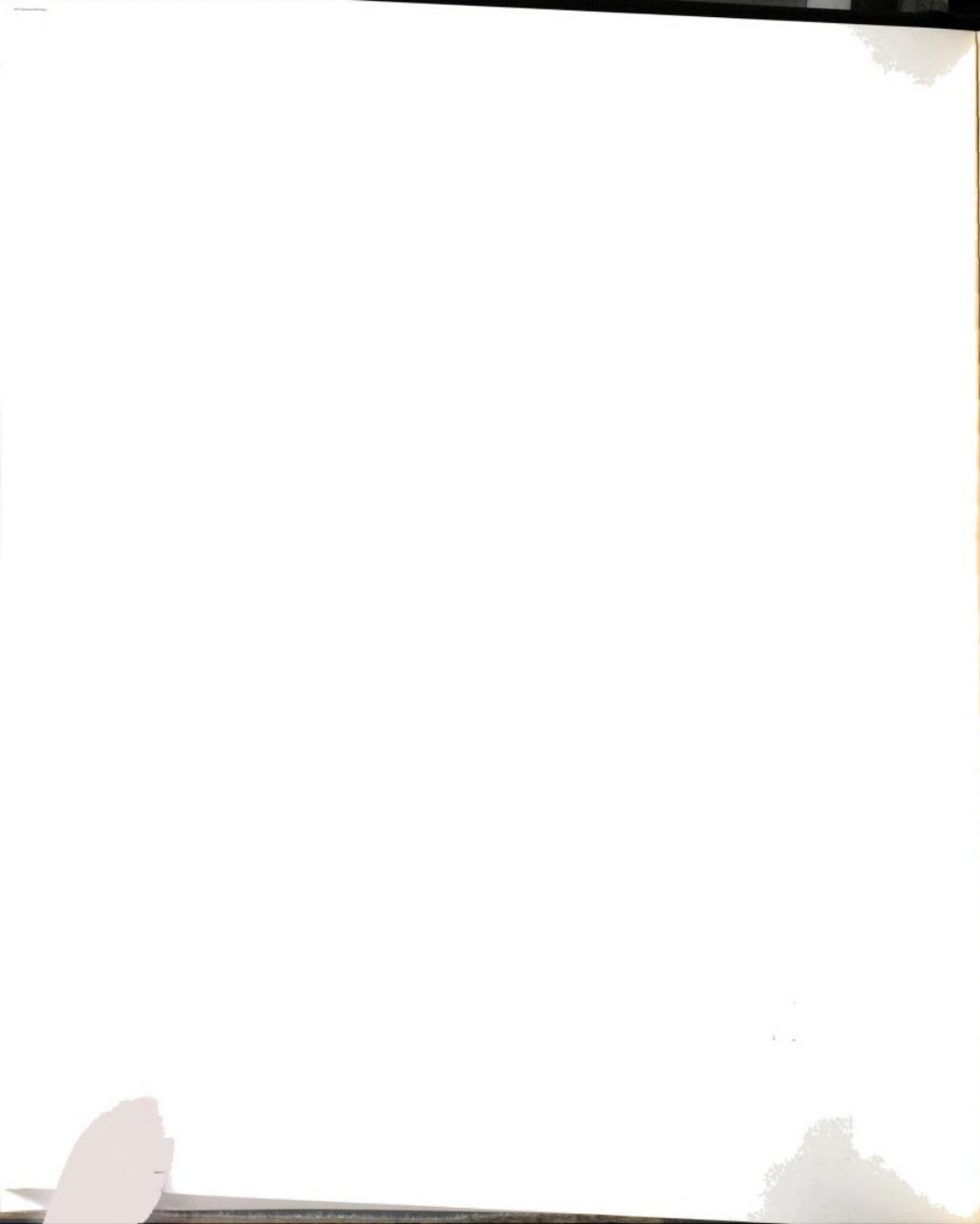
Human fibroblasts (SL66) were cultured in medium containing  $^{35}\text{SO}_4^{2-}$  to label the glycosaminoglycans. The cells were then detached from the culture dish to leave radioactively-labeled components of the extracellular matrix, hereafter termed  $^{35}\text{S}$ -labeled substrate-attached material. When unlabeled SL66 fibroblasts were plated onto this  $^{35}\text{S}$ -labeled substrate-attached material, the cells mediated two distinct events: (a) release of radioactivity from the substrate-attached material into the medium; and (b) degradation of certain glycosaminoglycans into radioactive components of very low molecular weight, including free radioactive sulfate. In the presence of mannose 6-phosphate, however, the degradation of the substrate-attached material by SL66 cells was partially inhibited. Analyses of this effect in terms of the dose-response curve, saccharide specificity, ammonium chloride sensitivity, and the requirement for cells suggest that both an intracellular compartment and the mannose 6-phosphate receptor that binds lysosomal enzymes at the cell surface may play important roles in the turnover and degradation of certain proteoglycans in substrate-attached material.



## INTRODUCTION

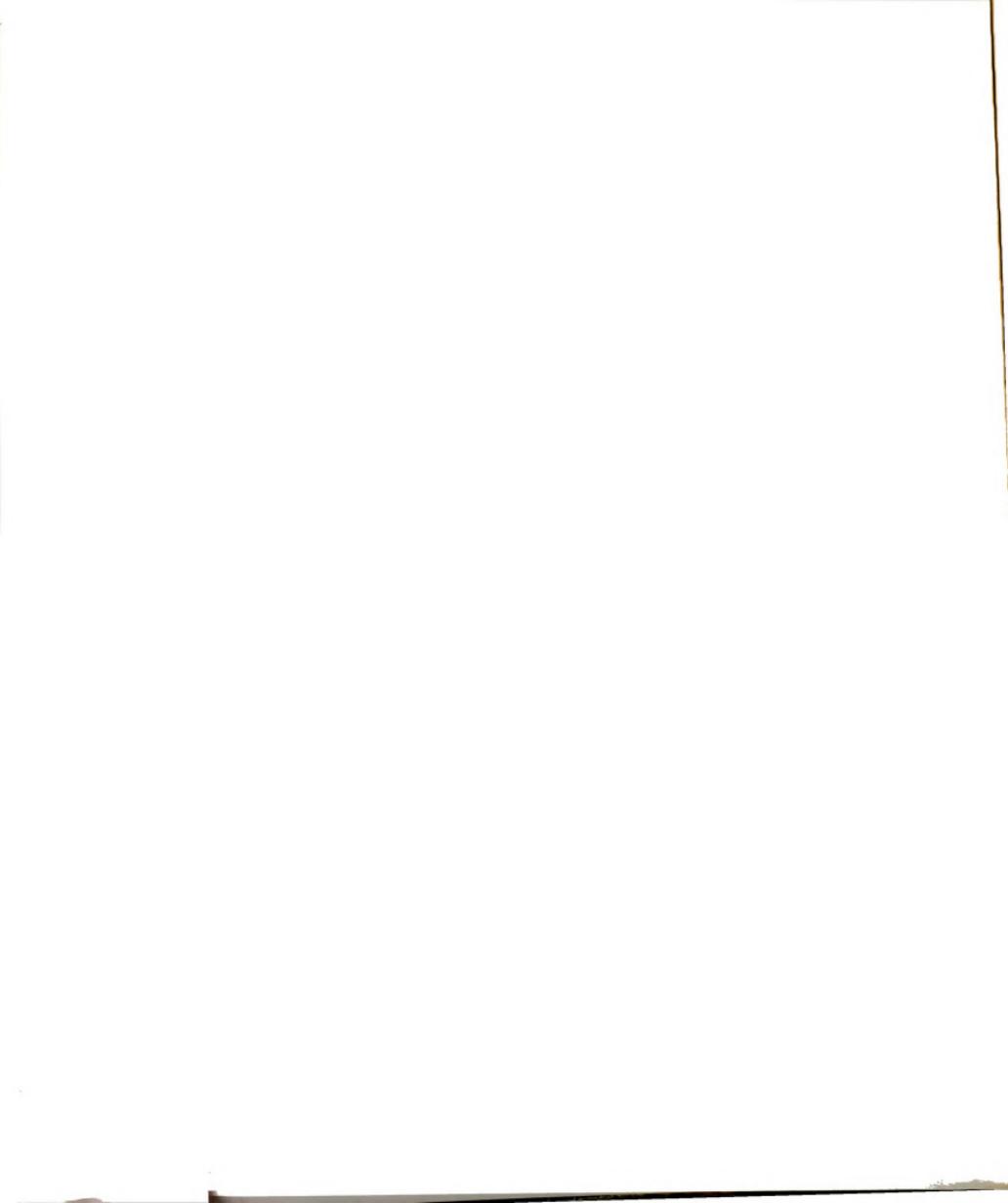
Fibroblasts adhere to tissue culture substrata at certain regions of the cell undersurface called "foot pads" (1). Upon cell movement over the substratum, these foot pads are pinched off from the posterior of the cell and remain bound to the substratum. Culp and co-workers showed that the chemical components of adhesion sites from 3T3 fibroblasts could be isolated as substratum attached material (SAM) upon EGTA-mediated detachment of the cells (2-4). Biochemical analysis of the 3T3 cell adhesive material indicates that it is composed of the glycosaminoglycans (GAGs) heparan sulfate, chondroitin sulfate, and hyaluronic acid, as well as cytoskeletal proteins and cellular fibronectin. Both the quantity and the types of proteoglycans found in SAM change with time in cell culture, possibly as a result of cell movement and reattachment of new adhesion sites (5,6). Therefore, the turnover and metabolism of the GAGs may play an important role in determining the anchorage and motility of cells.

Although it is generally thought that the primary site of the breakdown of the GAGs is the lysosome (7), some evidence has accumulated that partial degradation of the GAGs might occur in the extracellular space. Lark and Culp have reported the existence of a low molecular weight, single chain heparan sulfate in the SAM of cultured Balb/cSVT2 cells (6). It has also been reported that B16 melanoma cells release heparan sulfate from an isolated extracellular matrix as fragments

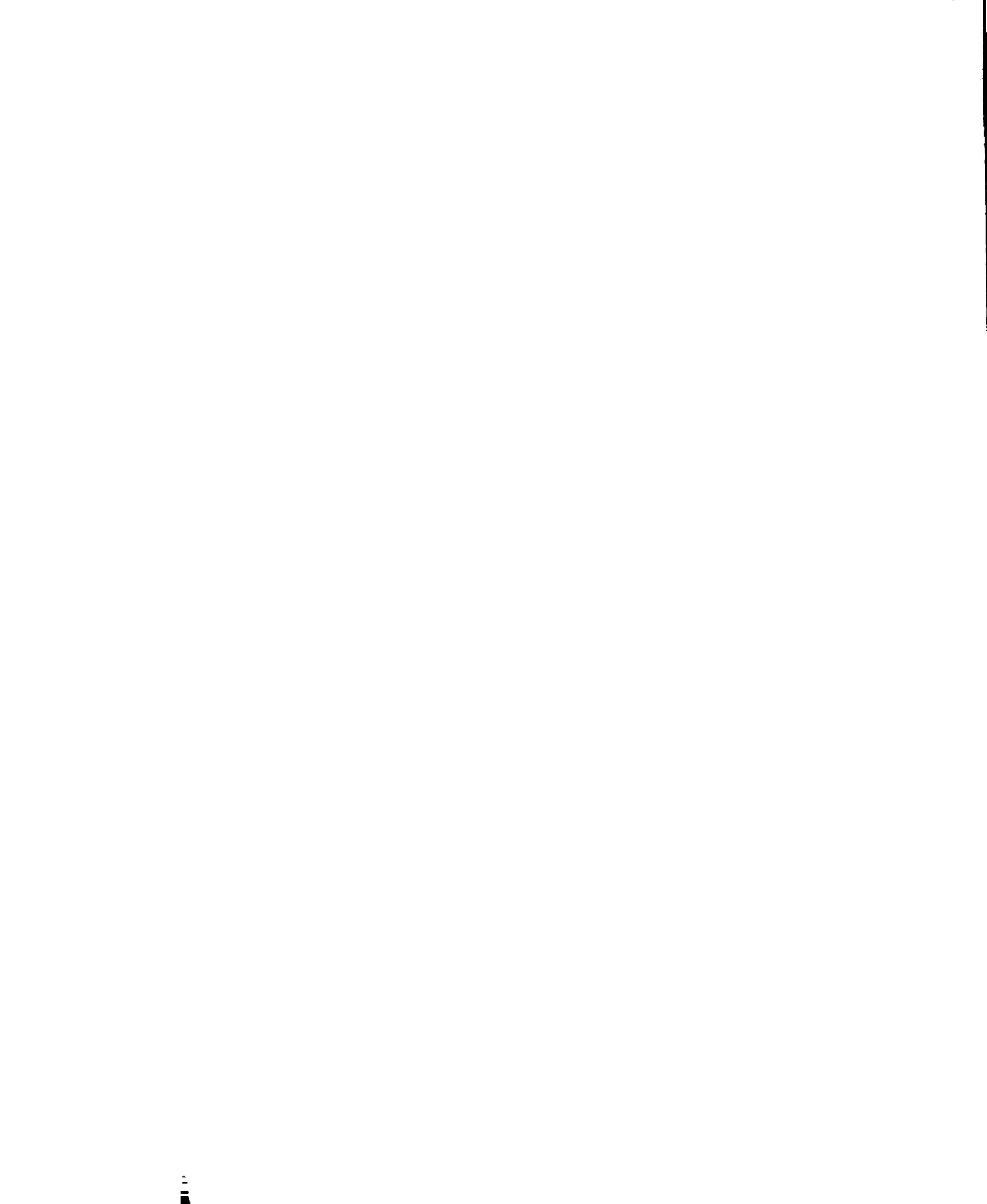


approximately one-third their original size (8). Apparently, this degradation is extra-lysosomal since passage of the GAGs through the lysosome would be expected to result in complete degradation of the molecules. This process is nevertheless thought to involve lysosomal enzymes inasmuch as the pH optimum for this activity was found to be approximately pH 5. Extracellular acid hydrolase activity is not an unprecedented idea; several tumor cell lines have been shown to secrete active lysosomal enzymes (9-12).

Recent work by several laboratories has clearly shown that the cellular distribution of membrane bound lysosomal enzymes includes the cell surface (13-15). Moreover, it has been shown that these enzymes contain D-mannopyranoside 6-phosphate (M6P) residues (16-18) and can bind to a specific carbohydrate binding protein at the cell surface called the mannose 6-phosphate receptor (19,20). In previous studies, we had observed that treatment of normal human fibroblasts with 10 mM M6P decreased the turnover of sulfated GAGs from the cell surface (pericellular compartment) into the surrounding fluid medium (extracellular compartment) of the culture dish (21,22). It was suggested that normal turnover of some pericellular GAGs requires cell surface associated lysosomal enzymes and that dissociation of lysosomal enzymes by M6P caused an increased retention of GAGs at the cell surface. Since the labeling conditions of these experiments did not differentiate between plasma membrane and SAM associated GAGs, the present effort was undertaken to determine if the turnover of sulfated GAGs in SAM is affected when lysosomal enzymes are dissociated from the cell surface by M6P. The data presented in the present communication show that M6P



inhibits the degradation by human fibroblasts of a fraction of the sulfated GAGs in isolated SAM.



## MATERIALS AND METHODS

### Chemicals

Carrier free  $H_2^{35}SO_4$  was obtained from ICN. All monosaccharides were purchased from Sigma and were of the D-configuration. Phosphorylated sugars were purchased as mono- or disodium salts. Pronase was from Calbiochem-Behring Corp. EGTA was from Sigma.

### Cell Lines and culture conditions

The normal human foreskin fibroblast cell line SL66 (23) was a gift of Dr. V. Maher and Dr. J. McCormick (Carcinogenesis Laboratory, Michigan State University). Fibroblast lines from mucopolidosis II subjects (GM 2933, I-cells) were from the Human Mutant Cell Repository. All cells were maintained in Eagle's MEM (K.C. Biologicals) with 20% fetal bovine serum (FBS, Gibco), 100 ug/ml streptomycin (Sigma), and 159 IU/ml penicillin (Sigma), at 35°C in humidified air containing 5% CO<sub>2</sub>. Stock SL66 cells were not allowed to reach confluence and medium was changed two times per week. Medium for the GM 2933 cell line was changed every two days and was supplemented with two times the normal concentrations of amino acids and vitamins. GM 2933 cells were split 1:2 every 6-9 days. Cells were used between passages 10 and 30.



### Preparation of Radiolabeled SAM

Procedures for preparation and digestion of labeled SAM are illustrated in fig. 1. Cultures were labeled by seeding  $1.0 \times 10^6$  cells in streptomycin-free MEM (20% FBS, 40-50 uCi/ml  $H_2^{35}SO_4$ ), with  $MgCl_2$  substituted for  $MgSO_4$ , onto 100 mm tissue culture dishes. When M6P was included in the radiolabeling medium, the medium was brought to 10 mM M6P by addition of concentrated M6P in MEM. Some experiments reported here used SAM prepared in the presence of M6P; similar results were obtained irrespective of whether the SAM was prepared in the presence or absence of the saccharide. After 72 hours, the radiolabeling medium was removed, the monolayers were washed two times with 5 ml of 5 mM EGTA in phosphate buffered saline (PBS), and incubated for 30-45 minutes in 10 ml of the 5 mM EGTA in PBS on a rotary shaker (60 cycles/minute) at 35°C to remove the cells. Undetached cells were removed by gentle pipeting. The isolated SAM was washed two times with 5 ml of 5 mM EGTA in PBS, followed by two washes with 5 ml sterile, glass distilled water to lyse any remaining cells, and stored at 35°C in 5 ml MEM for not more than 30 minutes.

### Digestion of SAM by Unlabeled Cells or Conditioned Medium

Unlabeled cells used to digest SAM were removed from stock flasks by adding EDTA (.02% in PBS) for 10 minutes followed by 10 minutes in 0.04% trypsin (Gibco). After the cells were completely detached, the trypsin solution was brought to 20% FBS by addition of FBS and centrifuged for 3 minutes at 1330 X g. The cell pellet was suspended in a small volume of MEM containing 5% FBS and recentrifuged. The pellet was resuspended in MEM and  $3.0 \times 10^6$  cells were added to each dish of

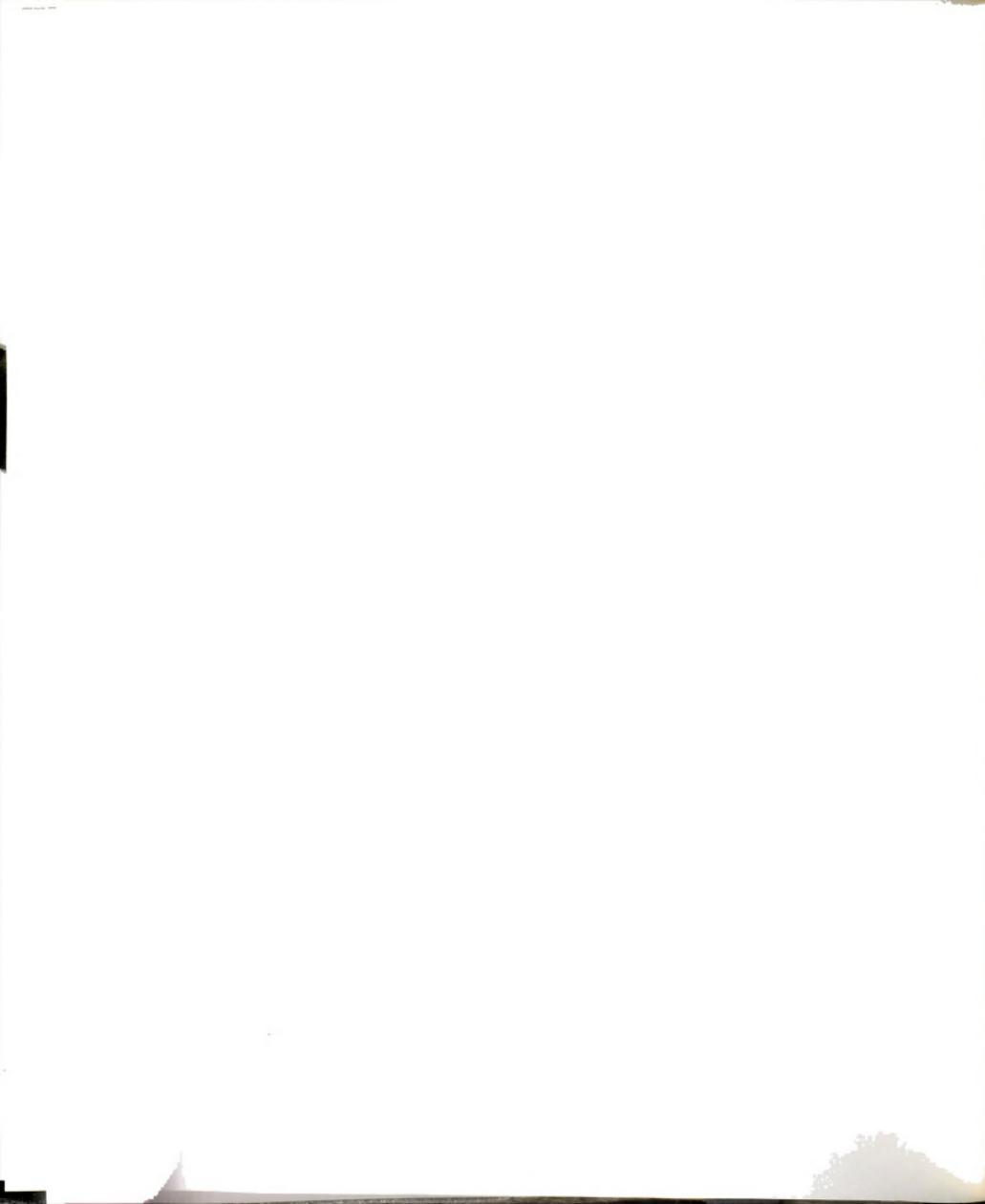
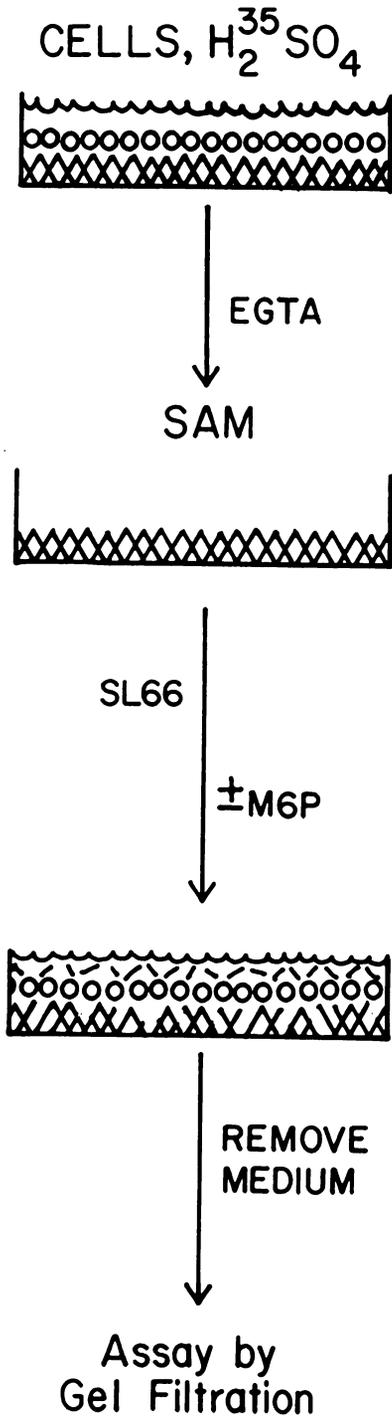
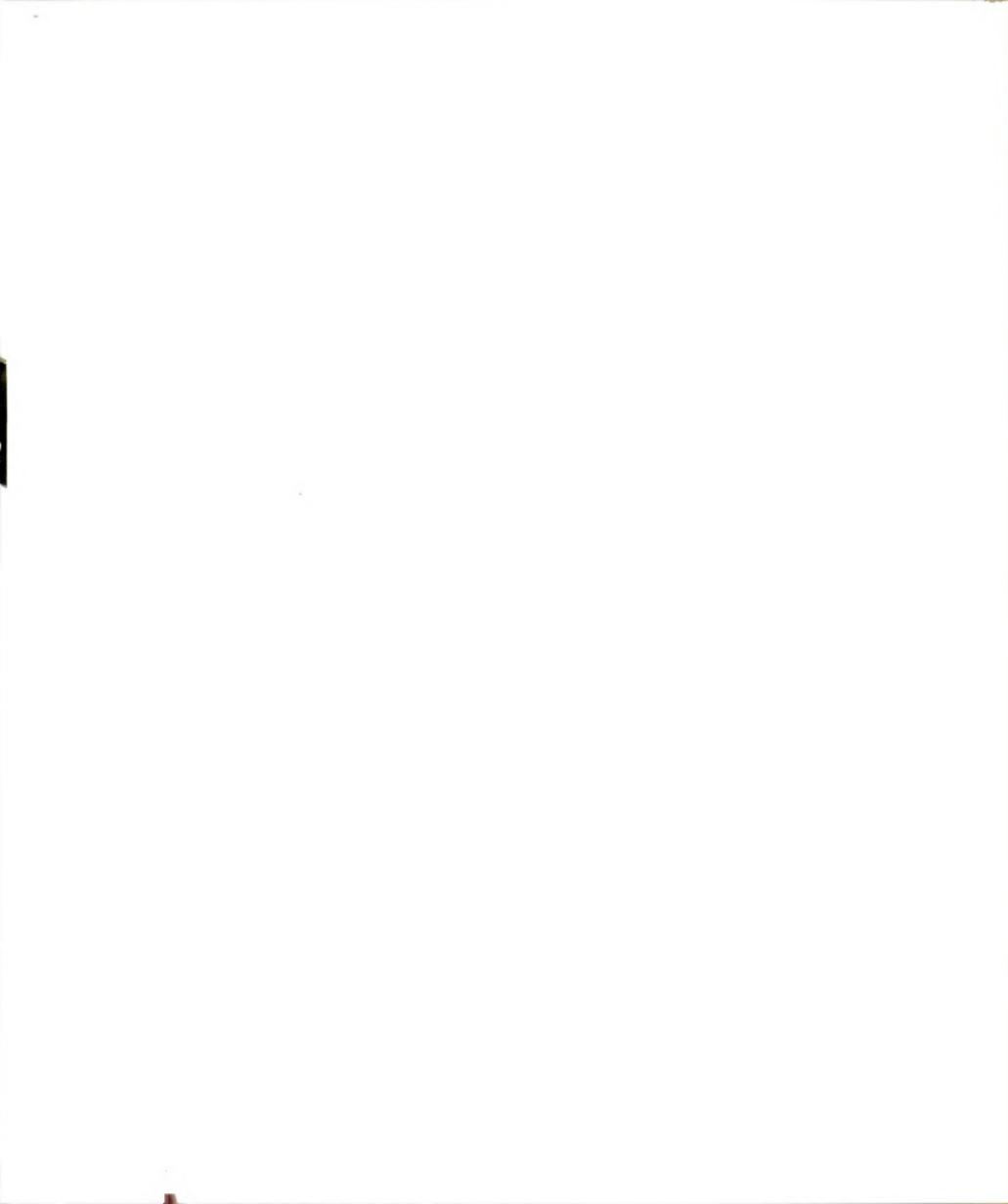




Figure 1: Protocol for preparation of radiolabeled SAM and subsequent digestion by SL66 cells. Cells ( $1 \times 10^6$ ) were incubated with 40-50  $\mu\text{Ci H}_2^{35}\text{SO}_4$  for 72 hours and then detached with EGTA. Unlabeled cells were plated on the radiolabeled SAM in the presence or absence of M6P. After 24 hours, the medium was collected and analyzed by column chromatography.





isolated SAM in 4 ml MEM (without FBS). Saccharides and  $\text{NH}_4\text{Cl}$  were prepared as stock solutions in MEM at 50 mM or 5 mM concentrations, passed through 0.45  $\mu\text{m}$  filters (Millipore), and added to the culture dish to give the desired final concentration in a total volume of 5 ml. After completion of the digestion period, the soluble radioactivity was harvested for analysis by gel filtration (fig. 1).

Medium was conditioned by exposure to confluent monolayers of SL66 fibroblasts. Cells were grown to confluence in 150  $\text{cm}^2$  flasks in 20 ml MEM containing 20% FBS. The medium was removed and 10 ml of fresh medium was added. After 24 hours, the medium was decanted into 10 ml plastic centrifuge tubes and centrifuged for 3 minutes at 1330 X g to remove cellular debris. This preparation was called conditioned medium (CM) and was used to digest  $^{35}\text{S}\text{O}_4$ -labeled SAM in the absence of cells. Five ml of the CM were added to [ $^{35}\text{S}$ ]SAM for 24 hours after which the solubilized radioactivity was analyzed by gel filtration.

#### Harvest and Bulk Quantitation of Radioactivity

The culture dishes were divided into three compartments called the SAM, cellular, and soluble compartments. The soluble material was defined as the radioactivity removable with the medium. The cellular compartment was defined as the radioactivity in the EGTA solution used to remove the cells. The SAM was defined as the radioactivity left on the dish after removal of the cells by EGTA. SAM was harvested with 0.2% sodium dodecyl sulfate (SDS, Sigma) in glass distilled water. Alternatively, the SAM and cellular compartments were harvested together using 0.2% SDS and 0.05 M NaOH in glass distilled water, and called the insoluble compartment. Total radioactivity consisted of soluble radio-



activity plus insoluble (cellular and SAM) radioactivity. Aliquots from each fraction were solubilized at a ratio of 1:9 (sample: scintillation cocktail) in scintillation fluid consisting of 7 g 2,5-diphenyloxazole (Research Products International Corp.), 338 ml Triton X-100 and 667 ml toluene. Radioactivity was determined on a Packard Tricarb 300C liquid scintillation counter and expressed as counts per minute (cpm) or disintegrations per minute (dpm).

#### Quantitation of Degraded Material

The soluble fraction was harvested, boiled for 5 minutes, and stored at  $-20^{\circ}\text{C}$  until analyzed. After thawing, 1.5 ml of the soluble fraction were boiled for 1 minute; 0.5 ml of 0.6 M Tris, pH 7.4, 0.8% SDS and 4 mM  $\text{NaN}_3$  were added and the sample boiled for 1 minute more. After cooling, 0.5 ml were diluted in 4.5 ml of scintillation cocktail and counted to determine total dpm applied to the column. The remaining 1.5 ml were loaded on a Sephadex G-25 column (1.3 X 35 cm) equilibrated with 0.15 M Tris, pH 7.4, 0.2% SDS and 1 mM  $\text{NaN}_3$ . Fractions of 1.0 ml were collected directly in scintillation vials. The radioactivity in each fraction was determined by liquid scintillation counting. The radioactivity that was not excluded from the column was called degraded [ $^{35}\text{S}$ ]proteoglycan ([ $^{35}\text{S}$ ]PG). This material eluted at the same position as free  $^{35}\text{SO}_4^{2-}$ . When free  $^{35}\text{SO}_4^{2-}$  containing a known amount of radioactivity was chromatographed in the presence of SL66 conditioned medium, greater than 95% of the radioactivity was recovered. Percent degraded [ $^{35}\text{S}$ ]PG was determined by the calculation:

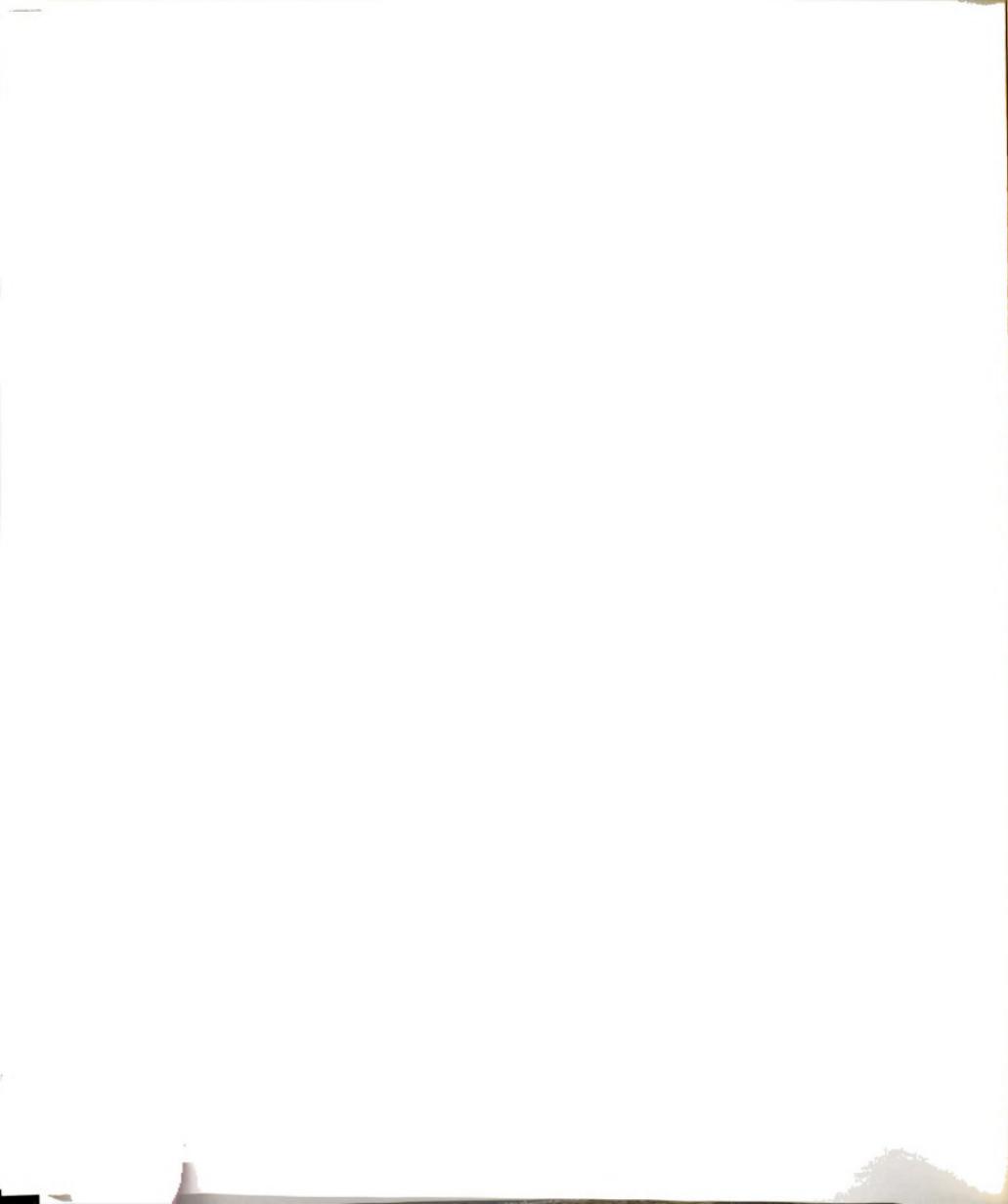
$$\% \text{ Degraded} = \frac{\text{dpm degraded}}{\text{dpm loaded}} \times 100$$



### Analysis of SAM Fractions by Column Chromatography

The SAM fraction was harvested after completion of the radiolabeling period, boiled for 5 minutes and lyophilized. The dried material was suspended in 1 ml of 0.1 M acetic acid-NH<sub>4</sub>OH, pH 5.7, 0.013 M EDTA, and 0.5 mM NaN<sub>3</sub> and the pH was adjusted to 5.7 with acetic acid. Pronase (4 mg) was added and the mixture was incubated at 50°C for 24 hours. More pronase (4 mg) was added, and the incubation was continued for an additional 24 hours. During the digestion, the pH was adjusted to 5.7 when necessary. The samples were neutralized with 1 M NaOH and stored at -20°C.

Samples were chromatographed on columns (110 x 1 cm) of Sepharose CL6B equilibrated with 0.2% SDS, 0.15 M Tris, pH 7.4, 1 mM NaN<sub>3</sub>. Fractions of 1.0 ml were collected and the radioactivity was determined by scintillation counting.



## RESULTS

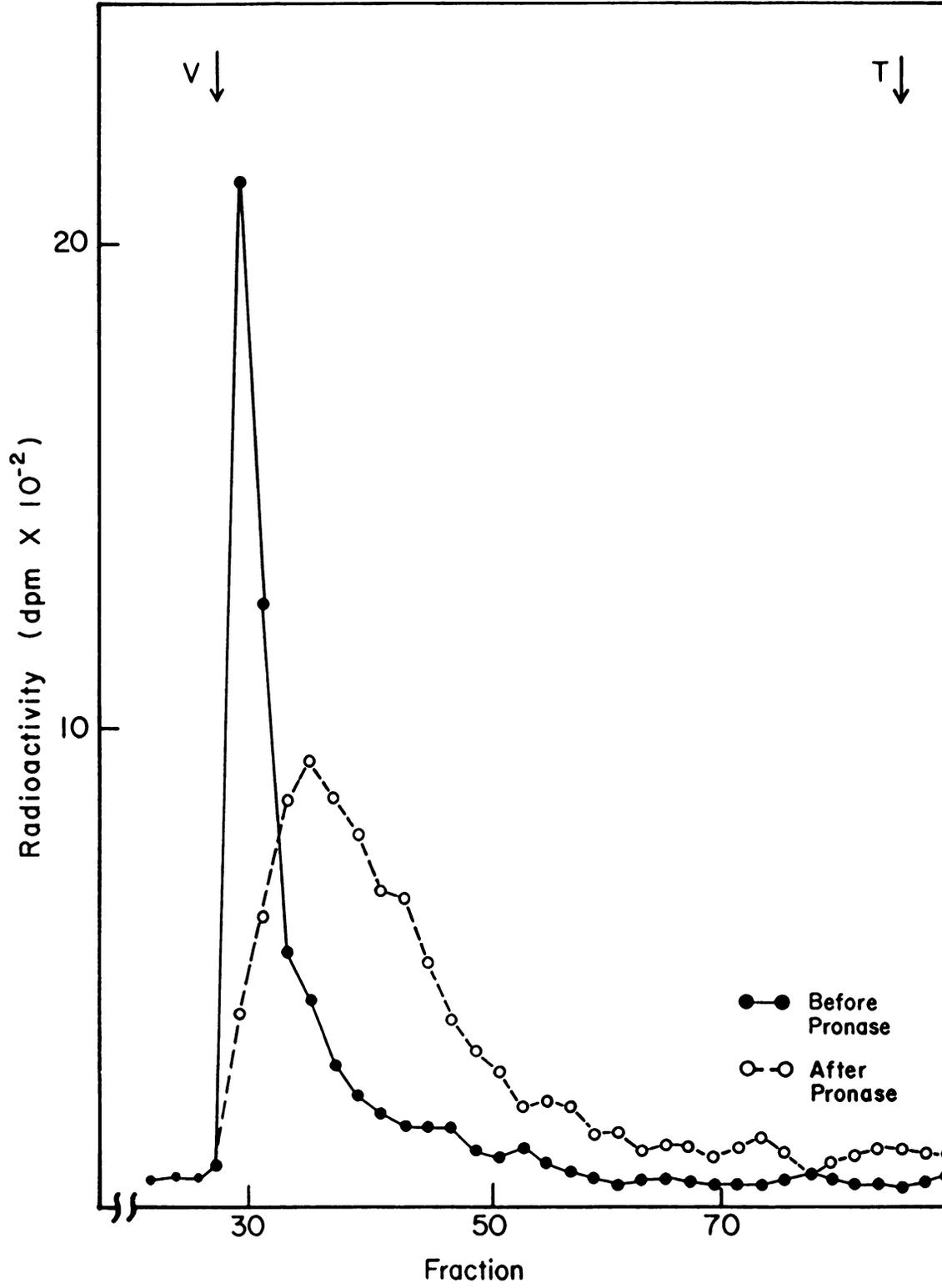
### Characterization of $^{35}\text{SO}_4$ -labeled SAM by Column Chromatography

The main goal of this study was to test the hypothesis that fibroblasts can degrade radioactive components in SAM and whether this digestion is sensitive to M6P. To determine if the turnover of components of SAM is affected by M6P, the chromatographic properties of SAM labeled with  $^{35}\text{SO}_4^{2-}$  were analyzed. Radioactive SAM produced by SL66 cells in the presence or absence of M6P was chromatographed on Sepharose CL6B columns. The chromatographic profile of SAM prepared in the presence of M6P showed a large peak of radioactivity, representing  $^{35}\text{S}$ -labeled GAGs of high molecular weight, near the void volume of the column (fig. 2). Pronase treatment of the same material resulted in a reduction of the molecular weight of the components. Therefore, the majority of the GAGs in SAM were associated with protein, presumably as proteoglycans (PG). There was little or no radioactivity eluted at the total volume of the column.

Similar chromatographic profiles were obtained when SAM prepared in the absence of M6P was analyzed on Sepharose CL6B columns. Moreover, I-cells, which produce lysosomal enzymes lacking the M6P marker required for intracellular transport and therefore are deficient in certain hydrolases in the lysosome (24), also yielded similar results.



Figure 2: Chromatographic profile of SAM components before (●—●) and after (○----○) digestion with pronase. Fibroblasts (SL66) were labeled with  $^{35}\text{SO}_4^{2-}$  for 72 hours in the presence of M6P (10 mM), after which the cells were removed using EGTA. The radioactive SAM was solubilized in 0.2% SDS. Samples were incubated with and without pronase and then analyzed on a Sepharose CL6B column (106 x 1 cm) equilibrated with 0.2% SDS in 0.15 M Tris Buffer, pH 7.4, 1 mM  $\text{NaN}_3$ . Fractions of 1.0 ml were collected. Void volume, V; total volume, T.





### Solubilization of SAM Components into the Medium

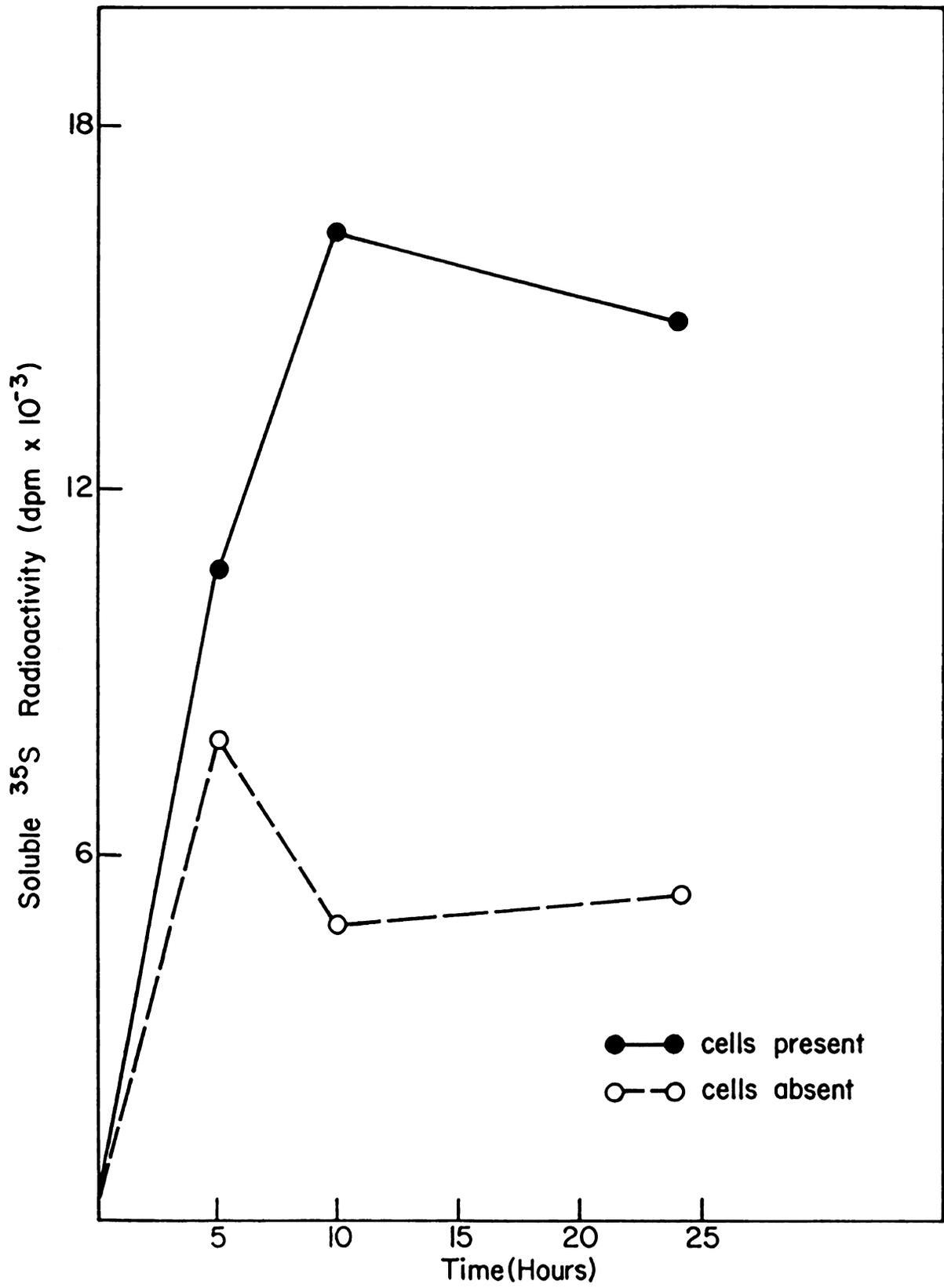
Perturbation by M6P of the turnover of PG by fibroblasts might be expected to affect the quantity or composition of molecules released from the SAM into the surrounding medium. To test this possibility,  $^{35}\text{SO}_4^{2-}$  labeled SAM was subjected to digestion by SL66 cells. At various times, the medium was harvested and the amount of bulk radioactivity released from the SAM into the medium was determined (fig. 3).

Over a period of 10 hours, SL66 cells solubilized more than 40% (15,000 dpm) of the radioactivity originally associated with SAM and released it into the medium (fig. 3). This effect required the presence of cells since only 15% (6,000 dpm) of the radioactivity can be released over the same time period when SAM was incubated with culture medium alone (MEM). After 24 hours, the level of radioactivity released by the cells did not change significantly. The kinetics of the release of radioactivity from SAM shown in fig. 3 is representative of all conditions employed. The amount of radioactivity released depended only on the presence of cells; it was not affected by the addition of saccharides.

### Column Chromatography of Solubilized SAM Components

Analysis of the solubilized SAM components by Sepharose CL6B chromatography separated the radioactivity into two distinct peaks (fig. 4). The first peak (component A) eluted at the void volume of the column ( $M_r > 1 \times 10^6$ ). This peak is believed to consist of high molecular weight PG since pronase treatment of the sample reduced the molecular weight of component A (data not shown). The second peak (component B) eluted at the total volume of the column. Several lines

Figure 3: Time course of release of radioactivity from SAM. Radiolabeled SAM was prepared as described in the legend to Figure 2. It was then treated with medium with (●—●) or without (○----○) SL66 cells and the radioactivity released into the medium was analyzed at various times as described in Materials and Methods.





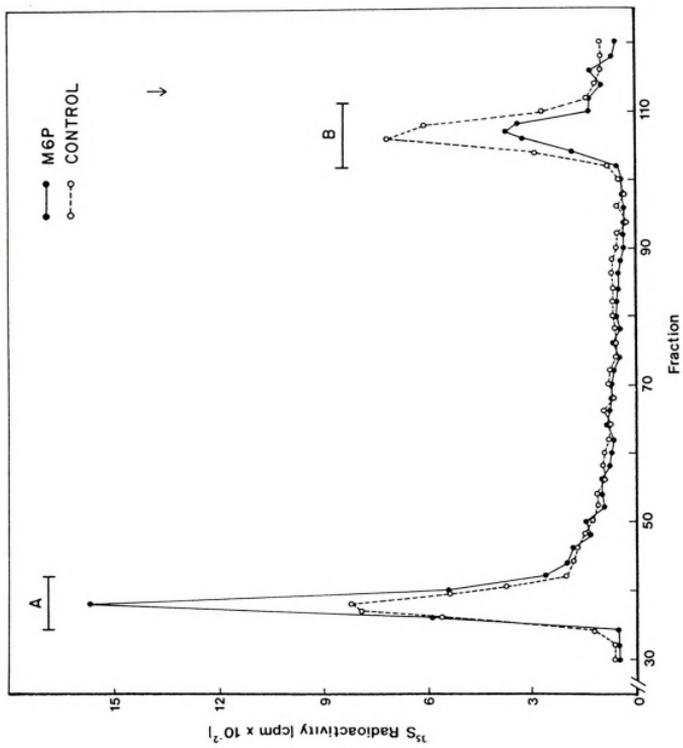
of evidence indicate that component B consists of  $^{35}\text{S}$ -labeled material of very low molecular weight, including free  $^{35}\text{SO}_4^{2-}$ . First, cetylpyridinium chloride failed to precipitate the radioactivity, indicating that the material was not polyanionic (25). Second, the radioactive material in component B was dialyzable in dialysis tubing with a molecular weight cut off limit of 12,000-14,000. Third, Sephadex G-25 columns, which are used to remove unincorporated  $^{35}\text{SO}_4^{2-}$  from preparations of metabolically labeled GAGs (26), also resolved the material released from SAM into two peaks having relative proportions identical to those observed on Sepharose CL6B columns. Finally, free  $^{35}\text{SO}_4^{2-}$  eluted at the same position as Component B on both Sephadex G-25 and Biogel P2 columns. Component B was observed only in the material released from SAM when SL66 cells are present; incubation of SAM with medium alone failed to yield this low molecular weight material (see below). Therefore, Component B represents SAM material extensively degraded by cells and will be referred to as degraded [ $^{35}\text{S}$ ]PG.

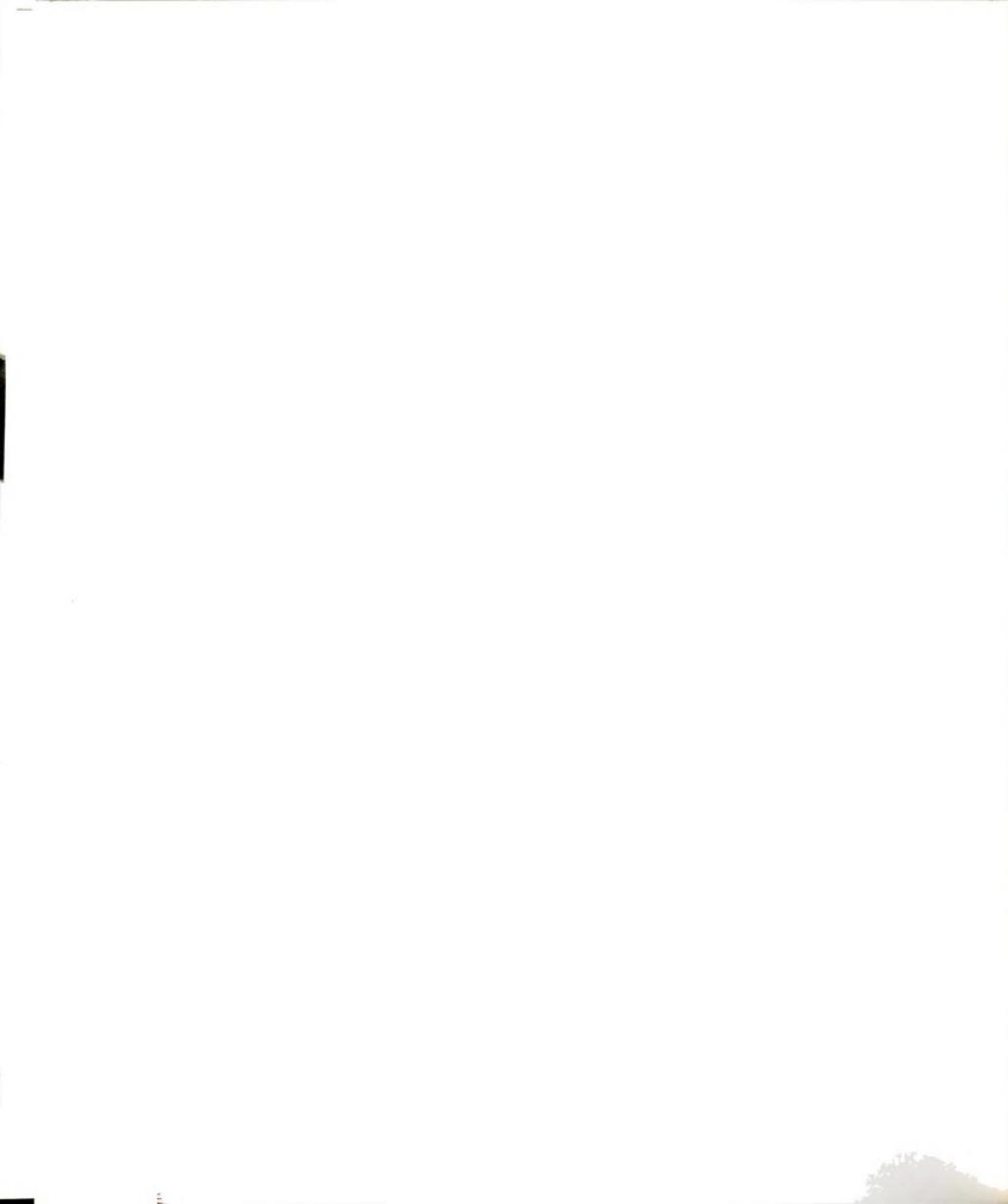
When SL66 cells were plated on labeled SAM in the absence of M6P, the amount of degraded [ $^{35}\text{S}$ ]PG was always greater than the corresponding fraction from M6P-treated cultures (fig. 4). This difference was offset by a corresponding decrease in the quantity of Component A in control cultures compared to M6P treated cultures. However, there was no net difference in total amount of radioactivity released, as pointed out previously.

Degraded [ $^{35}\text{S}$ ]PG was quantitated by analysis of the radioactive material released from SAM on Sephadex G-25 columns and is expressed as a percent of the total radioactivity loaded onto the column. Analysis of samples from 24-hour incubations showed that the material released



Figure 4: Sepharose CL6B chromatography of soluble radiolabeled material released from SAM by SL66 cells. Radiolabeled SAM was prepared and subjected to digestion by SL66 cells in the presence (●—●) or absence (o---o) of 10 mM M6P. After 24 hours the medium was removed and chromatographed on a Sepharose CL6B column (110 x 1 cm) equilibrated with 0.2% SDS, 0.15 M Tris, pH 7.4, 1 mM NaN<sub>3</sub>. Fractions of 1.0 ml were collected. The arrow indicates the total volume of the column.





from cultures without M6P contained about 20% degraded [ $^{35}\text{S}$ ]PG, whereas that from M6P treated cultures contained about 10% degraded [ $^{35}\text{S}$ ]PG (table 1, line A). This represents about a 50% inhibition in conversion of PG to low molecular weight degradation products.

#### Kinetics, Dose Response Curve and Saccharide Specificity of the M6P Effect

The amount of radioactivity found in the medium in the form of degraded [ $^{35}\text{S}$ ]PG was quantitated at various times after the addition of SL66 fibroblasts (fig. 5). A rapid increase in degraded [ $^{35}\text{S}$ ]PG was observed during the first 10 hours, followed by a slower, gradual increase. At all time points, digestion of SAM by SL66 cells in the absence of M6P always produced a higher percentage of degraded [ $^{35}\text{S}$ ]PG than digestion of SAM by the cells in the presence of M6P.

Analysis of the dose-response curve of the M6P effect on the production of degraded [ $^{35}\text{S}$ ]PG by SL66 cells showed that the effect was saturable at a saccharide concentration of 1 mM or above (fig. 6). The concentration of M6P required for half-maximal inhibition was estimated to be 60  $\mu\text{M}$ .

The saccharide specificity of the effect of M6P on the production of degraded [ $^{35}\text{S}$ ]PG from SAM by SL66 fibroblasts was studied by quantitating the percentage of degraded [ $^{35}\text{S}$ ]PG in cultures containing various saccharides (table 2). Cultures containing fructose 1,6-diphosphate and M6P showed the effect (38 and 45% inhibition, respectively) to a much greater extent than both phosphorylated (mannose 1-phosphate) and non-phosphorylated (mannose) analogs of M6P (15-19% inhibition).



---

 TABLE 1 Quantitation of degraded [ $^{35}\text{S}$ ]PG after various incubations.<sup>a</sup>


---

<u>INCUBATIONS</u>	<u>DEGRADED [<math>^{35}\text{S}</math>]PG</u>	
	<u>M6P</u>	<u>CONTROL</u>
A. SL66	9.9 $\pm$ 0.35 <sup>b</sup>	19.7 $\pm$ 1.80 <sup>b</sup>
B. MEM	0.0	0.0
C. CM	0.0	0.0
D. I-cell	1.0	4.0
E. SL66 + $\text{NH}_4\text{Cl}$	9.1 $\pm$ 2.3 <sup>b</sup>	9.9 $\pm$ 0.21 <sup>b</sup>

---

<sup>a</sup> $^{35}\text{S}$ -labeled SAM was prepared as described in materials and methods. The SAM was incubated in the presence of the various solutions for 24 hours, after which the medium was harvested and degraded [ $^{35}\text{S}$ ]PG determined by gel filtration. The concentrations of  $\text{NH}_4\text{Cl}$  and M6P were 10 mM.

<sup>b</sup>Standard deviations calculated for triplicate determinations.





Figure 5: Time course of the generation of degraded [ $^{35}\text{S}$ ]PG by SL66 cells from SAM. Radiolabeled SAM was subjected to digestion by SL66 cells for various times in the presence (●—●) or absence (○----○) of 10 mM M6P, after which the medium was removed and chromatographed on Sephadex G-25 and the % degraded [ $^{35}\text{S}$ ]PG was determined as described in Materials and Methods.

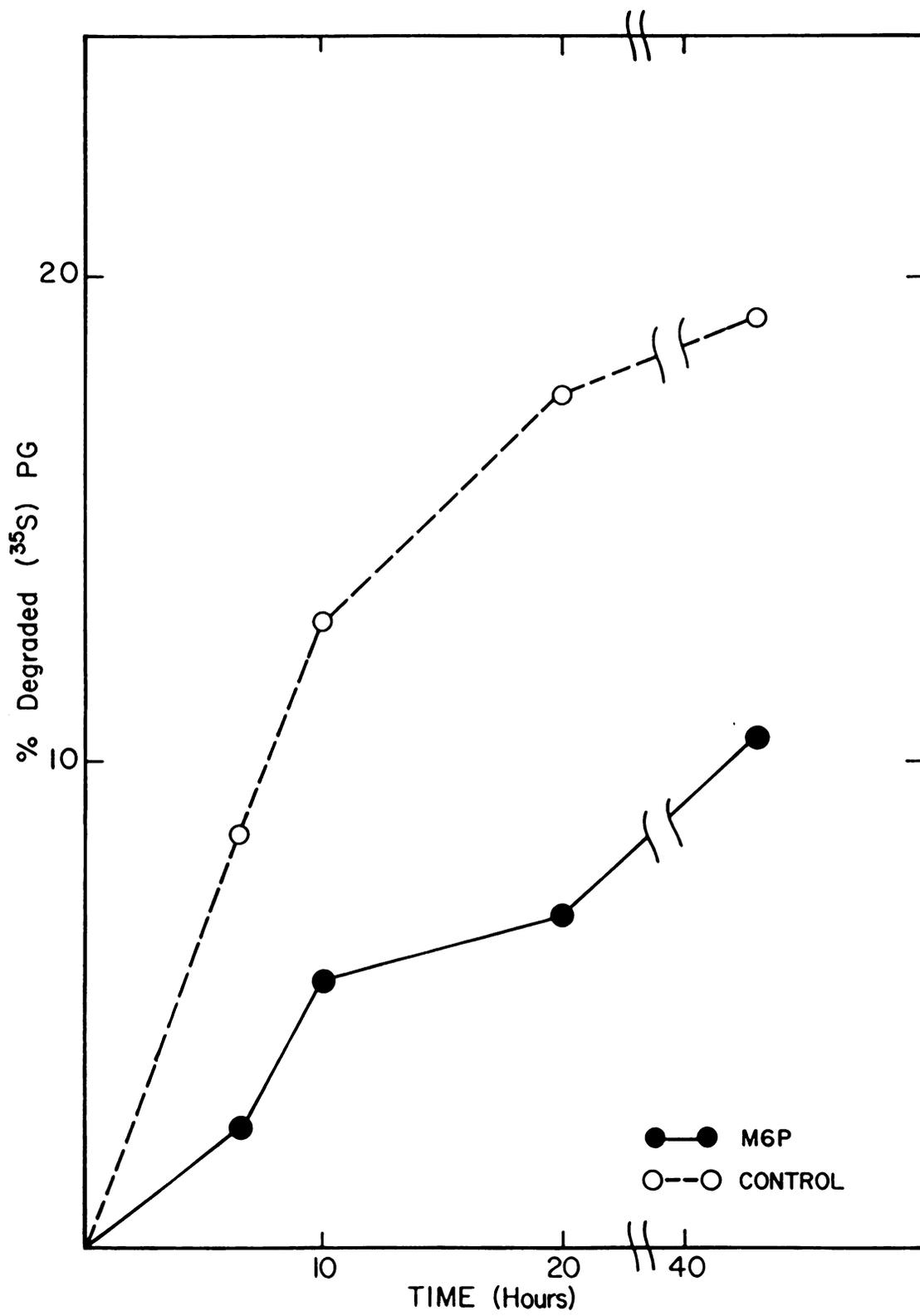


Figure 6: Dose response of M6P effect on the generation of degraded [ $^{35}\text{S}$ ]PG from SAM by SL66 cells. Radiolabeled SAM was digested by SL66 cells in the presence of various concentrations of M6P. After 24 hours, the medium was removed and chromatographed on Sephadex G-25 and the % degraded [ $^{35}\text{S}$ ]PG was determined as described in Materials and Methods.

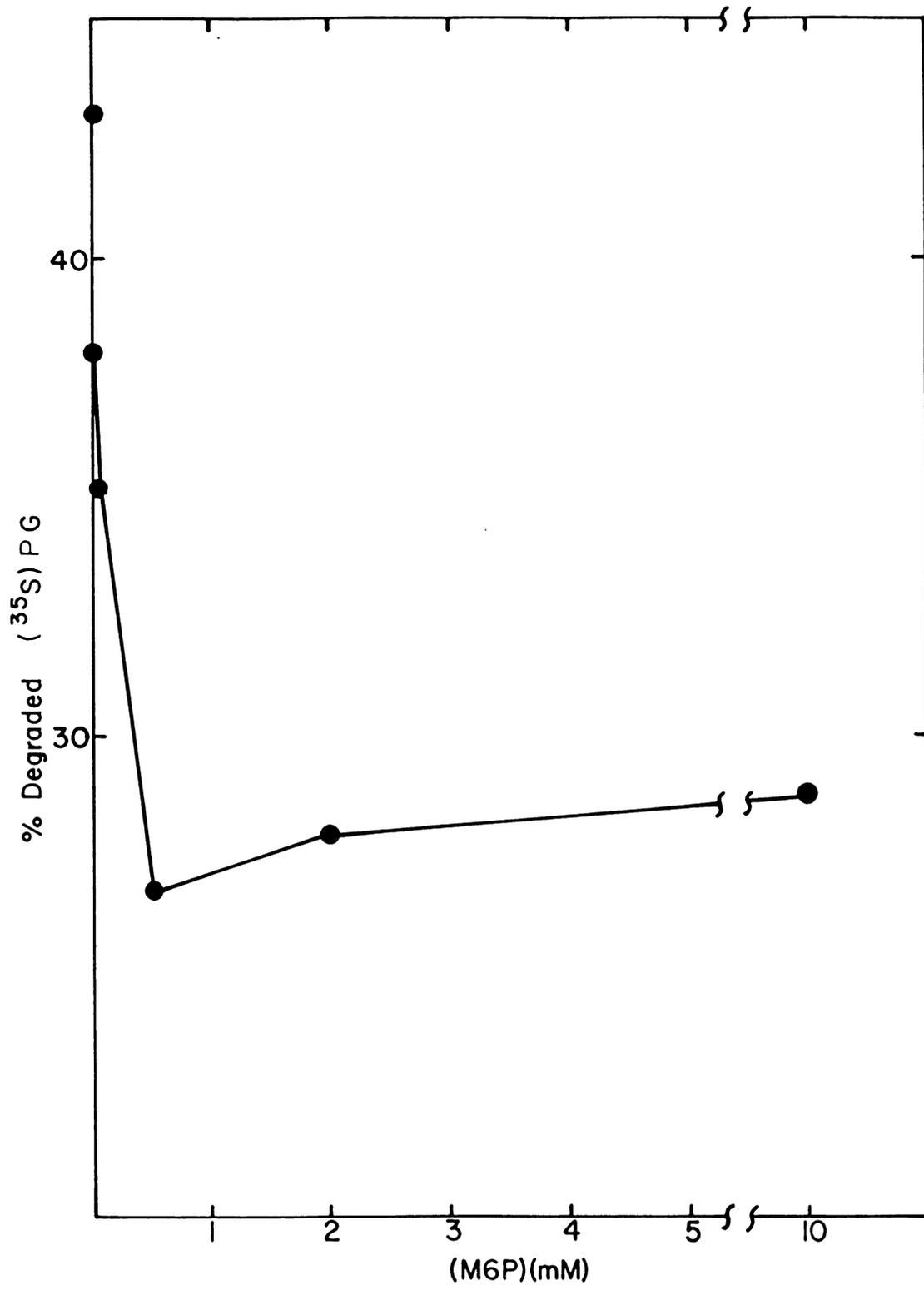




TABLE 2 Quantitation of degraded [<sup>35</sup>S]PG produced by SL66 cells in the presence of various saccharides.<sup>a</sup>

<u>Saccharides</u>	<u>Degraded [<sup>35</sup>S]PG (%)<sup>b</sup></u>	<u>% Inhibition<sup>c</sup></u>
None	24.2	-
mannose	20.6	14.8
mannose 1-phosphate	19.5	19.4
glucose 1-phosphate	19.7	18.5
fructose 1,6-diphosphate	15.0	38.0
M6P	13.4	44.6

<sup>a</sup><sup>35</sup>S-labeled SAM was prepared as described in materials and methods. The radiolabeled SAM was incubated in the presence of SL66 cells for 8 hours, after which the medium was collected and the % degraded [<sup>35</sup>S]PG determined by gel filtration. The concentration of saccharides was 1 mM.

<sup>b</sup>Mean of duplicate cultures.

<sup>c</sup>% inhibition was calculated using the formula:

$$\frac{\% \text{ degraded (saccharide)}}{\% \text{ degraded (control)}}$$



### Evidence that M6P Affects a Cell-Associated Compartment

When  $^{35}\text{S}$ -labeled SAM was incubated with culture medium alone, there was a release of radioactivity from the SAM into the medium (fig. 3). However, all of this released radioactivity consisted of high molecular weight material; no degraded [ $^{35}\text{S}$ ]PG was produced (table 1, line B). The SAM used in these experiments contained no radioactive material eluting at the position of degraded [ $^{35}\text{S}$ ]PG on Sepharose CL6B columns (fig. 2). Therefore, the lack of degraded [ $^{35}\text{S}$ ]PG after SAM had been incubated with culture medium suggests the generation of degraded [ $^{35}\text{S}$ ]PG was not due to an autolytic process within the SAM.

When  $^{35}\text{S}$ -labeled SAM was incubated with CM, about 30-40% of the total radioactivity was released into the medium in 24 hours. This is similar to the level of radioactivity released from SAM by SL66 cells. Presumably, this is the result of either enzymatic activity in the CM or the exchange with unlabeled SAM components accumulated in the CM. In any case, no degraded [ $^{35}\text{S}$ ]PG was observed under these conditions (table 1, line C). These results suggest that when SAM is incubated with SL66 cells, the resultant production of degraded [ $^{35}\text{S}$ ]PG is not due to soluble factors secreted into the medium.

When I-cells were plated on  $^{35}\text{S}$ -labeled SAM, only low amounts of degraded [ $^{35}\text{S}$ ]PG were produced (table 1, line D). The I-cells are known to lack hydrolases in the lysosomes and to have a high level of secretion of the same enzymes (24). Therefore, the present results indicate that even in the presence of elevated extracellular lysosomal enzymes, very little degraded [ $^{35}\text{S}$ ]PG is produced.

Gonzalez-Noriega et al. (27) reported that treatment of cells with  $\text{NH}_4\text{Cl}$  caused elevated secretion of lysosomal enzymes. Moreover,  $\text{NH}_4\text{Cl}$

is a potent inhibitor of intracellular lysosomal hydrolase activity because it increases intralysosomal pH (28). When SL66 cells were plated on  $^{35}\text{S}$ -labeled SAM in the presence of  $\text{NH}_4\text{Cl}$  (10 mM), the degradation and release of  $[\text{}^{35}\text{S}]\text{PG}$  was reduced in a fashion similar to that observed with M6P (Table 1, line E). The presence of both  $\text{NH}_4\text{Cl}$  and M6P did not have any effects beyond that observed with either reagent alone.

## DISCUSSION

The experiments presented here document several key features concerning the turnover of GAG components in SAM. First,  $^{35}\text{SO}_4^{2-}$ -labeled molecules from SAM produced by SL66 cells in the presence and absence of M6P consist mainly of proteoglycans, similar in molecular weight to those isolated from the SAM of mouse 3T3 fibroblasts (1). Second, when SL66 fibroblasts are plated onto SAM, GAG containing components are released from the SAM. Some of these remain in the medium as macromolecules and others are degraded. Degradation of the GAGs is mediated by cells and not by secreted acid hydrolases, since incubation with conditioned media or with cells oversecreting lysosomal enzymes (I-cells and  $\text{NH}_4\text{Cl}$  treated SL66 cells) actually results in decreased degradation of GAGs. Third, degradation of GAGs from SAM is inhibited by 40-50% when M6P is included in the medium.

Several lines of evidence indicate that the effect of M6P is specifically mediated through the cell surface M6P-receptor that binds to lysosomal enzymes. Analysis of the dose-response curve of M6P inhibition showed that it was a saturable effect and the value of  $K_i$ , estimated at half-maximal inhibition, was 60  $\mu\text{M}$ . This is in close agreement with that reported for the inhibition of binding and uptake via the M6P receptor of  $\alpha$ -L-iduronidase by iduronidase deficient cells (13,16) and with that reported for the binding of  $\beta$ -galactosidase to the M6P receptor purified from liver plasma membranes (19). In previous



experiments (21,22), we had estimated a value of  $K_i$  for the effect of M6P on the turnover of the GAGs of the cell surface of SL66 fibroblasts. This value (60  $\mu$ M) is in agreement with the  $K_i$  estimated by the present assay.

In addition, the specificity of the effect was restricted to M6P and fructose 1,6-diphosphate. Both of these phosphorylated saccharides have low  $K_i$  values and are strong inhibitors of the binding and uptake of lysosomal enzymes via the cell surface M6P receptor (13). In contrast, phosphorylated and non-phosphorylated analogs of M6P failed to yield the same effect, in accord with the specificity of M6P receptor-lysosomal enzyme interaction (13,14,16). Glucose 1-phosphate, which does inhibit lysosomal enzyme binding and uptake, but with a high  $K_i$  value (13), did not mimic the effect of M6P at the concentration (1 mM) tested.

Finally, when the radioactive SAM was subjected to digestion by I-cells, which lack lysosomal enzymes carrying the M6P marker (18,24), there was little degradation of high molecular weight GAGs into material of lower molecular weight. The I-cells secrete high levels of lysosomal enzymes that do not bind to cell surface M6P receptors. Similarly,  $\text{NH}_4\text{Cl}$ , which raises the pH of intracellular compartments, inhibits lysosomal function, disrupts turnover of cell surface receptors, and increases secretion of lysosomal enzymes (27,28), reduced the degradation of proteoglycans to the same extent as M6P. The presence of both  $\text{NH}_4\text{Cl}$  and M6P yielded effects no different than those of either reagent alone. Therefore, the results indicate that the generation of degraded [ $^{35}\text{S}$ ]PG from high molecular weight molecules is not mediated by elevated levels of extracellular, soluble lysosomal enzymes.

It should be noted that M6P inhibits the degradation of some, but not all, of the sulfated PG derived from SAM. In the presence of M6P, a significant quantity of [<sup>35</sup>S]PG is still degraded by cells to low molecular weight components. This represents ~60% of the total degraded [<sup>35</sup>S]PG produced by control cells. It is likely that the effect of M6P is exerted at the population of M6P receptor-acid hydrolase complexes residing at the cell surface. In fibroblasts, sorting of newly synthesized lysosomal enzymes into a pathway leading to lysosomes is mediated by M6P receptors (14-19). This appears to be an intracellular process, unaffected by incubating cells in the presence of M6P. Therefore, the majority of receptor-enzyme complexes most likely do not reach the plasma membrane. Nor does M6P enter the cell and dissociate receptor-enzyme complexes at sites prior to commitment of the enzymes into a pathway leading to lysosomes. Consistent with this is the finding that M6P treatment of cells does not cause intracellular accumulation of undegraded GAGs (21). In fibroblasts, some acid hydrolase-M6P receptor complexes are found at the cell surface, due either to transport of complexes from internal membranes or binding of secreted enzymes to unoccupied surface receptors (14,15). This binding is prevented and dissociation is facilitated by inclusion of M6P in the medium.

How M6P prevents degradation of a population of GAGs derived from SAM is unknown. The saccharide does not inhibit endocytosis of GAGs derived from SAM (Brauker, J.H., Roff, C.F. and Wang, J.L., unpublished observation). Instead, M6P may cause accumulation of lysosomal enzymes in the medium; some of these activities may result in the removal of recognition markers on PGs required for binding and endocytosis by cells.

Alternatively, surface bound hydrolases may be required to solubilize GAGs in a form that is endocytosed by fibroblasts.

Degradation requires internalization of the GAGs. Recently, compartments other than lysosomes, possibly including endocytotic vesicles, have been demonstrated to be involved in degradation of GAGs (29,30). Endocytic vesicles contain acid hydrolases and become acidic. Therefore, if endocytosed GAGs enter these vesicles, conditions for degradation may exist. In fibroblasts, a major pathway of entry of acid hydrolases into endocytic vesicles may be via cell surface receptors. If this and the concept that endocytic vesicles function as sites of degradation are true, then displacement of lysosomal enzymes from the cell surface M6P receptors would be the basis for the observed reduction in the rate of degradation of PG in the presence of M6P. Furthermore, this would support the original notion of the secretion-recapture hypothesis for the transport of lysosomal enzymes proposed by Neufeld et al. (31). The only modification is that transport and sorting vesicles, rather than lysosomes, are the primary target for delivery of lysosomal enzymes by a cell surface pathway.

#### ACKNOWLEDGEMENTS

This work was previously published in *Experimental Cell Research*, Volume 164 (1986) pp. 115-126, and is reproduced with permission from the publisher. Copyright © 1986 by Academic Press Inc. All rights of reproduction in any form reserved  
0014-4827/86 \$03.00.

## REFERENCES

1. Culp, LA, Rollins, BA & Rollins, BJ, J supramol struct 11 (1979) 281.
2. Culp, LA, Biochemistry 15 (1976) 4094.
3. Rollins, BA & Culp, LA, Biochemistry 18 (1979) 141.
4. Rollins, BA & Culp, LA, Biochemistry 18 (1979) 5627.
5. Lark, MW & Culp, LA, Biochemistry 22 (1983) 2289.
6. Lark, MW & Culp, LA, J biol chem 259 (1984) 212.
7. Roden, L, The biochemistry of glycoproteins and proteoglycans (ed WJ Lennarz) p. 267. Plenum Press, New York (1980).
8. Nakajima, M, Irimura, T, DiFerrante, D, DiFerrante, N, & Nicolson, GL, Science 220 (1983) 611.
9. Recklies, AD, Poole, AR & Mort, JS, Biochem j 207 (1982) 633.
10. Sylven, B, Eur j cancer 4 (1968) 463.
11. Weiss, L, Int j cancer 22 (1978) 196.
12. Poole, AR, Lysosomes in biology and pathology (ed JT Dingle) vol 3 p. 303. Elsevier/North Holland, Amsterstam (1978).
13. Sando, GN & Neufeld, EF, Cell 12 (1977) 619.
14. Fischer, HD, Gonzalez-Noriega, A, Sly, WS, & Moore, DJ, J biol chem 255 (1980) 9608.
15. von Figura, D & Voss, B, Exp cell res 121 (1979) 267.
16. Kaplan, A, Achord, DT & Sly, WS, Proc natl acad sci US 74 (1977) 2026.

17. von Figura, D & Weber, F, *Biochem j* 176 (1978) 943.
18. Tabas, I & Kornfeld, S, *J biol chem* 255 (1980) 6633.
19. Sahagian, GG, Distler, J & Jourdian, GW, *Proc natl acad sci US* 78 (1981) 4289.
20. Steiner, AW & Rome, LG, *Arch biochem biophys* 214 (1982) 681.
21. Roff, CF, Wozniak, RW, Blenis, J & Wang, JL, *Exp cell res* 144 (1983) 333.
22. Roff, CF & Wozniak, RW, *The extracellular matrix* (eds SP Hawkes & JL Wang) p. 329. Academic Press, New York (1982).
23. Drinkwater, NR, Corner, RC, McCormick, JJ & Maher, VM, *Mutat res* 106 (1982) 277.
24. Reitman, ML, Varki, A & Kornfeld, S, *J clin invest* 67 (1981) 1574.
25. Saarni, H, & Tammi, M, *Anal. biochem* 81 (1977) 40.
26. Oohira, A, Wright, T & Bornstein, P, *J biol chem* 258 (1983) 2014.
27. Gonzalez-Noriega, A, Grubb, JH, Talkad, V & Sly, WS, *J cell biol* 85 (1980) 839.
28. Dean, RT, Jessup, W & Roberts, CR, *Biochem j* 217 (1984) 27.
29. Yanagishita, M & Hascall, VC, *J biol chem* 249 (1984) 10270.
30. Yanagishita, M & Hascall, VC, *J biol Chem* 260 (1985) 5445.
31. Neufeld, EF, Sando, GN, Garvin, AJ & Rome, HJ, *J supra struct* 6 (1977) 95.

Chapter 3

Turnover of Cell Surface Proteoglycans  
in Cultured Fibroblasts: Comparison  
between Normal and I-cells

James H. Brauker and John L. Wang

Department of Biochemistry  
Michigan State University  
East Lansing, MI 48824

## SUMMARY

The metabolism of cell-associated proteoglycans (PG) was studied in I-cell fibroblasts and normal (SL66) skin fibroblasts cultured in  $^{35}\text{SO}_4^{-2}$  to label the glycosaminoglycans (GAGs). Kinetic data from label-chase experiments and gel filtration analysis of the molecular weight distribution of the radiolabeled GAGs yielded the following main conclusions. a) The amount and GAG composition (75% heparan sulfate) of the  $^{35}\text{SO}_4^{-2}$ -labeled cell surface PG of I-cells do not differ significantly from those of normal cells. b) The rates of shedding into the medium and internalization of cell surface PG are unaffected in I-cells. c) I-cells retain partially degraded GAG chains intracellularly during a chase in unlabeled medium, reflecting their known deficiency of lysosomal enzyme activities. d) Two classes of GAG chains are stored in I-cells. One group of large molecules is composed mainly (90%) of galactosaminoglycans, and makes up 2/3 of the total intracellular radioactivity. The other group of small molecules is composed of mainly (90%) heparan sulfate (HS) and makes up 1/3 of the total intracellular radioactivity. e) The galactosaminoglycan GAG chains are delivered to the degradative compartment only during the

radiolabeling period, and not during the chase, consistent with the observation that few galactosaminoglycan PG are found at the cell surface. f) The HS GAG chains are internalized continuously from the cell surface during the chase; g) The internalized HS GAG chains are acted on by a HS endoglycosidase which reduces the size of the GAG to about 1/6 of its original size.

It is likely that this HS endoglycosidase activity is mediated by an enzyme that is delivered to its site of action by a pathway which is not dependent on mannose 6-phosphate recognition markers. Thus, it is unlikely that it is a lysosomal enzyme.

## INTRODUCTION

Cultured human fibroblasts synthesize sulfated proteoglycans (PG) which are secreted into the medium, transported to the cell surface, or transported directly to degradative compartments of the cell (1). The cell surface is rich in proteoglycans which contain heparan sulfate (HS) glycosaminoglycan (GAG) chains (2-6). The PG may leave the cell surface by two pathways; they may be shed into the medium, or they may be internalized by the cells and transported to degradative compartments (3-7). After internalization of the PG, the GAG chains are acted on by endoglycosidase and exoglycosidase enzymes to completely degrade them to their constituent monomers (8,9). This activity has been generally hypothesized to occur in lysosomes. Recently, non-lysosomal protease and endoglycosidase activity against internalized HSPG have been postulated to occur in rat ovarian granulosa cells (10,11). This hypothesis is based on evidence that the protease and endoglycosidase activities are not interrupted when cells are treated with the lysosomotropic agent chloroquine. However, this evidence is inconclusive because some lysosomal enzymes may still be active at pH 6 (9), which is the approximate pH of the intracellular acid compartments of cells treated with chloroquine (12).



Fibroblasts from I-cell (mucopolysaccharidosis II) patients have a deficiency of lysosomal enzyme activity in their lysosomes (13,14). The basic biochemical mechanism has been characterized as an inability of I-cells to phosphorylate mannose residues on the oligosaccharide chains of lysosomal enzymes. The normal mode of transport of lysosomal enzymes requires that the oligosaccharide chains of the enzyme be phosphorylated, so that they can be recognized by specific receptors in the Golgi called mannose 6-phosphate receptors (15,16). The enzymes bind to these receptors and are transported directly to lysosomes via an intracellular route (17-19). Lack of phosphorylated mannose residues on lysosomal enzymes in I-cells results in the enzymes being secreted into the medium, [where, at neutral pH they do not degrade GAGs (24)], rather than being transported to lysosomes (13,14). Thus, although I-cells synthesize precursor lysosomal enzymes, they have a deficiency of the enzymes needed for degradation of GAGs. Undegraded GAGs are stored in the lysosomes of these cells.

Like the genetic disorders involving the deficiency of single enzymes (mucopolysaccharidoses) (9,20), it would be expected that the GAGs which are stored in the intracellular degradative compartment would consist predominantly of catabolites which are substrates for the first missing enzyme in the pathway of degradation. Therefore, we studied the internalization and degradation of cell surface PG in I-cells, in comparison to normal

cells, to determine whether some of the early steps in PG degradation are affected by the lysosomal dysfunction. We show evidence that the steps of internalization, separation of GAG chains from protein cores, and heparan sulfate (HS) endoglycosidase action are all unaffected in I-cell fibroblasts. These data support the hypothesis that the early steps in the degradation of PG occur in non-lysosomal compartments.

## MATERIALS AND METHODS

### Cell culture and radiolabeling procedures

Fibroblast lines from I-cell (GM 2273B, GM 2013A), heterozygous I-cell (GM 2014) and Sanfilippo (GM 0312) subjects were from the Coriell Institute for Medical Research (Camden, NJ). SL66 cells, derived from normal skin fibroblasts, were from Drs. J. McCormick and V. Maher (21). All cells were maintained in Eagle's Minimal Essential Medium (MEM) (Gibco, Grand Island, New York) with 20% fetal bovine serum (FBS) (Hazelton Research Products, Denver, PA), 100 µg/ml streptomycin and 159 IU/ml penicillin (Sigma, St. Louis, MO) at 35°C in humidified air. Medium for stock SL66 cultures was changed three times a week; these cells were passaged before the cultures reached confluence. Medium for the other cell lines was changed every two days and the cultures were split 1:2 or 1:4 upon reaching confluence. Confluent monolayers of cells were removed from the dish with trypsin (400 µg/ml) in buffer A [11.5 mM sodium-potassium phosphate (pH 7.2), 140 mM NaCl, 2.68 mM KCl, and 0.01 mM EDTA], and plated in 6 well dishes (8 cm<sup>2</sup>/well, Costar, Cambridge, MA) for 1-2 days before labeling. All cultures were confluent at the time of labeling.

Cells were radiolabeled in streptomycin-free MEM with  $MgCl_2$  substituted for  $MgSO_4$ , 20% dialyzed FBS (Gibco), 50  $\mu Ci/ml$   $H_2^{35}SO_4$  (carrier free, ICN, Irvine, CA).

Harvest of cell fractions during label-chase experiments

The radioactivity was harvested either at the end of the radiolabeling period (0 hour) or after "chasing" in unlabeled medium for various times, and designated as extracellular, pericellular, or intracellular radioactivity (22). The extracellular compartment was the medium, including cell secretions, which was removable by pipetting. The pericellular material was the radioactivity in the cell monolayer which could be removed from the cells by trypsinization. The intracellular fraction was the trypsin resistant radioactivity remaining with the cells after trypsinization. To harvest these cell compartments, the medium (extracellular fraction) was removed and centrifuged for 3 minutes at 1330 g to remove cellular debris. The cell monolayers were washed several times with MEM; the washes were discarded. Pericellular and intracellular material were obtained by removal of the washed monolayer with trypsin (400  $\mu g/ml$  in buffer A for 20 minutes at 35°C); this removed all the radioactivity from the dish. Cells which remained loosely attached to the dish were dislodged by gentle pipetting. The cell suspension was centrifuged for 3 minutes at 1330 g. The supernatant, defined as the pericellular fraction, was decanted. The remaining cell pellet, or intracellular

fraction, was dissolved in H<sub>2</sub>O and frozen at -20°C. The intracellular material was analyzed for cell protein by the Bradford method (23). Alternatively, for gel filtration experiments to compare the size of pericellular and intracellular <sup>35</sup>SO<sub>4</sub>-labeled material, the whole cell monolayer (sum of intracellular and pericellular), was harvested at once, after removal of medium and washing, by addition of 0.2% sodium dodecyl sulfate (SDS), 1 mM NaN<sub>3</sub>. Harvest by this method does not affect the molecular weight of the pericellular PG, whereas trypsin reduces their molecular weight. Aliquots from extracellular, pericellular, intracellular, or whole cell samples were solubilized at a ratio of 1:9 (sample:scintillation cocktail) in scintillation cocktail consisting of 7 g 2,5-diphenyloxazole (Research Products International Corp.), 338 ml Triton X-100 and 667 ml toluene. Radioactivity was determined on a Packard Tricarb 300CD liquid scintillation counter and expressed as disintegrations per minute (dpm). Protease inhibitors were added to all of the cell fractions to make final concentrations of 1mM benzamidine HCl, 0.5 mM EDTA, 5 mM N-ethyl maleimide, 25 mM 6-aminohexanoic acid, and 1 mM phenyl methyl sulfonyl fluoride.

For "chase" experiments, the monolayers were washed after removal of radiolabeling medium and incubated with MEM containing 20% FBS, and harvest procedures were performed at various times thereafter. Alternatively, the cells were pretreated for 15 minutes with PBS alone, or PBS

containing 30 µg/ml trypsin, to remove pericellular GAGs without removing cells from the dish. In some experiments the cells were removed from the dish with trypsin (400 µg/ml in buffer A), and replated in growth medium for one hour. The adherent cells were then washed and "chased" in unlabeled medium. At various times thereafter, the extracellular, pericellular, and intracellular compartments were harvested as above. Low molecular weight (LMW) and high molecular weight (HMW) components of medium were quantitated by loading 200 µl aliquots on columns containing 2 ml of DEAE cellulose (Sigma) equilibrated and eluted with 0.05 M sodium acetate, 0.15 M NaCl, 0.5% Triton X-100, pH 6. Under these conditions the HMW components bound to the gel, whereas the LMW components eluted in the first 2 ml of buffer. The LMW components migrated on Sephadex G-25 columns at the position of free sulfate and therefore were considered to be degraded material (24).

#### Chemical and enzymatic degradation of GAGs

Samples were used directly from pericellular fractions, whereas intracellular fractions were first eluted on CL6B columns in 4 M guanidine HCl (see below). The radioactivity eluting in regions B or C of the CL6B column was pooled and precipitated in 2 volumes of cold 90% ethanol, heated for 2 minutes in a 95° C water bath, followed by 5 minutes on ice. The samples were then centrifuged for 10 minutes at 12,000 X g. The supernatant

was removed and the precipitate was suspended in H<sub>2</sub>O. Aliquots of 0.3 ml of the pericellular and intracellular samples were treated as follows: a) For digestion with chondroitin ABC lyase (C'ase , Miles, Elkhart, IN), 0.3 ml of a solution containing 0.1 M Tris, 0.1 M Na acetate, 0.05 unit/ml C'ase (pH 7.3), were added and incubated for 3 hours at 37° C; b) For nitrous acid degradations, 37.5 µl of 2.4 M NaNO<sub>2</sub>, and 37.5 µl glacial acetic acid were added for 80 minutes at 25° C, followed by addition of 75 µl of 3 M sodium sulfamate to stop the reaction; c) For alkaline degradations, 0.3 ml of 0.4 N NaOH were added for 24 hours at 37° C, followed by addition of acetic acid to neutralize the sample. The degradation products were analyzed by gel filtration on Sepharose CL6B or Sephadex G-50 columns (see below).

#### Gel filtration

Samples were chromatographed on columns of Sepharose CL6B (0.7 x 110 cm) or Sephadex G-50 (0.7 x 50 cm) at a flow rate of 5 ml/hour. The elution buffer was 0.2 M lithium acetate, 0.2 % SDS, and 1 mM NaN<sub>3</sub>, pH 5, or 0.05 M Tris, 0.05 M Na<sub>2</sub>SO<sub>4</sub>, 4M guanidine HCl, 0.5 % triton-X 100, pH 7. Fractions of 0.8 ml were collected and analyzed for radioactivity.



## RESULTS

### Turnover of $^{35}\text{SO}_4^{-2}$ -labeled components in cells

I-cells and SL66 cells were labeled with  $^{35}\text{SO}_4^{-2}$  for 24 hours. After washing to remove unincorporated radioactivity, the labeled cultures were chased in unlabeled medium. The intracellular and pericellular fractions were isolated and the radioactivity quantitated (fig. 1). At the beginning of the chase period (0 hours) the level of intracellular radioactivity in I-cells was more than 3 fold higher than that in normal cells (fig. 1a). Over a 48 hour chase period, the quantity of  $^{35}\text{S}$ -label in I-cells decreased to 63% of the initial amount. In contrast, the normal cells rapidly lost most of the initial radioactivity during the first 12 hours (24% of initial value). The accumulation of [ $^{35}\text{S}$ ]GAGs is consistent with the observation of Schmickel et al (25), and is likely due to dysfunctional lysosomal activity in these cells (26).

In both cell types, radioactivity in the pericellular compartment decreased with time (fig. 1b). No difference was observed at any time point between the two cell types. Thus, it appears that the turnover of the GAGs in the pericellular compartment of I-cells is not affected by the lysosomal dysfunction.

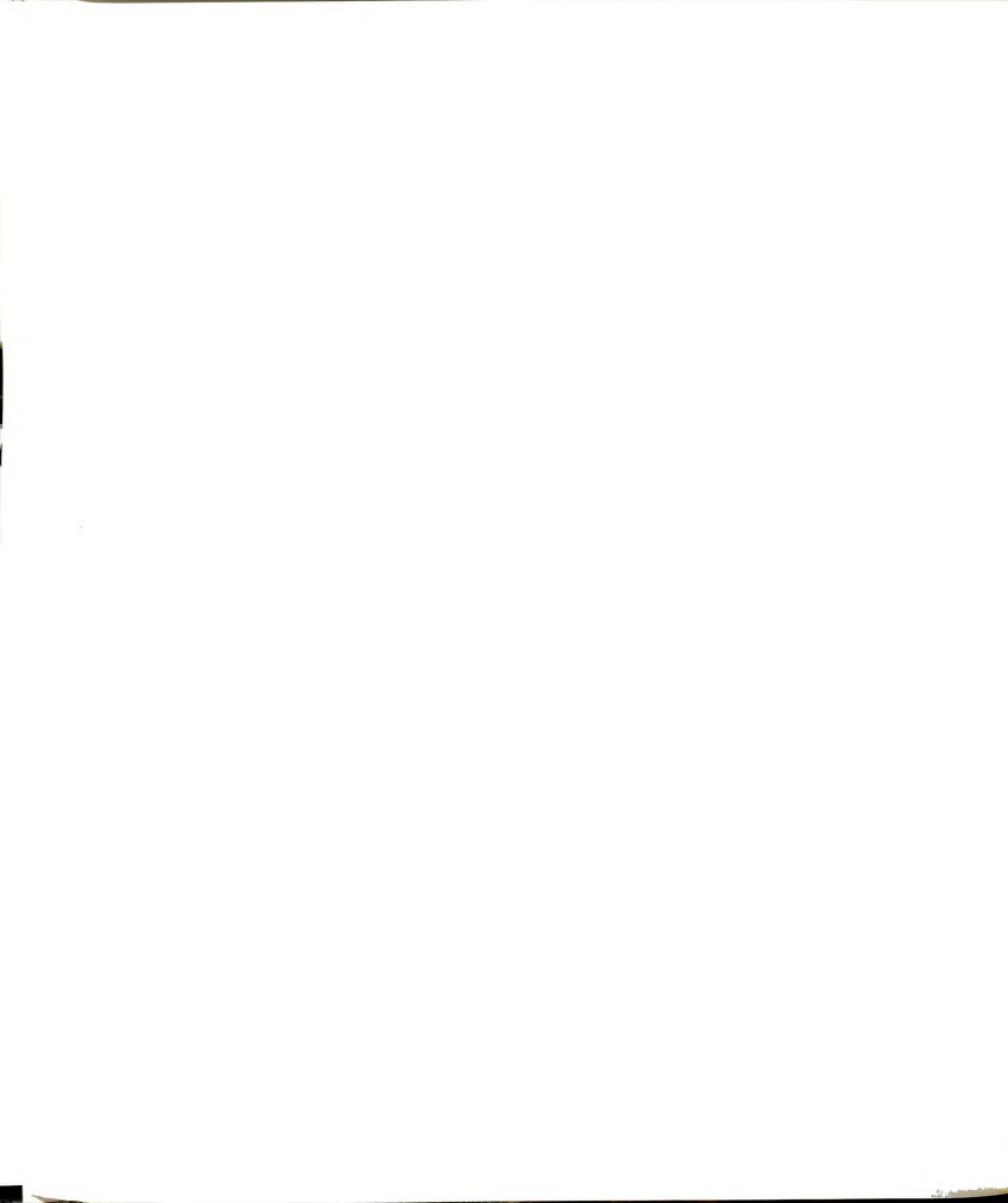
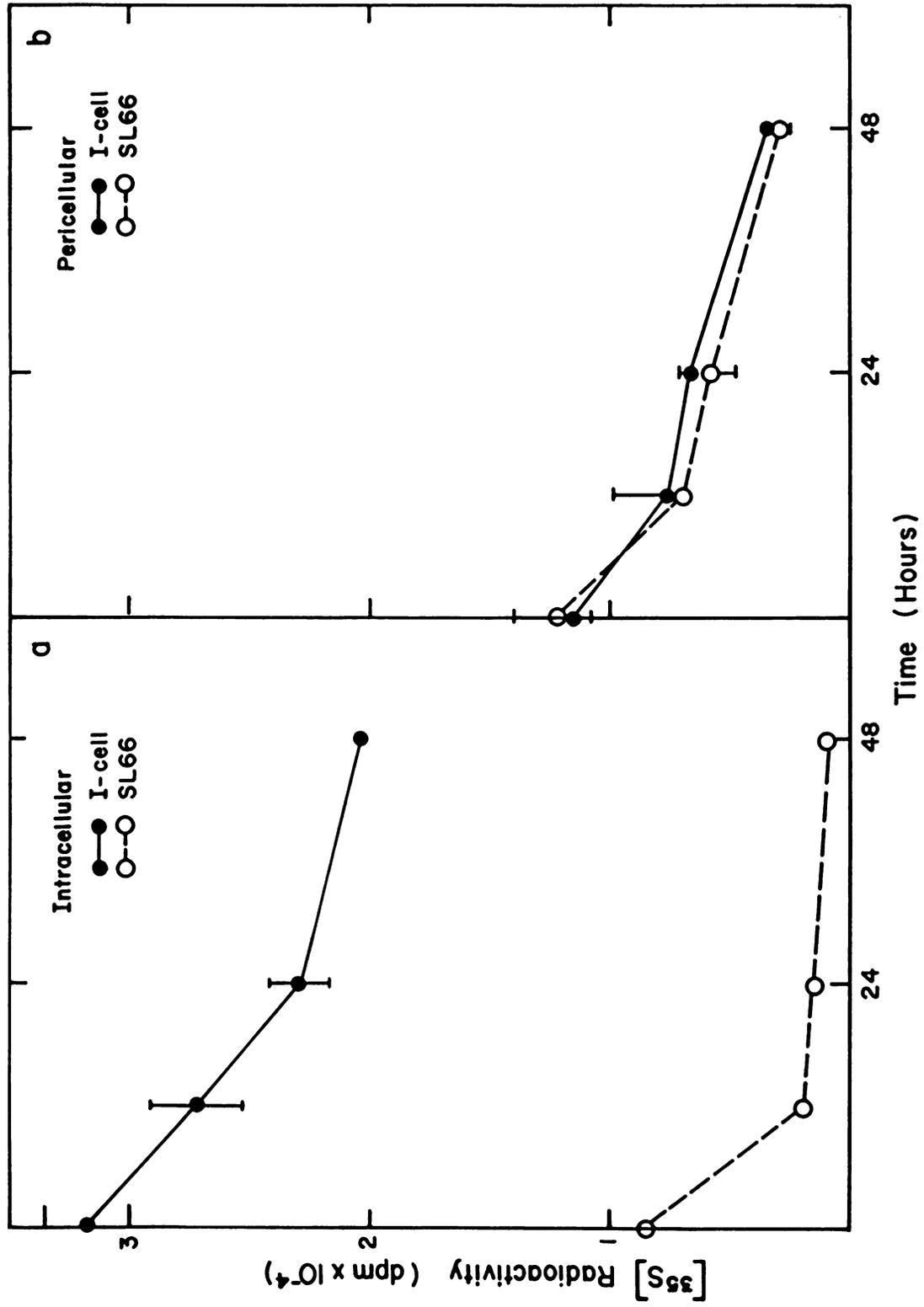


Figure 1. Kinetics of the turnover of  $^{35}\text{SO}_4$ -labeled components in the (a) intracellular and (b) pericellular compartments of SL66 (○) and I (●) cells. Cells were labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ ) for 24 hours, washed and chased in unlabeled culture medium. The intracellular and pericellular fraction were isolated as described in Materials and Methods and their radioactivity quantitated. The data represent the averages of duplicate determinations (error bars indicate standard deviations where larger than symbols).



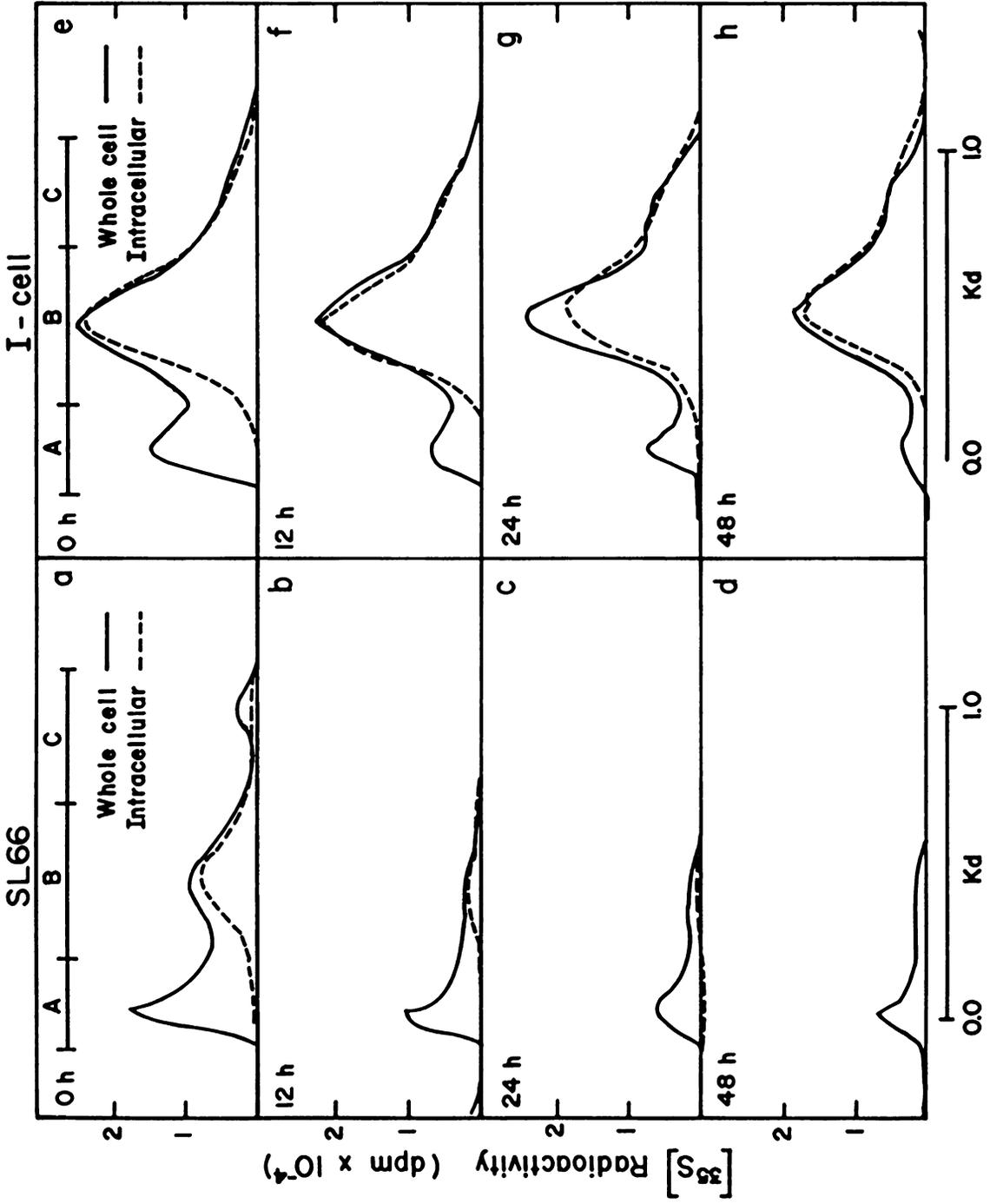


Molecular weight distribution of  $^{35}\text{SO}_4$ -labeled components in the cell monolayer

Radioactively labeled cells were removed from the culture dish with trypsin (400  $\mu\text{g}/\text{ml}$ , 15 minutes, 37 C) and the trypsin resistant pellet (intracellular) fraction was collected. Parallel cultures were extracted with 0.2% SDS which removed all of the radioactivity from the dish. This material was termed the whole cell monolayer. Gel filtration on columns of Sepharose CL6B was carried out under dissociative conditions for the intracellular fraction and the whole cell monolayer. A comparison of the profiles was made between material derived from various times during the label-chase experiment for normal SL66 cells (fig. 2 a-d) and for I-cells (fig. 2 e-h). The intracellular GAGs of both SL66 and I-cells have predominant peaks of radioactivity centered around 0.42 Kd on Sepharose CL6B (see, for example, region B of fig. 2a and 2e). In addition, I-cells develop a second "shoulder" of radioactivity during the chase period, centered around Kd = 0.72 (see, for example, region C of fig. 2 e-h).

At any given time, the radioactivity of the PGs of the pericellular fraction is represented by the difference between the profiles derived from the whole cell monolayer (solid line in fig. 2) and the intracellular fraction (dotted line in fig. 2). The pericellular material chromatographs near the void volume of a CL6B column. This

Figure 2. Gel filtration of  $^{35}\text{SO}_4^{-2}$ -labeled components derived from the whole cell monolayer (SDS extract, —) and the intracellular fraction (trypsin resistant cell pellet, ---) of SL66 cells (a - d) and I-cells (e - h) during a label (50  $\mu\text{Ci/ml}$ , 24 hour) and chase experiment. Radioactivity corresponding to 10  $\mu\text{g}$  of cell protein (using the intracellular fractions for protein determinations) was chromatographed on a column of Sepharose CL6B (0.7 X 110 cm) equilibrated with 0.2 M lithium acetate, 0.2% SDS, 1 mM  $\text{NaN}_3$ , pH 5. The horizontal bars designated A, B, and C highlight regions of interest discussed in the text.



material is in the form of proteoglycan, because its molecular weight is reduced after treatment with 0.2 M NaOH (see below). Thus, the bulk of the radioactivity of the pericellular fraction (region A, fig.2) has a higher molecular weight than that of the intracellular fraction. Similar results were obtained for both SL66 and I-cells. For the purpose of clarity of subsequent description and discussion, we define the regions A - C (fig. 2) of the chromatographic profile as follows: (A) high molecular weight material corresponding to the pericellular PG of the cell; (B) material with  $K_d$  0.42 corresponding to the majority of the intracellular GAGs; (C) low molecular weight material ( $K_d = 0.72$ ) corresponding to a class of GAGs that is accumulated in I-cells but not in normal fibroblasts.

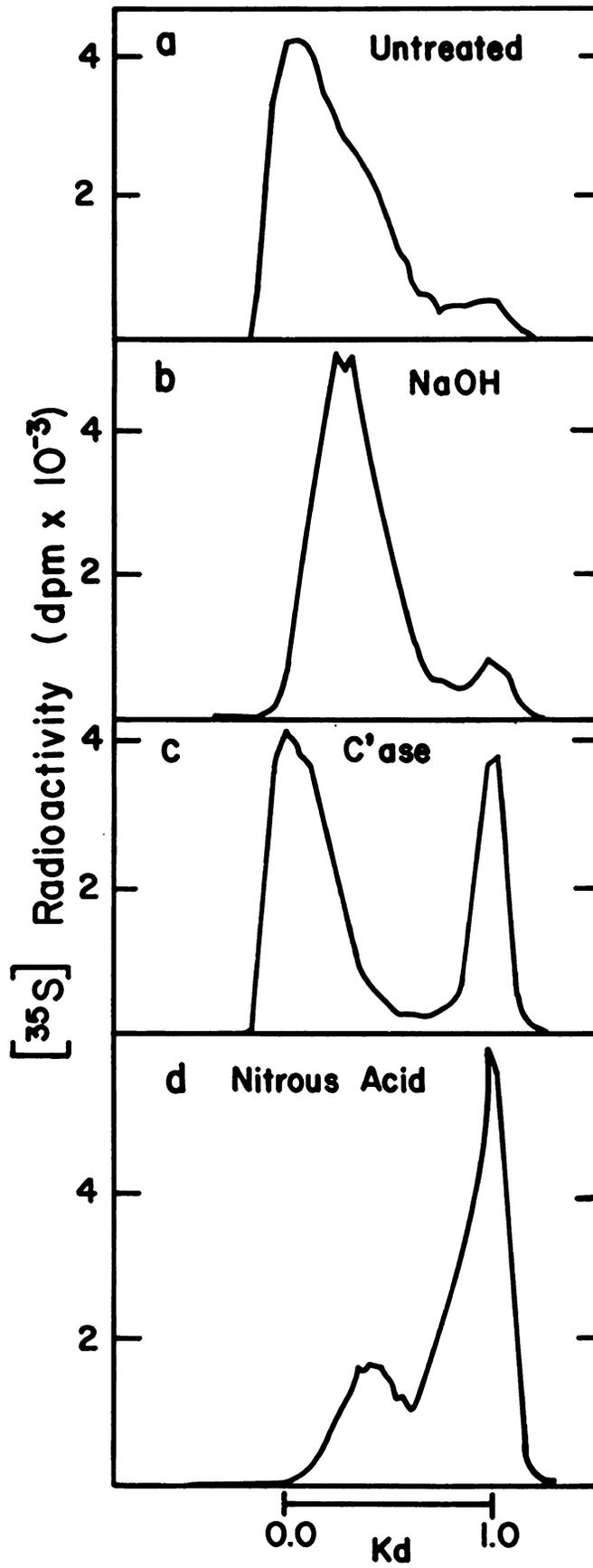
The results of both quantitative (fig. 1) and qualitative (fig. 2) experiments indicate that cell surface PG of I-cells turnover in a fashion comparable to that in normal fibroblasts. In addition, the gel filtration profiles reveal that, if I-cells internalize cell surface proteoglycans, these molecules are rapidly reduced in size, since the peak corresponding to cell surface PG does not seem to accumulate (fig. 2 e-h) as "storage" material in the intracellular compartment (1,9). Thus, it is likely that the first steps of degradation of cell surface PG by I-cells are not impaired.

### Characterization of the cell surface PG

I-cell fibroblasts were labeled with  $^{35}\text{SO}_4^{-2}$  for 24 hours. The radiolabeled cells were washed and then trypsinized. The radioactive material released by trypsin, representing the PG of the cell surface, was subjected to gel filtration on a column of Sepharose CL6B. This yielded a radioactive profile (fig. 3a) consisting mostly of high molecular weight material, with a broad shoulder extending into the more included regions of the column. The bulk of this material migrated at a position on the column (region A) consistent with our previous conclusion that the pericellular PGs are represented by the difference between the profiles derived from the whole cell layer (solid line, fig. 2a) and the intracellular fraction (dotted line, fig. 2a). However, trypsin treatment appears to reduce the molecular weight of some of the pericellular PG, resulting in the "shoulder" of radioactivity in fig. 3a.

When the pericellular radioactive material was subjected to treatment with 0.2 M NaOH, there was a reduction in the molecular weight (compare fig. 3a and 3b). This suggests that the majority of the pericellular label was in the form of PGs which lose the core proteins upon  $\beta$ -elimination. Approximately 25% of the pericellular PG was sensitive to C'ase (fig. 3c), suggesting that these are galactosaminoglycans. The majority of the pericellular PG was sensitive to nitrous acid (fig. 3d). Therefore, HSPG

Figure 3. Gel filtration of  $^{35}\text{SO}_4^{-2}$ -labeled components derived from the pericellular fraction (trypsin released) of I-cells labeled for 24 hours with 50  $\mu\text{Ci/ml}$  of  $^{35}\text{SO}_4^{-2}$ . (a) untreated pericellular fraction; (b) pericellular fraction treated with 0.2 N NaOH for 24 hours at 37°C; (c) pericellular fraction digested for 3 hours at 37°C with 0.025 unit/ml C'ase; and (d) pericellular fraction treated with 0.24 M nitrous acid for 80 minutes at 25°C.





represents the bulk of the  $^{35}\text{SO}_4$ -labeled GAGs of the cell surface.

The results described above were from I-cells. Essentially identical results were obtained when the same analyses were carried out on the pericellular material of SL66 cells.

#### Effect of trypsin on the turnover of pericellular PG

In order to examine the relationship between the cell surface pool of PG and the intracellular (degradative) compartment, the turnover of cell associated GAGs was analyzed with and without pretreatment of the cells with trypsin. The cells were labeled with  $^{35}\text{SO}_4^{-2}$  for 24 hours, and then treated with trypsin (30  $\mu\text{g}/\text{ml}$ ) in PBS, or with PBS alone for 15 minutes. Under these conditions of trypsin treatment, the cells were beginning to round up but they did not become detached from the dish. The cell layers were then washed and chased in unlabeled growth medium. The trypsinized cells reestablished flattened morphology within 1 hour.

Treatment with trypsin removed about 80% of the pericellular PG from both I-cells and normal cells (fig. 4b). This conclusion is derived by comparing the levels of radioactivity at the initial time point (0 hour) in fig. 4b (compare, for example, open circles and closed circles for SL66 cells). Some radioactivity was lost from the intracellular pool of both cell types when treated with trypsin (note lower radioactivity at each time point in

Figure 4. Kinetics of the turnover of  $^{35}\text{SO}_4^{-2}$ -labeled components derived from the (a) intracellular and (b) pericellular compartments of SL66 and I-cells. Cells were labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ , 24 hours). Parallel cultures were treated with trypsin (30  $\mu\text{g/ml}$  in PBS, 15 minutes) or incubated in PBS as controls. The wells were then chased in unlabeled culture medium and the radioactivity in the intracellular and pericellular fractions was quantitated. The data represent the averages of duplicate determinations (error bars indicate standard deviations where larger than symbols).

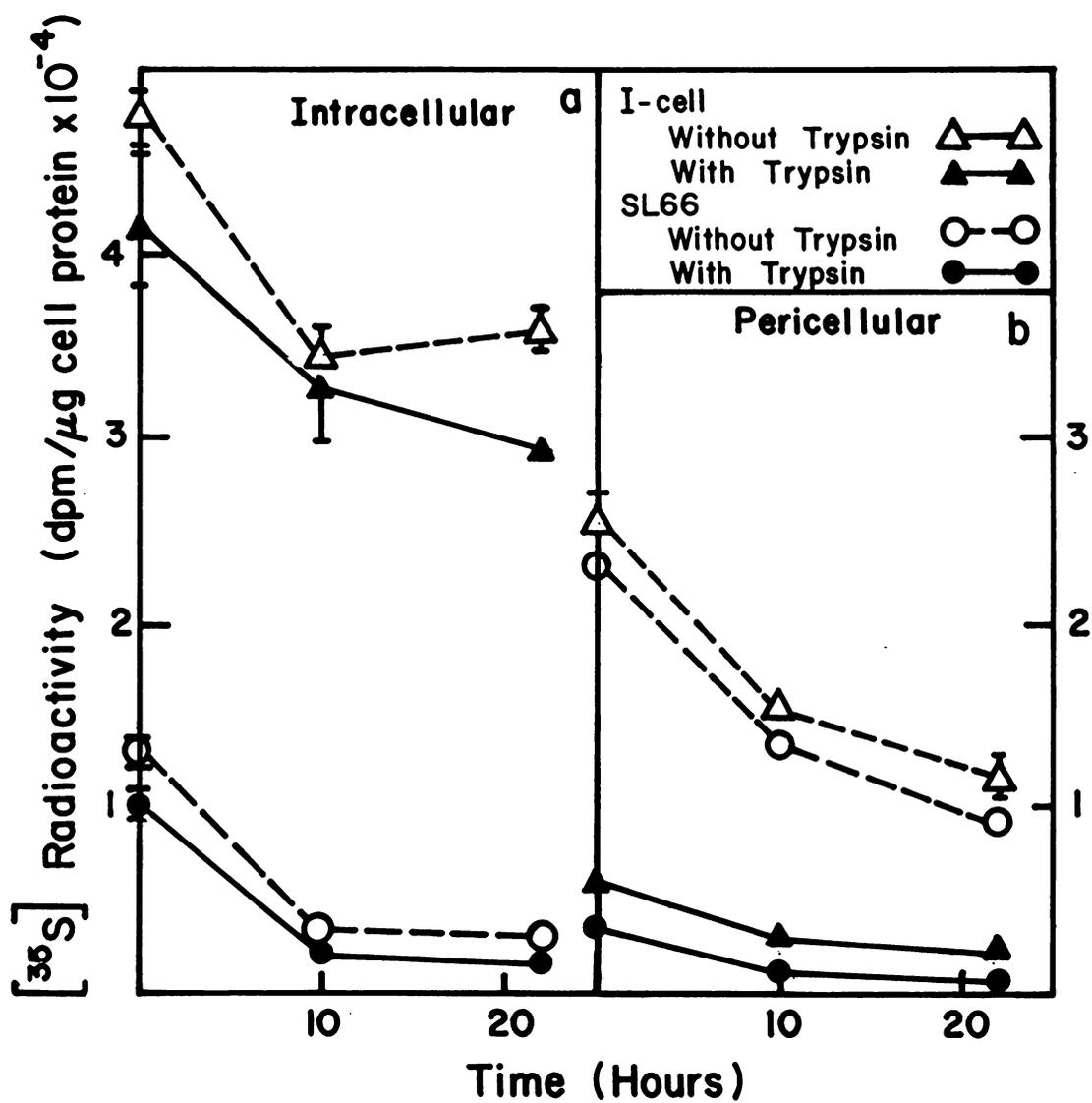


fig. 4a). This was thought to be due to a small loss of cells during the washing procedures following the trypsin treatment. No difference was observed in the Sepharose CL6B gel filtration profiles of trypsinized and untrypsinized cells at the initial time point (data not shown). When the cells were chased in unlabeled medium, the radioactivity disappeared from the pericellular pool of untrypsinized I-cells and normal cells at approximately the same rate (fig. 4b), as described before, (fig. 1b). There was little loss of radioactivity from the pericellular pool of trypsinized cells, compared to that of untrypsinized cells.

The rate of loss of radioactivity from the intracellular pool of trypsinized normal cells paralleled that of untrypsinized cells (fig. 4a), suggesting that internalized cell surface molecules do not affect the steady state levels of intracellular PGs in these normal cells. In contrast, the rate of disappearance of radioactivity from the intracellular pool of untrypsinized I-cells paralleled that of trypsinized I-cells during the first 12 hours, but then diverged, resulting in higher intracellular levels of radioactivity after 22 hours (fig. 4a). We interpreted this to be due to the storage of internalized cell surface PG in the untrypsinized cells. This hypothesis is further supported by analyzing the rates of accumulation of low molecular weight degradation products (presumed to be free sulfate) in the medium, which

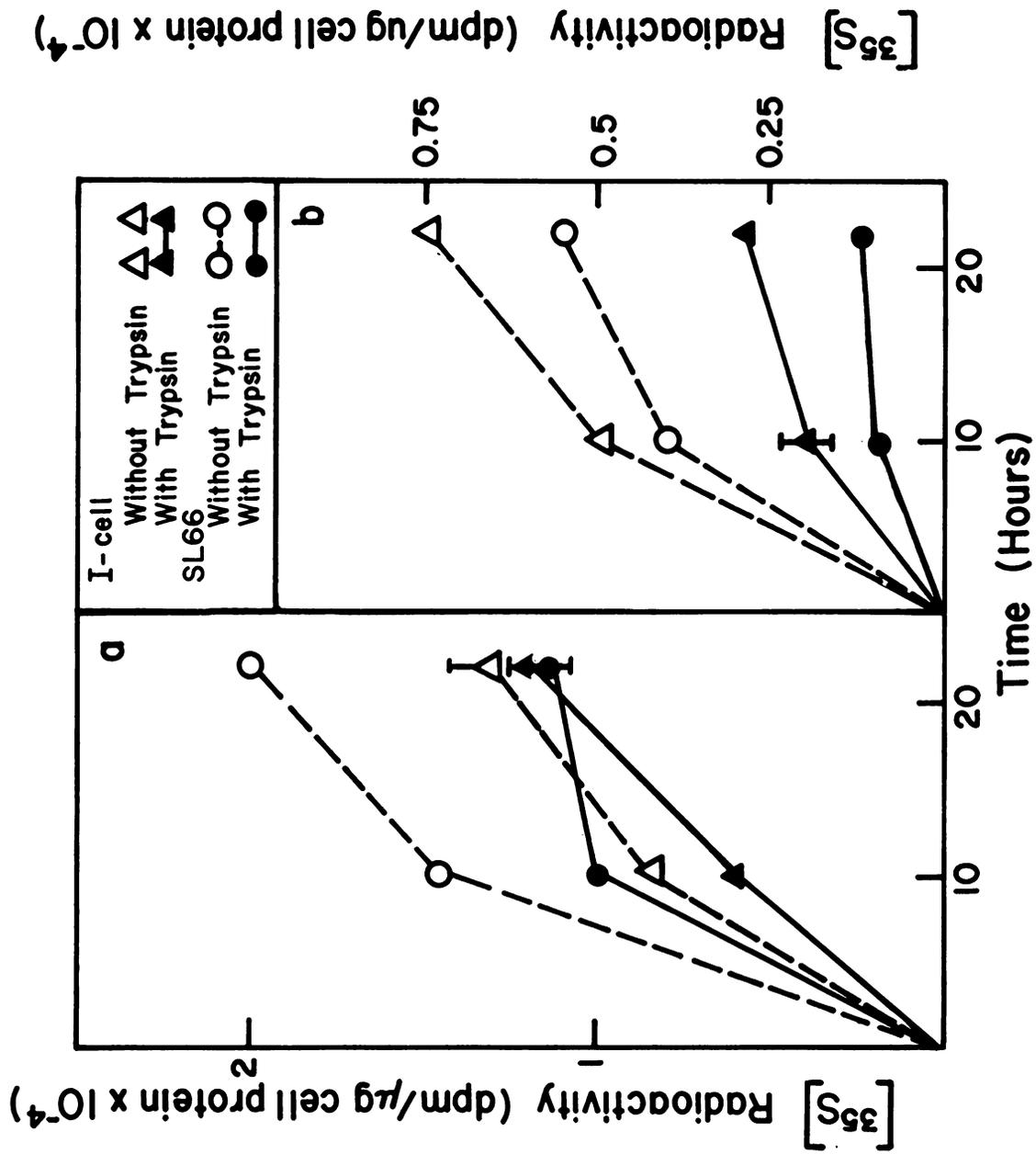
is an estimate of the overall degradation rate of the  $^{35}\text{S}$ -labeled PGs (fig. 5).

The untrypsinized SL66 cells released LMW degradation products at a higher rate than trypsinized normal cells during the first 12 hours. After 12 hours, little additional degradation occurred in trypsinized cells, while the untrypsinized cells continued to release degraded material (fig. 5a). In contrast, trypsinization had little effect on the rate of production of degraded (LMW) material in I-cell cultures (fig. 5a). The higher levels of LMW radioactive sulfate from untrypsinized normal cells compared to trypsinized normal cells probably is due to the internalization and degradation of pericellular PG initially at the cell surface of the untrypsinized cells. The corresponding material had been removed from the trypsinized cells. In contrast, I-cells internalized PG from the pericellular pool (note the higher radioactivity in the intracellular pool of untrypsinized I-cells at 22 hours, fig. 4a), but this additional substrate did not increase the rate of degradation, because in I-cells, the rate of degradation is limited by the intracellular lysosomal enzyme concentration.

#### Shedding and uptake of pericellular PG

Analysis of the HMW material in the medium of trypsinized and untrypsinized cells revealed that both I-cells and normal cells released substantial amounts of macromolecules into the medium during the chase (fig. 5b).

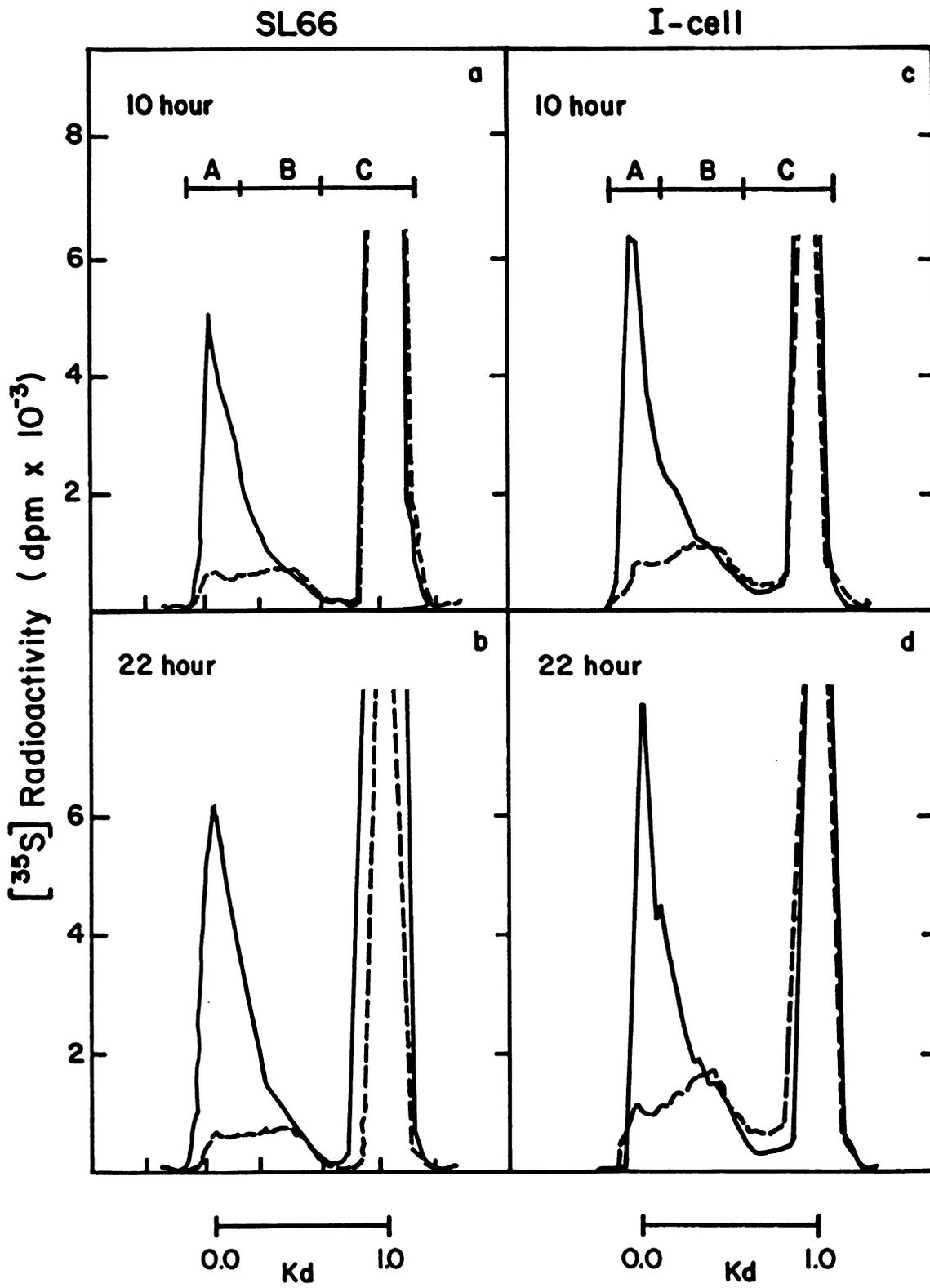
Figure 5. Kinetics of the appearance of (a) LMW radioactivity and (b) HMW radioactivity in the medium of SL66 and I-cells labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ , 24 hours). Parallel cultures were treated with trypsin (30  $\mu\text{g/ml}$ , 15 minutes) or incubated in PBS as controls. The cells were then chased in unlabeled culture medium and the radioactive components of the medium were fractionated on a column of DEAE cellulose (2ml total volume) equilibrated with 0.05 M sodium acetate, 0.5% Triton X-100, pH 6. LMW components represent material that did not bind to DEAE-cellulose (free sulfate) whereas HMW components represent material bound to the column, as defined in Materials and Methods.



Pretreatment of the cells with trypsin resulted in a reduction of the total radioactivity in the medium compared to untrypsinized cells. After 22 hours, I-cells accumulated higher amounts of macromolecules in the medium than did normal cells (fig. 5b). This was true of both untrypsinized and trypsinized cells. Gel filtration analysis of the radioactivity in the medium revealed a possible explanation for this difference. The bulk of the radioactivity released into the medium by untrypsinized normal cells (solid lines, fig. 6 a & b) chromatographed at the total volume of the column (large peak in region C), indicating that it consists of degraded material (24). The remaining radioactivity was of the same size class as pericellular GAGs (compare, for example, fig 6a with fig 2a), suggesting they were released from the cell surface. While trypsinized cells also yielded a high level of low molecular radioactivity (dotted lines, fig. 6 a & b) the macromolecules in the medium of trypsinized cells were predominantly of the size class of intracellular molecules (region B), suggesting that they were leaked or excreted from the cells.

Analysis of untrypsinized I-cell medium revealed similar data to that of normal cells, except that a broader shoulder of radioactivity was seen in the intermediate molecular weight range (region B, fig. 6 c & d). Analysis of the column profiles of the medium from trypsinized cells yielded higher amounts of radioactivity in region B of I-

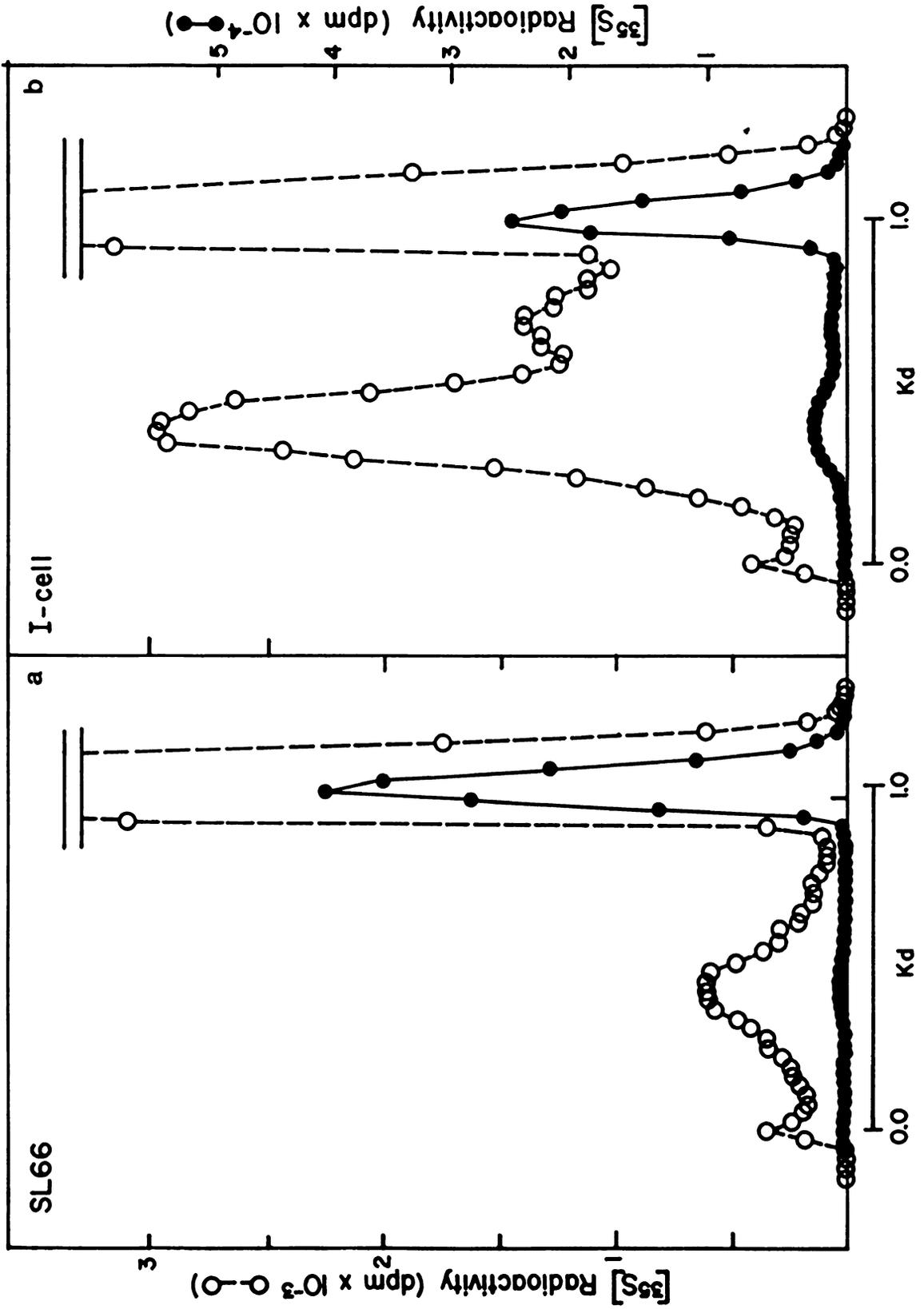
Figure 6. Gel filtration of  $^{35}\text{SO}_4^{-2}$ -labeled components in the medium of SL66 cells (a and b) and I-cells (c and d). Cells were labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ , 24 hours). Parallel cultures were treated with trypsin (30  $\mu\text{g/ml}$ , 15 minutes) or incubated in PBS as controls. The cells were then chased in unlabeled culture medium and the radioactive components of the medium were chromatographed on a column of Sepharose CL6B (0.7 X 110 cm) equilibrated with 0.2 M lithium acetate, 0.2% SDS, 1 mM sodium azide, pH 5. (—) material derived from cultures without trypsin treatment; (---) material derived from cultures with trypsin treatment. The horizontal bars designated A, B, and C highlight regions discussed in the text.



cells than in the corresponding region of normal cells. This observation supports the hypothesis that the region B molecules in the medium came from the degradative compartment. However, it is possible that the light trypsin concentration used in these experiments caused partial degradation of pericellular PG, which were then shed into the medium during the chase. Evidence against this possibility came from experiments in which cells were labeled, removed from the dish with trypsin, and replated (fig 7). The cells were trypsinized under the same conditions used to harvest cell compartments (400  $\mu\text{g/ml}$ ), which completely removed pericellular PG. Analysis of the Sepharose CL6B column profiles of the medium after a 24 hour chase revealed that the bulk of the released radioactivity migrates as degraded material (solid lines, fig. 7) [note, the same data are plotted at two scales to emphasize degraded radioactivity (closed circles) and macromolecules (open circles)]. The remaining radioactivity elutes in region B, and corresponds in size to the material that was inside the cells at the beginning of the chase period (open circles, fig. 7). About 25% of the radioactivity in I-cell medium migrates in region B (fig. 7 b), compared to only 5% in SL66 medium (fig. 7a). In normal cells, this represents a minor amount, compared to the amount of degraded material, as pointed out by Fratantoni and Neufeld (1). However, it represents a substantial fraction of the total macromolecules in the



Figure 7. Gel filtration of  $^{35}\text{SO}_4^{-2}$ -labeled components in the medium of (a) SL66 and (b) I-cells. Cells were labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ , 24 hours). The cells were then removed from the original dish by trypsin (400  $\mu\text{g/ml}$  in buffer A). They were replated for 1 hour in culture medium and then chased for another 24 hours in unlabeled culture medium. The medium was then chromatographed on a column of Sepharose CL6B (0.7 X 100 cm) equilibrated with 0.2 M lithium acetate, 0.2% SDS, 1 mM sodium azide, pH 5. The same radioactivity profile is plotted on two different scales to emphasize degraded radioactive material (•—•) and macromolecules (o---o).

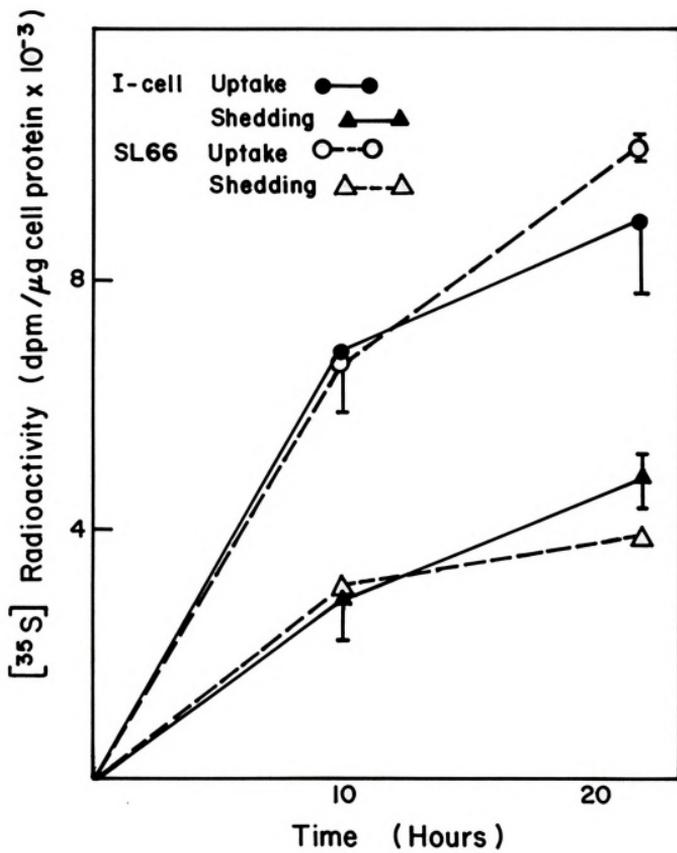


medium after a chase period without prior trypsinization (26% in normal cells and 40% in I-cells). These data indicate that quantitation of total macromolecules in the medium is not sufficient for an accurate measure of "shedding" from the cell surface. In light of these data "shedding" was defined as the difference in the quantity of macromolecules in the medium of untreated cells and those pretreated with 30  $\mu\text{g/ml}$  trypsin. Using this method, shedding from I-cells did not differ significantly from that in normal cells (fig. 8). The rate of uptake of pericellular PG could then be determined as the radioactivity lost from the pericellular pool but not shed (fig. 8). Uptake was not altered in I-cells compared to normal cells, and was about two fold higher than shedding in both cell types. (fig. 8).

#### Characterization of intracellular GAGs

We had observed in previous label-chase experiments that the intracellular GAGs of both SL66 and I-cells have, on gel filtration on Sepharose CL6B, predominant peaks around Kd 0.42 (region B, fig. 2a and 2e). In addition, I-cells develop a shoulder of radioactivity during the chase period (region C, fig. 2,e-h). A hint toward the chemical nature of the components in regions B and C of the Sepharose CL6B column was revealed by comparing the gel filtration profiles of the intracellular PG (derived from cells labeled for 24 hours and then chased for 24 hours) with the corresponding material derived from Sanfilippo

Figure 8. Quantitation of internalized (●,○) and shed (▲,△)  $^{35}\text{SO}_4^{-2}$ -labeled macromolecules from the pericellular fraction of SL66 (---) and I-cells (—). Cells were labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ , 24 hours). Parallel cultures were treated with trypsin (30 $\mu\text{g/ml}$  in PBS, 15 minutes) or incubated with PBS as controls. The cells were then chased in unlabeled culture medium and the radioactive components of the medium were fractionated on a column of DEAE cellulose to quantitate the HMW material (as described in fig.5) Shed material (▲,△) was derived by subtracting the amount of HMW material in the medium of trypsinized cultures from that in untrypsinized cultures. Uptake was derived by subtracting the amount of shed material from the total amount of radioactivity lost from the pericellular pool at each time point.

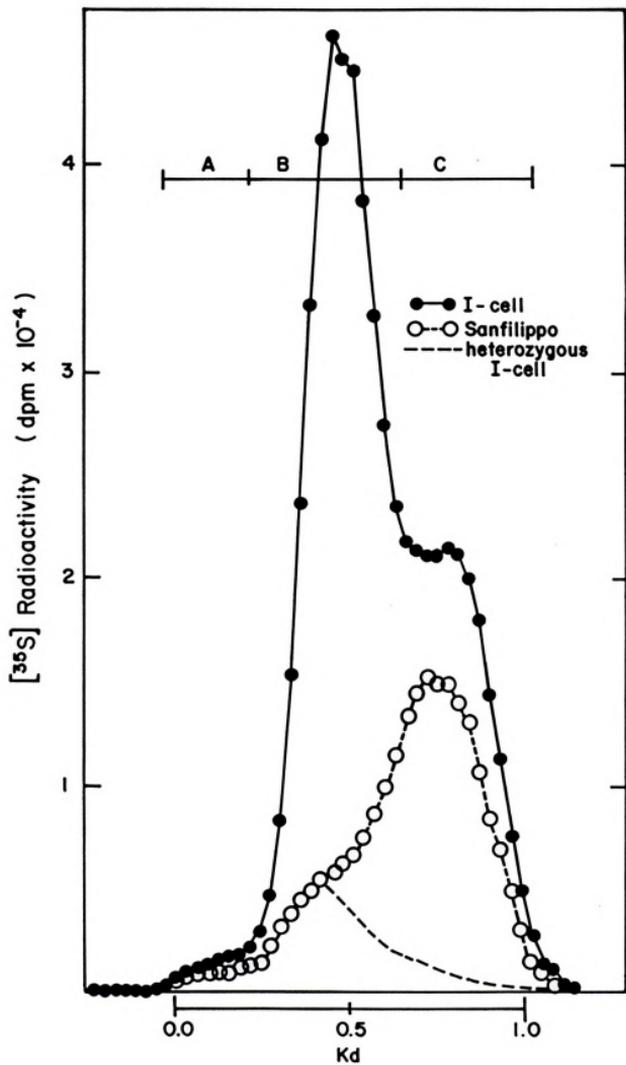


cells (fig. 9). The intracellular GAGs of Sanfilippo cells yielded a predominant peak in region C of the Sepharose CL6B column. Inasmuch as a previous study (27) had shown that Sanfilippo cells accumulate only HS GAGs, due to a defect in an enzyme in the degradation pathway of HS, these results suggest that the region C molecules stored in the intracellular pool of I-cells may be HS. We also analyzed the intracellular GAGs derived from cells of a subject characterized as an "I-cell heterozygote" (GM 2014). The Sepharose CL6B profile from these cells yielded a peak in region B of the column. The level of radioactivity was much lower than that of I-cells. Moreover, this profile was similar to that of normal (SL66) cells (compare with the dotted lines in fig. 2 a-c).

Additional information concerning the chemical nature of the intracellular material chromatographing in regions B and C of the Sepharose CL6B column was derived from studies on their sensitivities to chemical treatments. The intracellular material in region B (derived from I-cells labeled for 24 hours and chased for 24 hours) was sensitive to C'ase but resistant to nitrous acid (fig. 10 a-c). This suggests that the material consisted of galactosaminoglycans. In contrast, the bulk of the intracellular material from region C was resistant to C'ase but was reduced in molecular weight by treatment with nitrous acid. Coupled with the results derived from Sanfilippo cells, these data indicate that the material in



Figure 9. Gel filtration of  $^{35}\text{SO}_4^{-2}$ -labeled components of the intracellular fraction of I-cells (●—●), Sanfilippo cells (○--○), and heterozygous I-cells (---). Cells were labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ , 24 hours) and chased in unlabeled culture medium for 24 hours. The intracellular fraction was chromatographed on a column of Sepharose CL6B (0.7 X 110 cm) equilibrated with 0.2 M lithium acetate, 0.2% SDS, 1 mM sodium azide, pH 5.



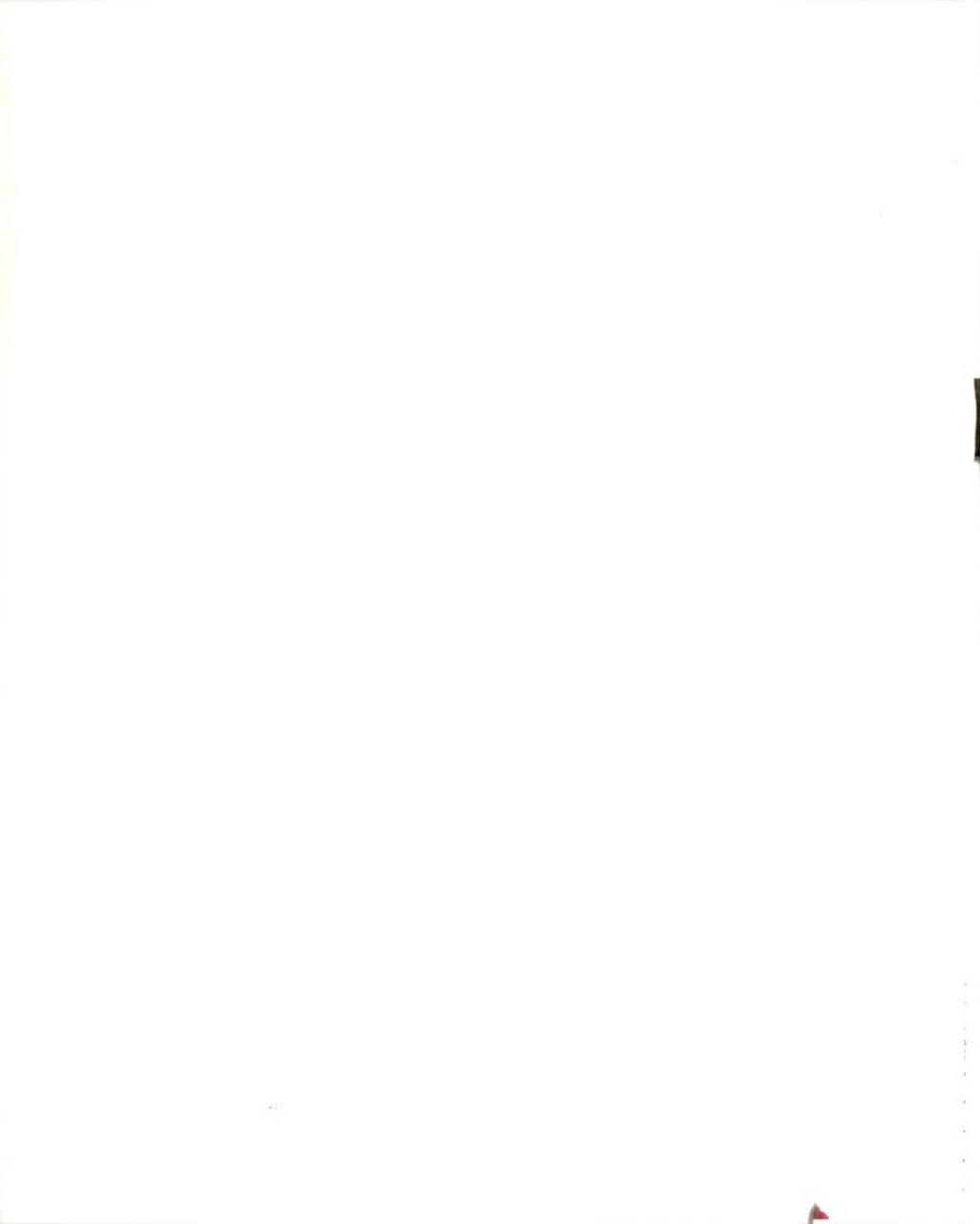
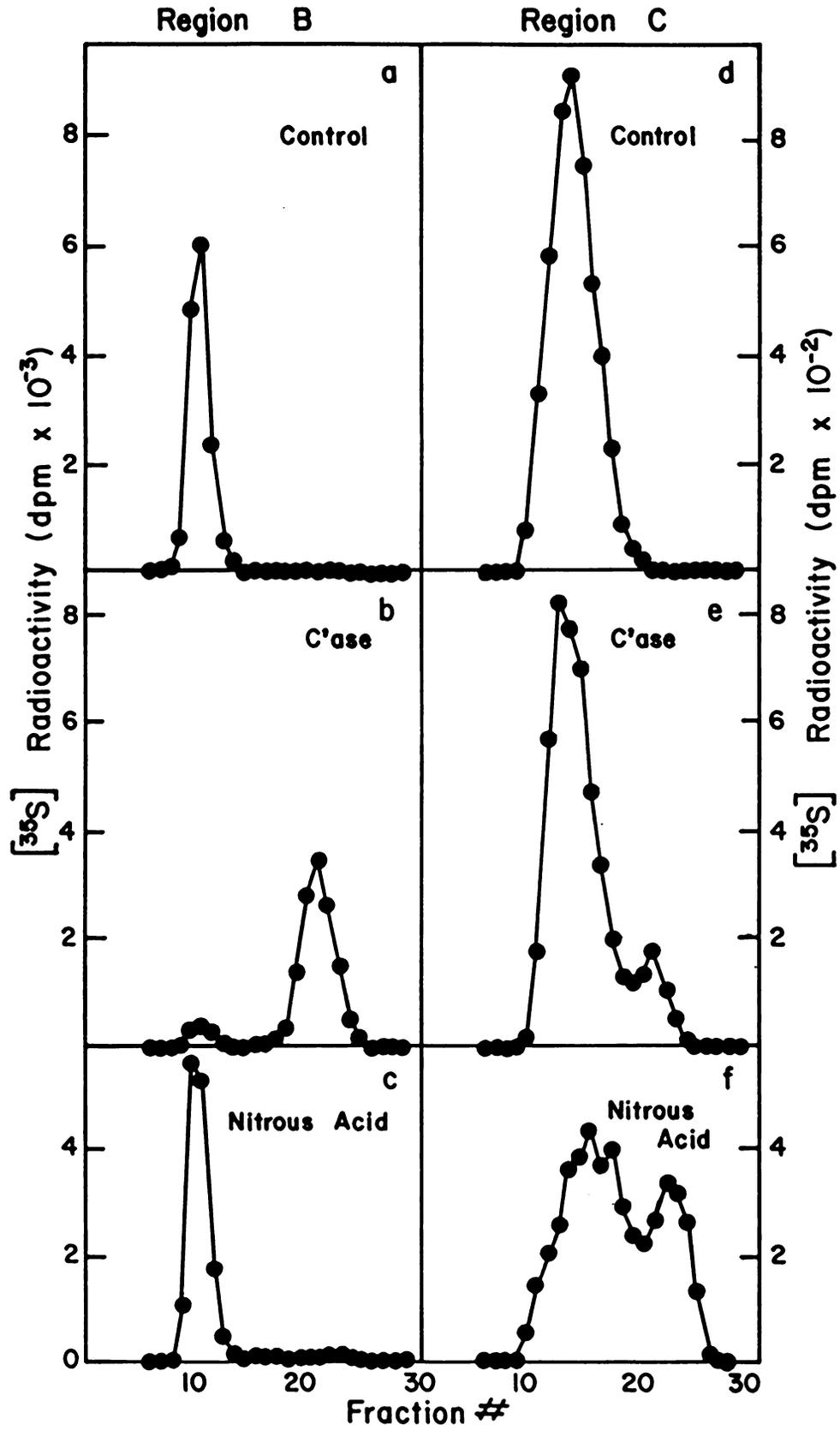


Figure 10. Gel filtration of  $^{35}\text{SO}_4^{-2}$ -labeled components corresponding to region B and C (figure 9) of the intracellular fraction of I-cells after chemical treatments. Cells were labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ , 24 hours) and chased for 24 hours. The intracellular radioactivity was fractionated on columns of Sepharose CL6B as described in legend to fig. 9. The pooled fractions corresponding to regions B and C were chromatographed without treatment (a, d), after C'ase digestion (0.025 unit/ml, 3 hours, 37°C) (b, e), and after nitrous acid treatment (0.24 M, 80 minutes, 25°C) on columns of Sepharose G-50 (0.7 X 50 cm) equilibrated with 0.2 M lithium acetate, 0.2% SDS, 1 mM sodium azide, pH 5.



region B of the column profile consists mainly of galactosaminoglycans and the material in region C of the column profile is predominantly HS.

#### Turnover of intracellular GAGs

The intracellular  $^{35}\text{SO}_4$ -labeled GAGs from cultures of I-cells labeled for 24 hours and chased in unlabeled medium, was harvested and analyzed on Sepharose CL6B columns. The radioactivity migrating in regions B and C was quantitated at various times (fig. 11). To determine the impact of the pericellular PG on the size of these pools, the cultures were trypsinized and replated before the chase (fig. 11b) or were left untreated (fig. 11a). The radioactivity disappeared from region B to around 60% of the initial (0 hour) value during a 48 hour chase period, and this disappearance of radioactivity was unaffected by pretreatment of the cells with trypsin (compare open circles in fig. 11a and 11b). In contrast, the rate of loss of radioactivity from region C was slower in untreated cultures compared to cultures pretreated with trypsin (compare closed circles in fig. 11a and b). In the trypsinized cultures, region C diminished to less than 70% of the initial value, whereas, in the untrypsinized culture, the value at 48 hours was greater than 85% of the initial amount (fig. 11a). Similar data were obtained when cultures were treated with lower concentrations of trypsin (30  $\mu\text{g/ml}$ ), so that they were not removed from the dish (data not shown). These data suggest that the cell surface

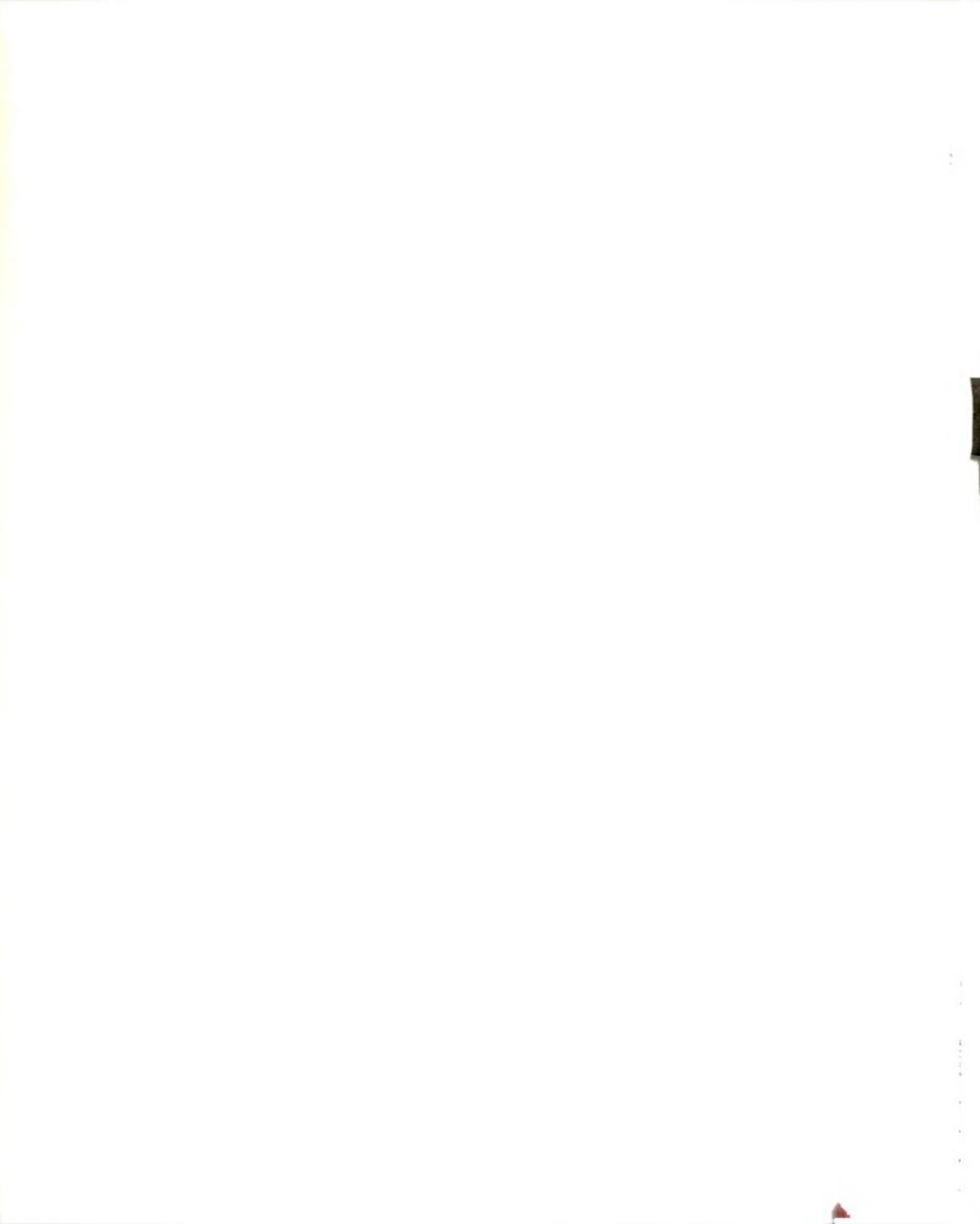
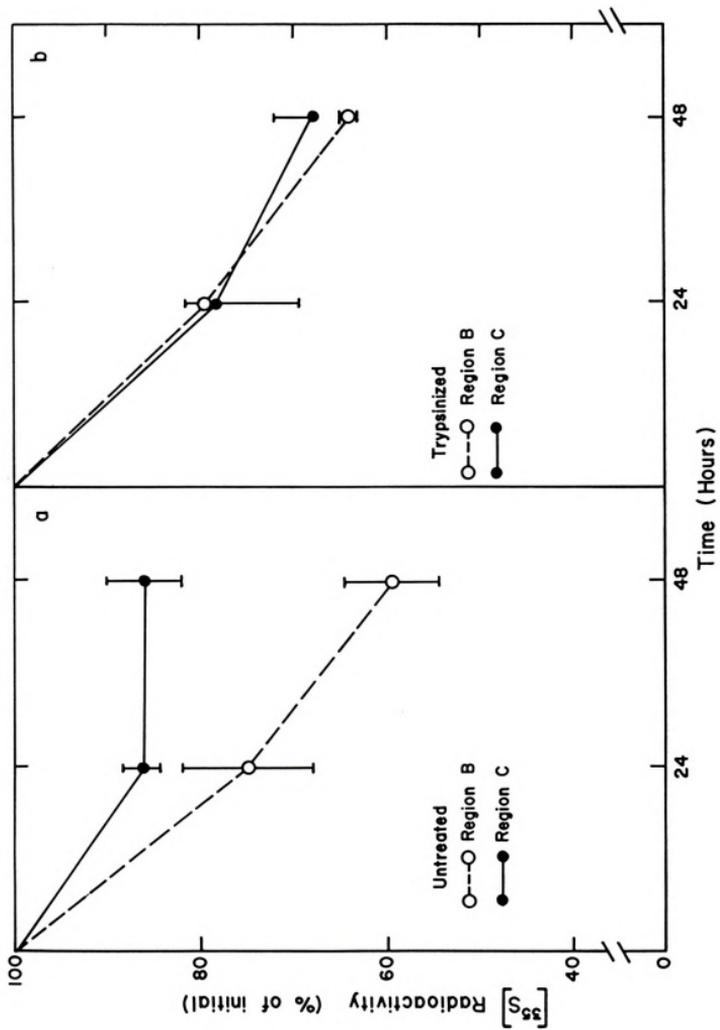


Figure 11. Kinetics of the turnover of  $^{35}\text{SO}_4^{-2}$ -labeled components corresponding to regions B and C (fig. 9) of the intracellular fraction of I-cells. Cells were labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ , 24 hours). Parallel cultures were trypsinized (400  $\mu\text{g/ml}$  in buffer A) or left untreated as controls. The cells were then chased in unlabeled culture medium. The intracellular fraction was fractionated on columns of Sepharose CL6B as described in legends to fig. 9. The radioactivity migrating in the fractions corresponding to region B and C was quantitated. The data represents the averages of duplicate determinations (error bars indicate standard deviations).



HSPG were internalized and that the GAG chains, which originally migrated at 0.42 Kd on Sepharose CL6B columns, were degraded to fragments migrating in region C of the column. Apparently, few galactosaminoglycans were internalized from the cell surface because pretreatment of the cells with trypsin does not alter the rate of turnover of the intracellular galactosaminoglycan (region B) pool. These data support the hypothesis that an HS degrading endoglycosidase activity is present in I-cells, and this activity is unaffected in the cells that lack functional lysosomes.

## DISCUSSION

The data reported here describe some characteristics of the PG of the cell surface and the degradative intermediates found in the interior of human fibroblasts. Comparison of the degradative intermediates of I-cells and normal cells illuminates our understanding of the early steps of degradation of cell surface PG, since those steps of PG degradation which are unaffected in I-cells are presumably mediated by non-lysosomal enzymes. Five key conclusions have been reached: a) The rates of shedding and internalization, and the composition of cell surface PG are not affected by the I-cell defect; b) The separation of GAG chains from protein cores is also unaffected; c) The endoglycosidic fragmentation of HS GAG chains is not affected by the I-cell defect; d) Internalization from the cell surface is a major pathway for delivery of HS GAGs to lysosomes; and e) The majority of intracellular storage GAGs are galactosaminoglycans. In contrast to HS GAGs, internalization from the cell surface pool is not a major pathway of entry for galactosaminoglycans into the degradative compartment.

In addition to the major conclusions concerning the degradation of GAGs in fibroblasts, we show evidence that a



substantial fraction (25% in normal cells and 40% in I-cells) of the  $^{35}\text{SO}_4$ -labeled macromolecules appearing in the medium during a chase (after a 24 hour labeling period) are derived from the intracellular (degradative) pool. This conclusion was derived by comparing the molecular weight distribution of macromolecules in the medium of trypsinized and untrypsinized cells (figs. 6 and 7). It is important to note that not all of the macromolecules in the medium of cultures after label-chase arrive by "shedding". An early report by Fratantoni and Neufeld (1), showed that 5% of the  $^{35}\text{SO}_4$ -labeled material liberated by normal fibroblasts (cells were labeled for 24 hours, replated, and chased) were macromolecules. The other 95%, under these conditions, consisted of degraded (free sulfate) molecules. We concur with these findings. However, our data also indicate that, under conditions where cells are chased without prior trypsinization, intracellular GAGs are also released without being degraded. This material makes up a small part of the total extracellular radioactivity, but makes up a relatively large part (>25%) of the macromolecules in the medium. Since these molecules are free GAG chains, they may need to be accounted for in interpreting the processes involved in shedding of PG from the cell surface (i.e. are GAG chains or whole PG shed into the medium from the cell surface?). A recent report by Bienkowski and Conrad (28) showed that free GAG chains were released from the intracellular compartment of a rat



hepatocyte cell line during a chase after a 24 hour label with  $^{35}\text{SO}_4^{-2}$ .

The presence of undegraded intracellular GAGs in the chase medium raises the question whether lysosomes fuse with the cell surface and release their contents into the culture medium. Alternatively, the GAG chains may have been liberated through cell death. Neither of these possibilities have been ruled out by our present experiments.

When the amounts of intracellular GAG chains released into the medium were taken into account, no difference was observed in rates of shedding of PG from the cell surface of I-cells when compared to normal cells (fig. 8). It has been previously suggested by others (4,11) that the bulk of the  $^{35}\text{SO}_4$ -labeled material released into the medium during a chase is by a mechanism involving proteolytic cleavage of the core protein of cell surface PG. If this is indeed the mechanism occurring in the fibroblasts, it is unlikely that lysosomal enzymes are involved, since the process is unaffected in I-cells.

An alternate fate of cell surface PG is internalization. Our data indicate approximately 60% of the total PG lost from the cell surface during a label-chase experiment are internalized. Although electron microscopic studies suggest that vesicular traffic in I-cells may be disrupted (29), it does not appear that traffic of cell surface PG to the cell interior is affected

inasmuch as no significant difference was observed in the rate of internalization by I-cells compared to normal cells. Analysis by gel filtration on Sepharose CL6B reveals that, in I-cells, none of the stored GAGs have the size characteristics of cell surface PG. In fact, none of the intracellular GAGs are associated with protein, whereas, virtually all of the cell surface GAGs are in the form of PG. These data suggest that the GAG chains are separated from core proteins, or that core proteins are degraded to such an extent that free GAG chains are produced. Furthermore, this process is unaffected in I-cells, suggesting it is a non-lysosomal enzyme activity.

Analysis of intracellular (storage) GAGs from I-cells on Sepharose CL6B columns reveals two major peaks of radioactivity, one large size class (region B, fig. 9) making up about 2/3 of the intracellular radioactivity, and one small size class (region C, fig. 9). The region B molecules are galactosaminoglycans and the region C molecules are HS. Kinetic evidence (fig. 11) shows that region C molecules (HS) enter the intracellular pool from the cell surface, whereas, region B molecules (galactosaminoglycans) are not derived from the cell surface. The size class, on Sepharose CL6B columns, of the HS GAG chains of cell surface PG (analyzed after alkaline  $\beta$ -elimination of the PG) was the same as that of the region B galactosaminoglycans stored inside the I-cell. However, after internalization, HS GAGs could be recovered only as

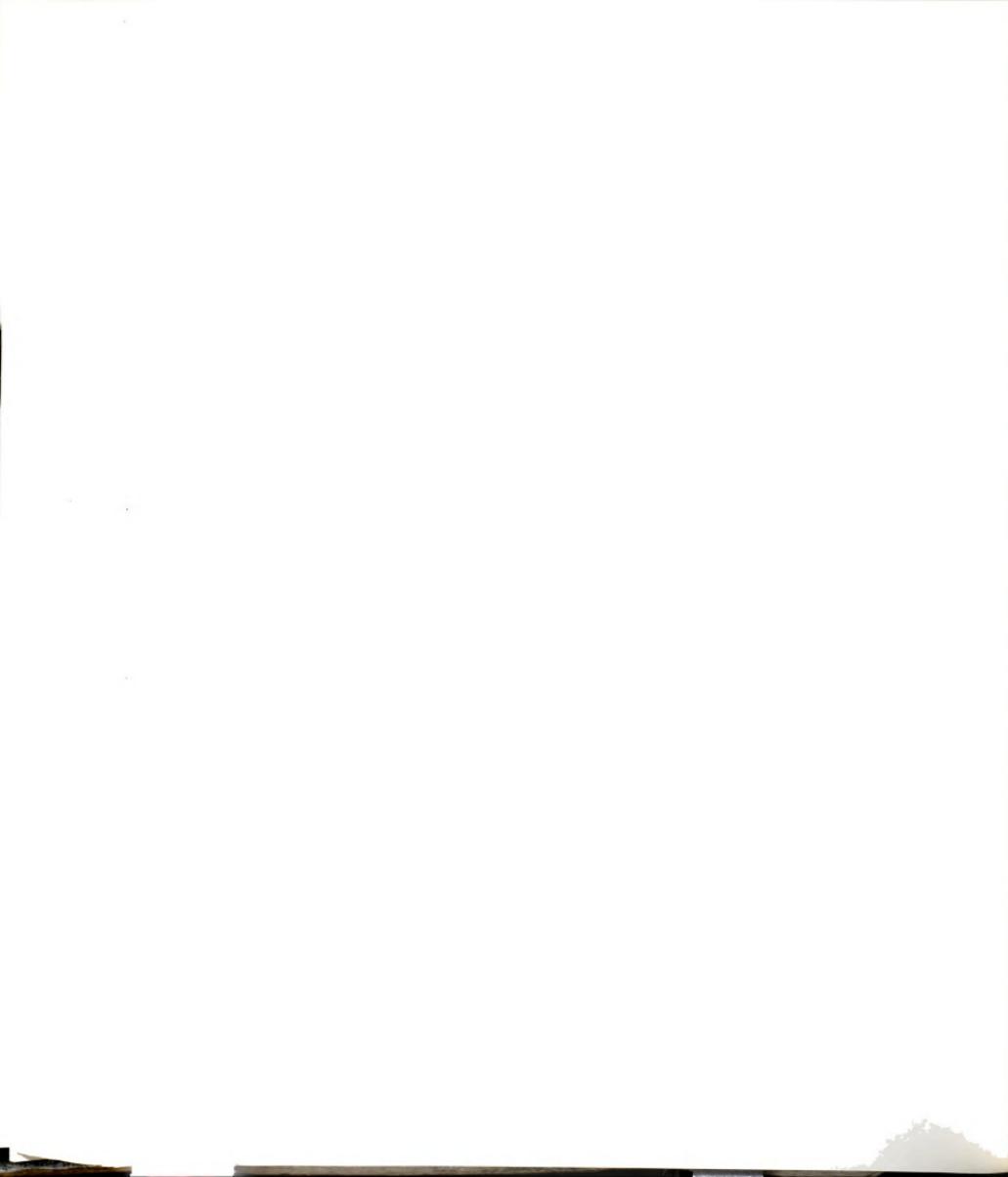
region C sized molecules. These data indicate that the HS GAG chains from the cell surface are reduced in size after internalization by a HS endoglycosidase. This observation is supported by analysis of Sanfilippo cells, which have a deficiency of a single lysosomal exoenzyme responsible for degradation of HS, and therefore store only HS GAGs in their lysosomes (8,27). The storage molecules of these cells migrate in region C of Sepharose CL6B columns, indicating the presence of a HS endoglycosidase activity which must have reduced the molecular weight of the GAGs (fig. 9). Hurler cells, which have a deficiency of a single lysosomal enzyme involved in the degradation of both galactosaminoglycans and HS, have two size classes of storage molecules identical to I-cells (Cowles, E.A., Brauker, J.H., Anderson, R.L., and Wang, J.L., unpublished data). Because the HS endoglycosidase activity is not affected by the I-cell defect, it is likely that it is a non-lysosomal enzyme activity.

Yanagishita and Hascall (10), studied the degradation of cell surface PG in rat ovarian granulosa cells. Their data indicate that there is a HS endoglycosidase activity which is not inhibited by the lysosomotropic amine chloroquine. Based on these observations it was suggested that the HS endoglycosidase was a non-lysosomal enzyme. Similar results were obtained by us, treating normal fibroblasts with  $\text{NH}_4\text{Cl}$  (Brauker, J.H., Laing, J.G., and



Wang, J.L., chapter 4, this thesis, manuscript in preparation).

HS endoglycosidase activity has been reported in liver cells (30), platelets (31-33), activated T-lymphocytes (34), and placenta (35,36). The pH optimum for the enzyme is around 6 (30,31). However, it has generally been described as a lysosomal enzyme. At least two enzymes (acid phosphatase and  $\beta$ -glucosidase) are present in I-cell fibroblasts in normal quantities (37,38). It is possible that the activity observed in our experiments is mediated by a lysosomal enzyme which can be transported to lysosomes by some mechanism other than the mannose 6-phosphate receptor transport pathway. It also can be argued, that the HS endoglycosidase is simply a lysosomal enzyme with a high pH optimum, and therefore was not affected by chloroquine or  $\text{NH}_4\text{Cl}$  treatments. Our present data, showing unimpaired HS endoglycosidase activity in I-cells, argues against this possibility. The enzyme would have to have two characteristics unlike other lysosomal enzymes (i.e., high pH optimum and an alternate transport mechanism). In addition, if the enzyme is a lysosomal enzyme having a high pH optimum, then it would not be expected to carry out the HS degradation efficiently in the low pH milieu of the lysosome even if it were transported to the lysosome of I-cells. The expected result would be that the amount of intracellular HS (migrating in region B) derived from I-cells and untreated normal cells would exceed the



corresponding amount in  $\text{NH}_4\text{Cl}$  treated normal cells. This was not the case (data not shown).

In summary, these data show that fibroblasts internalize HSPG from their cell surface, separate the GAG chains from core protein by an unknown mechanism, and degrade them to smaller fragments via an HS endoglycosidase. We hypothesize that, because these activities are unaffected by the I-cell defect, they occur in compartments of the cell other than lysosomes.

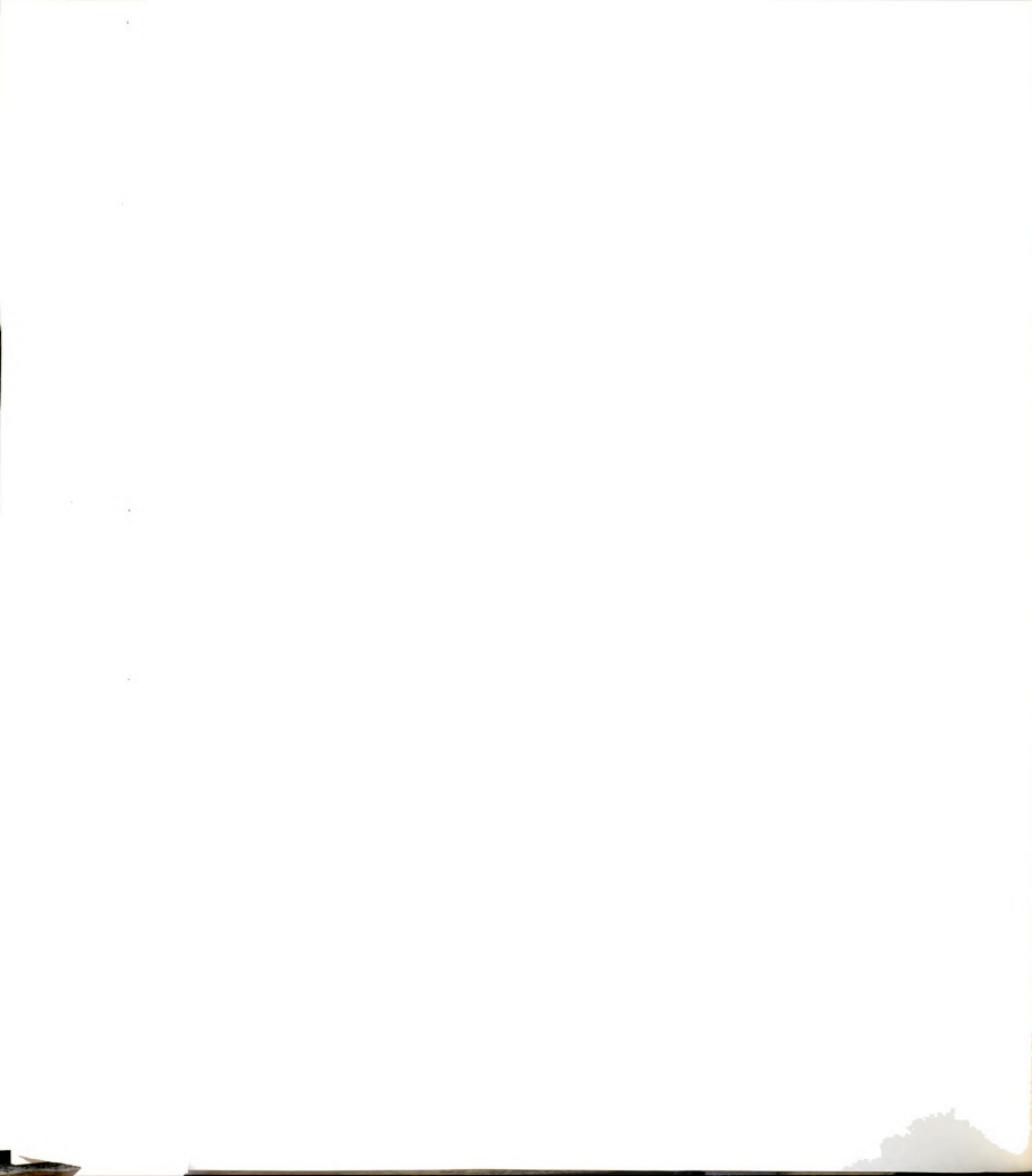


#### REFERENCES

1. Fratantoni, J.C., Hall, C.W., and Neufeld, E.F., (1968) Proc. Natl. Acad. Sci. 60, 699-706.
2. Hook, M., Kjellen, L., Johansson, S., and Robinson, J. (1984) Ann. Rev. Biochem. 53, 847-869.
3. Kresse, H., Teklof, W., von Figura, K., and Buddecke, E. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 943-952.
4. Johansson, S., Hedman, K., Kjellen, L., Christner, J., Vaheri, A., and Hook, M. (1985) Biochem. J. 232, 161-168.
5. Glimelius, B., Westermark, B., and Wasteson, A. (1977) Exp. Cell Res. 108, 23-30.
6. Vogel, K.G., and Peterson, D.W. (1981) J. Biol. Chem. 256, 13235-13242.
7. Cowles, E.A., Brauker, J.H., and Anderson, R.L. (1986) Exp. Cell Res. (In press).
8. Sly, W.S. (1980) In Metabolic Control and Disease (Brady, P.K., and Rosenberg, L.E. ed.) W.B. Saunders, Philadelphia. pp.545-581.
9. von Figura, K., and Klein, U. (1981) In Lysosomes and Lysosomal Storage Diseases (Dingle, J.T., Dean, R.T., ed.) Elsevier, New York. pp. 229-248.
10. Yanagishita, M., and Hascall, V.C. (1984) J. Biol. Chem. 259, 10270-10283.
11. Yanagishita, M. (1985) J. Biol. Chem. 260 11075-11082.
12. Dean, R.T., Jessup, W., and Roberts, C.R. (1984) Biochem. J. 217, 27-40.
13. Hickman, S., and Neufeld, E.F. (1972) Biochem. Biophys. Res. Comm. 49, 992-999.
14. Miller, A.L., Kress, B.C., Lewis, L., Stein, R., and Kinnon, C. (1980) Biochem. J. 186, 971-975.



15. Sahagian, G.G., Distler, J., and Jourdian, G.W. (1981) Proc. Natl. Acad. Sci. 78, 4289.
16. Steiner, A.W., and Rome, L.G. (1982) Arch. Biochem. Biophys. 214, 681-687.
17. von Figura, K., and Weber, E. (1978) Biochem. J. 176, 943-956.
18. Neufeld, E.F., Sando, G.N., Garvin, A.J., and Rome, L.H. (1977) J. Supramol. Struct. 6, 95-101.
19. Vladutiu, G.D., and Rattazze, M. (1979) J. Clin. Invest. 63, 595-600.
20. McKusick, V.A., and Neufeld, E.F. (1983) In The metabolic basis of inherited disease (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., and Brown, M.S., ed.) McGraw-Hill, New York. p.751-776.
21. Drinkwater, M.R., Corner, R.C., McCormick, J.J., and Maher, V.M. (1982) Mutat. Res. 106, 277.
22. Roff, C.F., Wozniak, R.W., Blenis, J., and Wang, J.L. (1983) Exp. Cell Res. 144, 333-344.
23. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
24. Brauker, J.H., Roff, C.F., and Wang, J.L. (1986) Exp. Cell Res. 164, 115-126.
25. Schmickel, R.D., Distler, J.J., and Jourdian, G.W. (1975) J. Lab. Clin. Med. 86, 672-682.
26. Neufeld, E.F., and McKusick, V.A. (1983) In The Metabolic Basis of Inherited Disease (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., and Brown, M.S., ed.) McGraw-Hill, New York. pp 778-787.
27. Klein, U., Kresse, H., and vonFigura, K. (1976) Biochem. Biophys. Res. Comm. 69, 158-166.
28. Bienkowski, M.J., and Conrad, E. (1984) J. Biol. Chem. 259, 12989-12996.
29. Brown, W.G., and Farquhar, M.G. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 5135-5139.
30. Hook, M., Wasteson, A., and Oldberg, A. (1975) Biochem. Biophys. Res. Comm. 67, 1422-1428.



31. Oosta, G.M., Favreau, L.V., Beller, D.L., and Rosenberg, R.D. (1982) *J. Biol. Chem.* 257, 11248-11255.
32. Wasteson, A., Hook, M., and Westermark, B. (1976). *FEBS lett.* 64, 218-221.
33. Yahalom, J., Elder, A., Fuks, Z., and Vlodavsky, I. (1984) *J. Clin. Invest.* 74, 1842-1849.
34. Naparstek, Y., Cohen, I.R., Guks, Z., and Vlodavsky, I. (1984) *Nature (London)* 310, 241-244.
35. Klein, U., and von Figura, K., (1979) *Hoppe-Seyler's Z. Physiol. Chem.* 360, 1465-1471.
36. Klein, U., and von Figura, K. (1976) *Biochem. Biophys. Res. Comm.* 73, 569-576.
37. Leroy, J.G., Ho, M.W., Mac Brinn, M.C., Zielke, K., Jacob, J., O'Brien, J.S. (1972) *Pediatr. Re.* 6, 752-757.
38. Berman, E.R., Kohn, G., Yatziv, S., and Stein, H. (1974) *Clin. Chim. Acta* 52, 115-124.



Chapter 4

Turnover of Cell Surface Proteoglycans in Cultured  
Fibroblasts: Internalization and  
Early Steps in Degradation

James H. Brauker, James G. Laing and John L. Wang

Department of Biochemistry  
Michigan State University  
East Lansing, MI 48824

## SUMMARY

The turnover of  $^{35}\text{SO}_4^{-2}$ -labeled glycosaminoglycans was studied in human fibroblasts labeled for 24 hours and chased in unlabeled medium with and without the lysosomotropic amine  $\text{NH}_4\text{Cl}$ . In the presence of  $\text{NH}_4\text{Cl}$ , the release of intracellular radioactivity from labeled cells was reduced, reflecting the known inhibitory action of  $\text{NH}_4\text{Cl}$  on intralysosomal enzyme activity. Analysis of the intracellular radiolabeled molecules revealed: a)  $\text{NH}_4\text{Cl}$  treated cells store two classes of GAG chains; one fraction of large molecules is composed mainly of galactosaminoglycans (75%), while another fraction of smaller molecules is composed mainly of heparan sulfates (HS); b) the large galactosaminoglycan GAG chains are delivered to the degradative compartment only during the labeling period, and not during the chase, consistent with the observation that there are few galactosaminoglycan PG at the cell surface; c) the small HS chains are internalized from the cell surface continuously during the chase; d) the internalized HS GAG chains are acted on by a HS endoglycosidase which reduces the GAG chain to about 1/6 of its original size. It is likely that the HS endoglycosidase is a nonlysosomal enzyme, since it is

active in cells treated with  $\text{NH}_4\text{Cl}$ , which raises the pH of lysosomes to pH 6 or higher.

Subcellular fractionation, on gradients of Percoll, of cells labeled with  $^{35}\text{SO}_4^{-2}$  in the presence of  $\text{NH}_4\text{Cl}$  yielded two species of GAG containing particles; a light fraction, which contained mainly the large GAG fragments, and a dense fraction, which contained smaller GAG fragments. Both the light and dense fractions contained galactosaminoglycans (60-70%) and HS (30-49%) as well as the lysosomal enzyme marker  $\beta$ -hexosaminidase. These results suggest that the initial steps in degradation of cell surface HSPG may not depend on an acidic lysosomal compartment, but may, in fact, involve a nonlysosomal endoglycosidase residing in an endocytotic vesicle.



## INTRODUCTION

Human fibroblasts synthesize glycosaminoglycans (GAGs) which are covalently linked to core proteins to make proteoglycans (PG). The PG are either secreted into the medium, transported to the cell surface (pericellular pool), or transported directly to a degradative compartment in the cell (1). Secreted PG contain mainly GAG chains of the class known as galactosaminoglycans [dermatan sulfate (DS) and Chondroitin sulfate (CS)], whereas cell surface PG contain mainly heparan sulfate (HS) GAGs (2-7). The intracellular (degradative) pool contains about 60-80% galactosaminoglycans and the remainder HS (2,5,6). In practically all cases studied, the intracellular GAGs are free polysaccharide chains, devoid of core proteins (2,5-7).

In the preceding paper (6)(chapter 3, this thesis), we have reported the turnover of cell surface PG in cultured fibroblasts from I-cell patients, which have a deficiency of hydrolases in the lysosomes (8). These I-cells store undegraded or partially degraded GAG chains in intracellular compartments (6,9). We have found that the early steps of degradation of the cell surface HS of I-cells are not defective even though the cells have a



generalized deficiency of intracellular lysosomal hydrolase activity. These early steps in the degradation of HSPG in I-cell include (6): (a) internalization of cell surface PG; (b) separation of GAG chains from core proteins (presumably by either endoglycosidase or protease activity); and (c) reduction in the size of the HS GAG chains by an endoglycosidase activity. We have also analyzed the degradation of galactosaminoglycan PGs. These PGs reach the cell surface either by a direct intracellular route from the Golgi, or by internalization from the soluble secreted pool. They are not a major component of the pericellular pool and are not internalized to any measurable extent from the pericellular pool. The galactosaminoglycans are recovered from the intracellular pool as free GAG chains of approximately the same size as newly synthesized GAG chains. Therefore, in I-cells, they are probably not acted on by an endoglycosidase.

In this paper, we provide evidence that the HS endoglycosidase activity of human fibroblasts is not affected by treatment with the lysosomotropic amine  $\text{NH}_4\text{Cl}$ . This, in conjunction with the evidence that the enzyme activity is not defective in I-cells (6), implies that the HS endoglycosidase activity occurs in a compartment other than lysosomes.

## MATERIALS AND METHODS

### Cell culture and radiolabeling procedures

Normal skin fibroblasts (SL66) were from Drs. J. McCormick and V. Maher [10]. Cells were maintained in Eagle's Minimal Essential Medium (MEM) (Gibco, Grand Island, New York) with 20% fetal bovine serum (FBS) (Hazelton Research Products, Denver, PA), 100 µg/ml streptomycin and 159 IU/ml penicillin (Sigma, St. Louis, MO) at 35 °C in humidified air. Medium for stock cultures was changed three times a week; these cells were passaged before the cultures reached confluence. Confluent monolayers of cells were removed from the dish with trypsin (400 µg/ml) in buffer A [11.5 mM sodium-potassium phosphate (pH 7.2), 140 mM NaCl, 2.68 mM KCl, and 0.01 mM EDTA], and plated in 6 well dishes (8 cm<sup>2</sup>/well, Costar, Cambridge, MA) for 1-2 days before labeling. All cultures were confluent at the time of labeling.

Cells were radiolabeled in streptomycin-free MEM with MgCl<sub>2</sub> substituted for MgSO<sub>4</sub>, 20% dialyzed FBS (Gibco), 50 µCi/ml H<sub>2</sub><sup>35</sup>SO<sub>4</sub> (carrier free, ICN, Irvine, CA).

### Harvest of cell fractions during label-chase experiments

The radioactivity was harvested either at the end of the radiolabeling period (0 hour) or after "chasing" in

unlabeled medium for various times, and designated as extracellular, pericellular, or intracellular radioactivity (11). The extracellular compartment was the medium, including cell secretions, which was removable by pipetting. The pericellular material was the radioactivity in the cell monolayer which could be removed from the cells by trypsinization. The intracellular fraction was the trypsin resistant radioactivity remaining with the cells after enzyme treatment. To harvest the radioactivity from these cell compartments, the medium (extracellular fraction) was removed and centrifuged for 3 minutes at 1330 g to remove cellular debris. The cell monolayers were washed several times with MEM; the washes were discarded. Pericellular and intracellular material were obtained by removal of the washed monolayer from the dish with trypsin (400 µg/ml in buffer A for 20 minutes at 35°C). Cells which remained loosely attached to the dish were dislodged by gentle pipetting. This procedure removed all the radioactivity from the dish. The cell suspension was centrifuged for 3 minutes at 1330 g. The supernatant, defined as the pericellular fraction, was decanted. The remaining cell pellet, or intracellular fraction, was dissolved in H<sub>2</sub>O and frozen at -20°C. Protease inhibitors were added to all of the cell fractions to make final concentrations of 1mM benzamidine HCl, 0.5 mM EDTA, 5 mM N-ethyl maleimide, 25 mM 6-aminohexanoic acid, and 1 mM phenyl methyl sulfonyl fluoride. Aliquots from

extracellular, pericellular, intracellular samples were in scintillation cocktail (1:9 v/v), which consisted of 7 g 2,5-diphenyloxazole (Research Products International Corp.), 338 ml Triton X-100 and 667 ml toluene. Radioactivity was determined on a Packard Tricarb 300CD liquid scintillation counter and expressed as disintegrations per minute (dpm).

For "chase" experiments, the monolayers were washed after removal of radiolabeling medium and incubated with MEM containing 20% FBS.  $\text{NH}_4\text{Cl}$  (1.0 M) in PBS was added to make a final concentration of 10 mM. Controls received PBS alone. Harvest procedures were performed at various times thereafter. In some experiments the cells were removed (prior to the chase) from the dish with trypsin (400  $\mu\text{g}/\text{ml}$  in buffer A), and replated in growth medium for one hour (6). The adherent cells were then washed and "chased" in unlabeled medium. At various times thereafter, the extracellular, pericellular, and intracellular compartments were harvested as above.

#### Gel filtration

Samples were chromatographed on columns of Sepharose CL6B (0.7 x 110 cm) or Sephadex G-50 (0.7 x 50 cm) at a flow rate of 5 ml/hour. The elution buffer was 0.2 M lithium acetate, 0.2 % SDS, and 1 mM  $\text{NaN}_3$ , pH 5, or 0.05 M Tris, 0.05 M  $\text{Na}_2\text{SO}_4$ , 4M guanidine HCl, 0.5% Triton-X 100, pH 7. Fractions of 1 ml were collected and analyzed for



radioactivity. Chromatographic profiles in these two buffers were identical.

#### Chemical and enzymatic degradation of GAGs

The radioactivity eluting in regions B or C (see fig. 1) of the Sepharose CL6B column (eluted with 0.05 M Tris, 0.05 M  $\text{Na}_2\text{SO}_4$ , 4 M guanidine HCl, 0.5% Triton X-100) was pooled and precipitated in 2 volumes of cold 90% ethanol, heated for 2 minutes in a 95° C water bath, and then cooled for 5 minutes on ice. The samples were then centrifuged for 10 minutes at 12,000 g. The supernatant was removed and the precipitate was suspended in  $\text{H}_2\text{O}$ . Aliquots of 0.3 ml of these samples were treated as follows: a) For digestion with chondroitin ABC lyase (C'ase, Miles, Elkhart, IN), 0.3 ml of a solution containing 0.1 M Tris, 0.1 M Na acetate, 0.05 unit/ml C'ase (pH 7.3), were added and incubated for 3 hours at 37° C; b) For nitrous acid degradations, 37.5  $\mu\text{l}$  of 2.4 M  $\text{NaNO}_2$ , and 37.5  $\mu\text{l}$  glacial acetic acid were added for 80 minutes at 25° C, followed by addition of 75  $\mu\text{l}$  of 3 M sodium sulfamate to stop the reaction. The degradation products were analyzed by gel filtration on Sephadex G-50 columns.

#### Cell fractionation

Post nuclear supernates were prepared from cells grown to confluency in 150  $\text{cm}^2$  flasks as previously described by Rome et al (12). Cells were removed from the flask with trypsin (400 $\mu\text{g}/\text{ml}$  in buffer A) pelleted at 1330 g for 3



minutes, suspended in 0.25 M sucrose, 10 mM EDTA, recentrifuged, and resuspended in 4 ml of the same buffer. This sample was disrupted in a nitrogen cavitation bomb (Parr Instrument Co., Moline, Ill.) for 15 minutes at 30 lbs/in<sup>2</sup>. The sample was then further homogenized with 3 strokes of a pestle in a 5 ml glass cell homogenizer. The nuclei were pelleted by centrifugation for 10 minutes at 1330 g, the supernatant was saved and the nuclear pellet was resuspended in 1.5 ml of the 0.25 M sucrose solution, rehomogenized with three strokes of the pestle, and recentrifuged. The second supernatant was combined with the first. This post nuclear supernatant was separated on a Percoll gradient. 27% Percoll gradients (13) were prepared as follows: 9 volumes of Percoll (as supplied by Pharmacia, Piscataway, NJ) were mixed with 1 volume of 2.5 M sucrose containing 10 mM EDTA, pH 6.8. Thirty ml of 27% Percoll was layered onto a cushion of 5 ml of sucrose (2.5 M containing 10 mM EDTA, pH 6.8) in a 39 ml quick seal centrifuge tube (Beckman Instruments, Fullerton, CA); 4 ml of sample was layered onto the Percoll. Centrifugation was performed in a Beckman vTi 50 vertical rotor for 1 hour at 33,000 g at 4°C. At the end of the centrifugation, a glass tube was inserted from the top of the centrifuge tube through the gradient to the bottom of the tube. Fractions (1 ml) were collected by drawing from the bottom of the tube with a peristaltic pump. Samples were stored at -20 C. The lysosomal enzyme  $\beta$ -hexosaminidase was assayed

as follows: Samples were thawed, and 50  $\mu$ l were added to 250  $\mu$ l 4-methylumbelliferyl- $\beta$ -D-glucoside (Sigma) (1.2 mM in 0.1 M sodium acetate, 0.1% Triton X-100, pH 4.4) for 30 minutes at 37°C. The reaction was stopped by addition of 1 ml 0.5 M glycine, 0.5 M Na<sub>2</sub>CO<sub>3</sub>. Fluorescence was measured on a Perkin-Elmer 650-40 fluorescence spectrophotometer.



## RESULTS

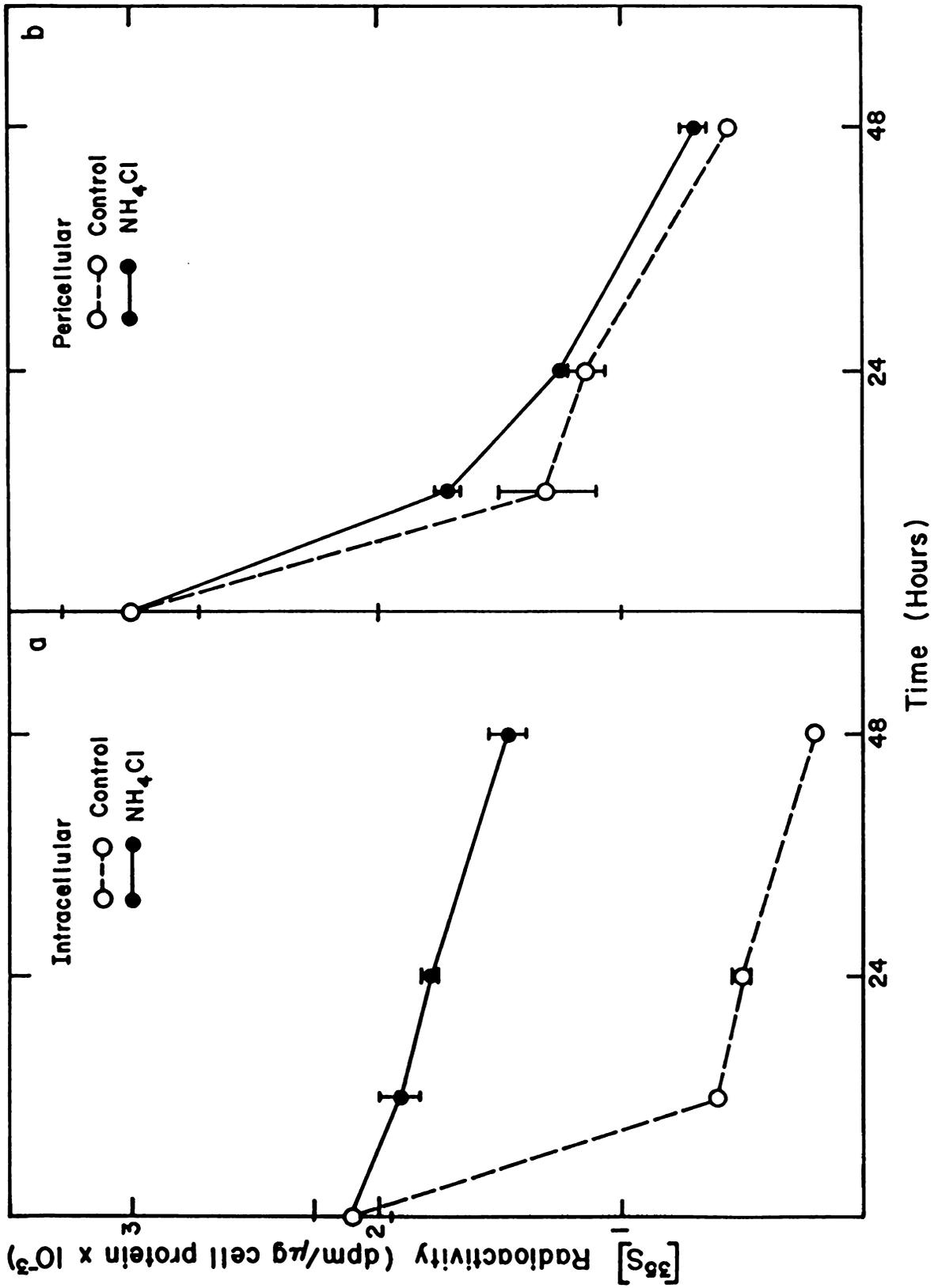
### The effect of $\text{NH}_4\text{Cl}$ on the turnover of $^{35}\text{SO}_4$ -labeled components

SL66 cells were labeled with  $^{35}\text{SO}_4^{-2}$  for 24 hours. The labeled cultures were chased in unlabeled medium in the presence and absence of  $\text{NH}_4\text{Cl}$ . The intracellular and pericellular fractions were isolated and the radioactivity quantitated (fig. 1). Cells in the absence of  $\text{NH}_4\text{Cl}$  rapidly lost most of the initial radioactivity during the first 12 hours (approximately 30% of initial value) (fig. 1a). In contrast, cells treated with  $\text{NH}_4\text{Cl}$  lost intracellular radioactivity at a slow rate; the overall loss during the 48 hour chase period was about 25%. This difference in the turnover of [ $^{35}\text{S}$ ]GAG is consistent with the known effect of  $\text{NH}_4\text{Cl}$ , a potent inhibitor of intracellular lysosomal hydrolase activity because it increases intralysosomal pH (14).

The radioactivity in the pericellular compartment decreased with time in cells treated both with and without  $\text{NH}_4\text{Cl}$  (fig. 1b). No difference was observed at any time point between the two conditions. Thus, it appears that the turnover of the GAGs in the pericellular compartment of cells is not affected by lysosomal dysfunction. This



Figure 1. Kinetics of the turnover of  $^{35}\text{SO}_4$ -labeled components in the (a) intracellular and (b) pericellular compartments of untreated ( $\circ$ ) and  $\text{NH}_4\text{Cl}$  treated ( $\bullet$ ) cells. Cells were labeled with  $^{35}\text{SO}_4^{-2}$  ( $50 \mu\text{Ci/ml}$ ) for 24 hours, washed and chased in unlabeled culture medium. The intracellular and pericellular fractions were isolated as described in Materials and Methods and their radioactivity quantitated. The data represent the averages of duplicate determinations (error bars indicate standard deviations where larger than symbols).



notion is consistent with the conclusion derived from our parallel study comparing GAG turnover in I-cells with normal cells (6).

Characterization of intracellular GAGs in  $\text{NH}_4\text{Cl}$  treated cultures

We had observed in previous label-chase experiments that the intracellular PGs of SL66 cells have, on gel filtration on Sepharose CL6B, a predominant peak around Kd 0.42 (region B, fig. 2a)(6). This peak decreases with time of the chase period (open circles, fig. 2 c,d), consistent with the kinetic data shown in fig. 1. In contrast,  $\text{NH}_4\text{Cl}$  treated cells (closed circles, fig. 2 b,c,d) retain radioactivity in the intracellular pool during the chase period. Moreover, in addition to the major 0.42 Kd peak, a shoulder of radioactivity becomes prominent during the chase period. This shoulder of radioactivity is centered around 0.72 Kd, and is referred to as region C material. This result mimics that obtained when labeled I-cells were chased and the intracellular radioactivity was analyzed by filtration on columns of Sepharose CL6B (6).

Radioactivity migrating in regions B and C of Sepharose CL6B columns (derived from SL66 cells labeled for 24 hours and chased for 24 hours in the presence of  $\text{NH}_4\text{Cl}$ ) were pooled and subjected to treatment with C'ase. The intracellular material in region B was sensitive to C'ase; 75% of the radioactivity was reduced in molecular weight by the enzyme treatment (fig. 3a,b). In contrast, only 25% of

Figure 2. Gel filtration of  $^{35}\text{SO}_4^{-2}$ -labeled components derived from the intracellular fraction of untreated cells (O—O) and  $\text{NH}_4\text{Cl}$  treated cells (●—●) during a label (50 uCi/ml, 24 hour) and chase experiment. Samples representing 10% of the material from the respective cultures were chromatographed on a column of Sepharose CL6B (0.7 X 110 cm) equilibrated with 0.2 M lithium acetate, 0.2% SDS, 1 mM  $\text{NaN}_3$ , pH 5. The horizontal bars designated A, B, and C highlight regions of interest discussed in the text. The 0 hour time point has open circles to point out that the cells were not exposed to  $\text{NH}_4\text{Cl}$  before the beginning of the chase.

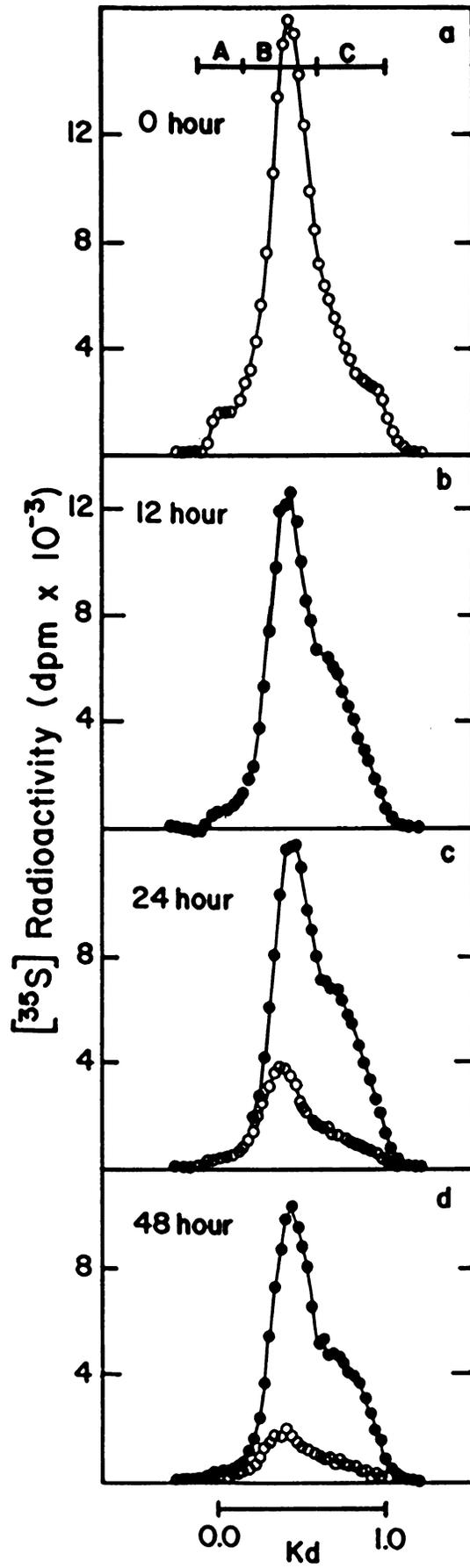
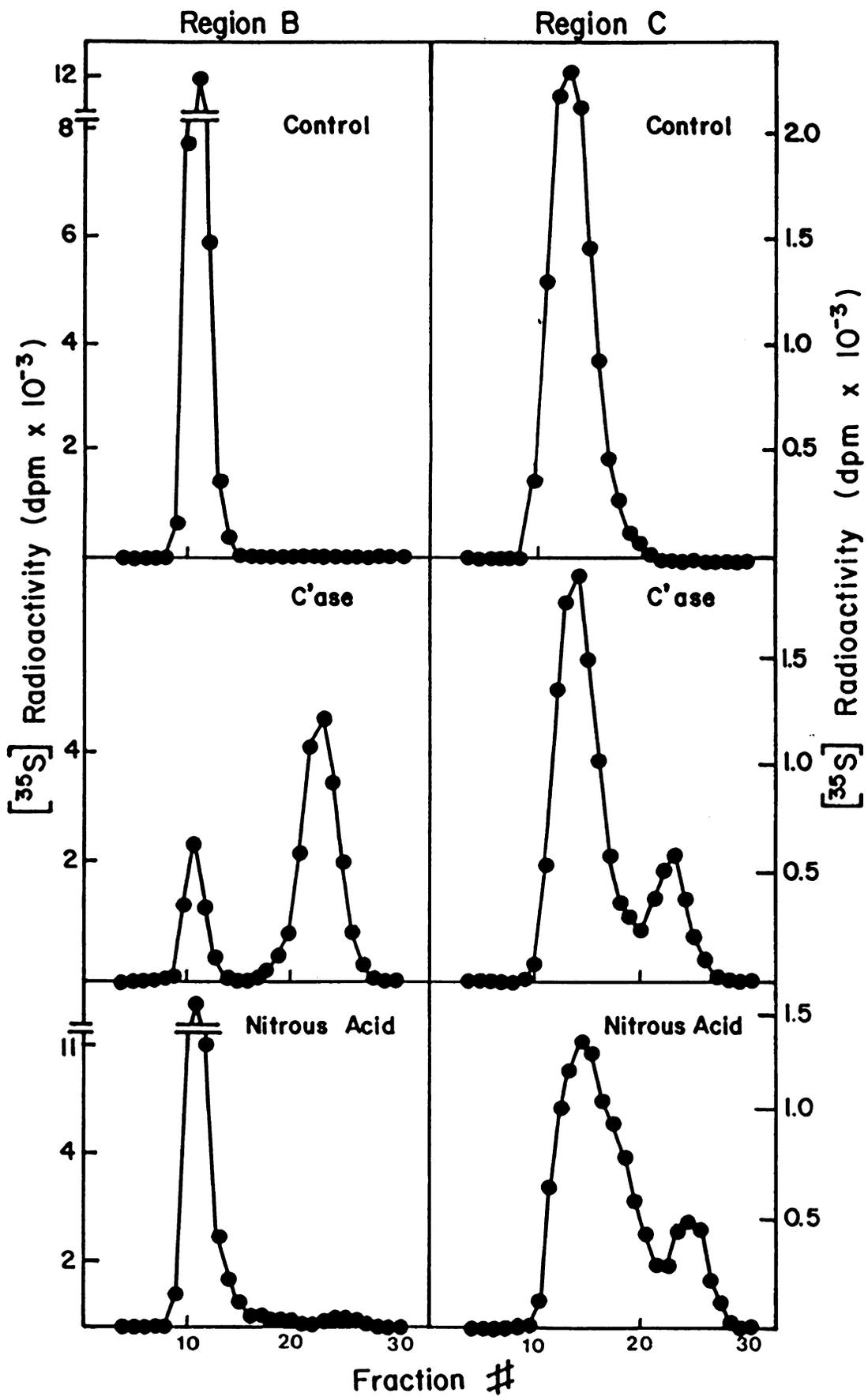




Figure 3. Gel filtration of  $^{35}\text{SO}_4^{-2}$ -labeled components corresponding to region B and C (figure 2) of the intracellular fraction of  $\text{NH}_4\text{Cl}$  treated cells after chemical treatments. Cells were labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ , 24 hours) and chased for 24 hours. The intracellular radioactivity was fractionated on columns of Sepharose CL6B as described in legend to fig. 2. The pooled fractions corresponding to regions B and C were chromatographed without treatment (a, d), after C'ase digestion (0.025 unit/ml, 3 hours, 37° C) (b, e), and after nitrous acid treatment (0.24 M, 80 minutes, 25° C) (c, f) on columns of Sephadex G-50 (0.7 X 50 cm) equilibrated with 0.2 M lithium acetate, 0.2% SDS, 1 mM sodium azide, pH 5.





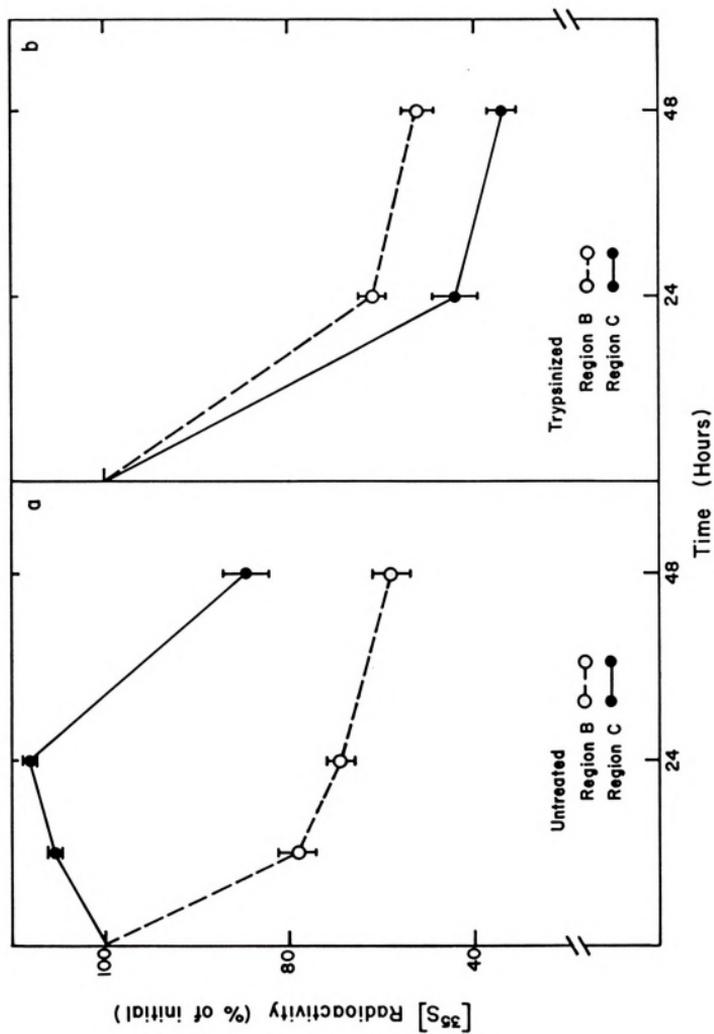
the intracellular material from region C was reduced in molecular weight by C'ase (fig. 3c,d). Conversely, the material from region B was not affected by nitrous acid treatment (fig. 3c), but the material from region C was sensitive to the chemical treatment (fig. 3f). These results suggest that region B material consisted mainly of galactosaminoglycans while region C was predominantly HS. We had previously shown that, for I-cells, the intracellular PG migrating in region B were galactosaminoglycans and the intracellular PG of region C was predominantly HS (6). Therefore, the chemical composition as well as the size distribution of storage material in  $\text{NH}_4\text{Cl}$  treated normal cells is similar to that of I-cells.

The effect of  $\text{NH}_4\text{Cl}$  on the turnover of the intracellular GAGs

Cells were labeled with  $^{35}\text{SO}_4^{-2}$  for 24 hours. The radiolabeled cultures were trypsinized to remove the pericellular PG and then chased in the presence of  $\text{NH}_4\text{Cl}$ . Control cultures, without any trypsin treatment, were chased with and without  $\text{NH}_4\text{Cl}$  in parallel. The intracellular GAGs in region B and C of Sepharose CL6B columns were quantitated (fig. 4). The radioactivity in region B of the Sepharose CL6B column decreased to approximately 50% of the initial value during the 48 hour chase period (fig. 4a). This loss of radioactivity was



Figure 4. Kinetics of the turnover of  $^{35}\text{SO}_4^{-2}$ -labeled components corresponding to regions B and C (fig. 2) of the intracellular fraction of  $\text{NH}_4\text{Cl}$  treated cells. Cells were labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ , 24 hours). Parallel cultures were trypsinized (400  $\mu\text{g/ml}$  in buffer A) and replated or left untreated as controls. The cells were then chased in unlabeled culture medium. The intracellular fraction was fractionated on columns of Sepharose CL6B as described in legend to fig. 2. The radioactivity migrating in the fractions corresponding to region B and C was quantitated. The data represents the averages of duplicate determinations (error bars indicate standard deviations).



unaffected by pretreatment with trypsin (compare open circles in fig. 4a and 4b). These results are similar to those obtained when the turnover of region B material of the intracellular GAGs of I-cells was quantitated (6). Apparently, few galactosaminoglycans are internalized from the cell surface because the intracellular galactosaminoglycan pool (region B) is not affected by prior trypsin treatment.

In contrast, the rate of loss of radioactivity from region C of the Sepharose CL6B column was sensitive to trypsin treatment (compare closed circles in fig. 4a and 4b). In untrypsinized cultures chased in the presence of  $\text{NH}_4\text{Cl}$ , the radioactivity in region C actually increased to a level beyond the initial value during the first 24 hours of the chase period. Subsequently, the level of radioactivity decreased (fig. 4a). In trypsinized cultures, the radioactivity of region C decreased monotonically to about 50% of the initial value (fig. 4b). These results suggest that intracellular GAGs migrating in region C of the column are derived from the cell surface. Trypsinization prior to the chase period deprived the intracellular compartment of the pool of PG originally at the cell surface. These results are again consistent with previous findings, based on a comparative analysis of normal and I-cells, that cell surface HSPG are internalized and that the GAG chains, which originally (while in the pericellular pool) migrated at 0.42 Kd on Sepharose CL6B



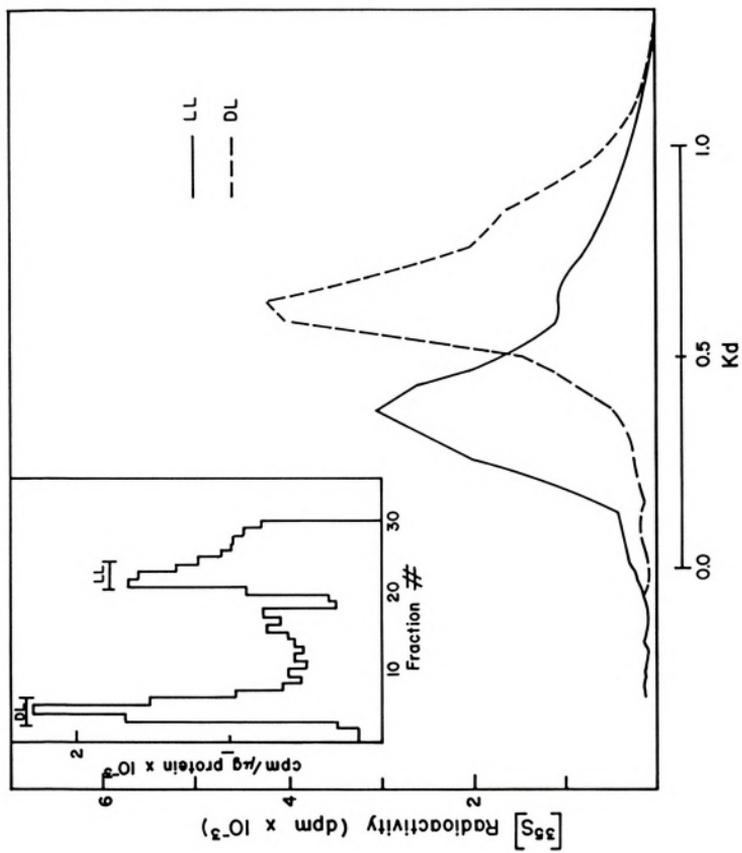
columns, were degraded to fragments migrating in region C of the column.

Analysis of the intracellular GAGs in subcellular fractions

Cells were labeled with  $^{35}\text{SO}_4^{-2}$ , in the presence of  $\text{NH}_4\text{Cl}$  for 24 hours. The cells were then harvested by trypsinization and disrupted in a nitrogen decavitator and subjected to subcellular fractionation in 27% Percoll gradients. When the radioactivity due to  $^{35}\text{SO}_4^{-2}$  was monitored in the fractions of the gradient, two major peaks of  $^{35}\text{S}$ -label were found (fig. 5, inset). When the lysosomal enzyme marker,  $\beta$ -hexosaminidase, was assayed in the individual fractions, two peaks of enzyme activity were found, corresponding in position to the radiolabeled peaks. The results are consistent with the subcellular fractionation studies of Rome and coworkers (12), who characterized these peaks of lysosomal enzyme activity. For this reason, these two peaks were designated as dense lysosomes (DL) and light lysosomes (LL) in accordance with the density of the Percoll fraction in which these two peaks were found and the nomenclature of Rome et al. (12).

The radioactive fractions corresponding to LL were pooled and analyzed on Sepharose CL6B columns (fig. 5). The majority of the [ $^{35}\text{S}$ ]GAGs chromatographed at a position corresponding to 0.42 Kd; there was also a shoulder at Kd = 0.62. In contrast, the pooled fractions derived from DL

Figure 5. Percoll density gradient fractionation of  $\text{NH}_4\text{Cl}$  treated cells. Cells were labeled with  $^{35}\text{SO}_4^{-2}$  (50  $\mu\text{Ci/ml}$ , 24 hours) in the presence of  $\text{NH}_4\text{Cl}$ . The intracellular fraction was collected, and the cells were disrupted by nitrogen cavitation at 30 lbs./in<sup>2</sup> for 10 minutes. The nuclei were removed by centrifugation and the post nuclear supernatant was layered onto 27% Percoll and centrifuged at 33,000 g for 1 hour. Fractions of 1 ml were collected and the radioactivity in each fraction was quantitated (inset). The fractions were collected from dense to light (left to right). Samples from dense (DL) and light (LL) peaks were pooled (indicated by bars) and chromatographed on columns of Sepharose CL6B (0.7 X 100 cm) in 0.2 M lithium acetate, 0.2% SDS, 1 mM  $\text{NaN}_3$ , pH 5. DL, (---); LL, (—).





yielded predominantly GAGs at 0.62 Kd. When samples of LL and DL were subjected to C'ase treatment, about 42% of the material in the LL, and about 32% of the material in the DL were resistant to degradation. These data indicate that the pooled LL fraction contains approximately 42% HS and 58% galactosaminoglycans and the pooled DL fraction contains approximately 32% HS and 68% galactosaminoglycans.



## DISCUSSION

The data presented here extend our previous results derived from studies of I-cell fibroblasts (6), which implicated a HS endoglycosidase activity that is functional in cells which have dysfunctional lysosomes. We further show here that: (a) the characteristic features of GAG storage by the I-cell can be mimicked in normal cells (SL66 fibroblasts) by treatment of the cells with  $\text{NH}_4\text{Cl}$ ; (b) the HSPG internalized from the cell surface are acted on by an HS endoglycosidase, and this activity is not inhibited by  $\text{NH}_4\text{Cl}$ ; and (c) two size classes of GAGs can be recovered at different densities after Percoll gradient fractionation of homogenized cells.

Both galactosaminoglycans and HS were recovered from the intracellular (storage) pool of  $\text{NH}_4\text{Cl}$  treated cells after a label-chase experiment. The HSPG of the cell surface contain GAG chains which migrate on Sepharose CL6B columns at 0.42 Kd (6). In contrast, the HS GAG chains found in the cell interior (storage pool) of cells treated with  $\text{NH}_4\text{Cl}$  are predominantly of a smaller size class (0.72 Kd). Thus, we concluded that the HS endoglycosidase activity is not inhibited by  $\text{NH}_4\text{Cl}$ , indicating that the enzyme is active at pH 6 or higher [the pH of  $\text{NH}_4\text{Cl}$  treated



lysosomes (14)], in contrast to acid hydrolases in the lysosome.

We have previously reported that I-cell fibroblasts [which are defective in the transport of lysosomal enzymes to lysosomes (8)] store HS GAG chains as fragments similar in size to those stored by  $\text{NH}_4\text{Cl}$  treated normal cells shown here (0.72 Kd) (6). Considered together, these data suggest that the HS endoglycosidase is active at pH 6 or higher and that it is transported to its site of action in the cell by some mechanism other than the known pathway for transport of lysosomal enzymes [mannose 6-phosphate receptor transport (8)]. If the enzyme were a lysosomal enzyme with a high pH optimum, as has been reported for HS endoglucuronidase in other systems (15,16), it would be expected to be active in  $\text{NH}_4\text{Cl}$  treated cells. In untreated normal cells, however, the activity of such an enzyme at low pH is expected to be reduced. Moreover, in I-cells, such a lysosomal enzyme is expected to be misdirected during transport and secreted. Analysis of the molecules migrating in region B of Sepharose CL6B columns from untreated cells,  $\text{NH}_4\text{Cl}$  treated normal cells, and I-cells, indicates no substantial difference in the amount of large (0.42 Kd) HS chains. This can be determined by comparing the area under the curve in region B of the Sepharose CL6B column profiles of  $\text{NH}_4\text{Cl}$  treated and untreated cells after a 24 hour chase. The total radioactivity of untreated cells is 32% of that of  $\text{NH}_4\text{Cl}$  treated cells (fig 2c), while

25% of the radioactivity of  $\text{NH}_4\text{Cl}$  treated cells is HS (fig. 3b). Thus, it is clear that HS GAG chains are not degraded at a slower rate under conditions in which the lysosomes are acidic. Otherwise, much higher amounts of radioactivity would be recovered in region B of untreated cells (fig. 2c).

Our observations are similar to those reported for ovarian granulosa cells in culture (17). These cells express both HSPG and DSPG on their cell surface. Both PGs are internalized by the cells, and the GAG chains are separated from protein cores. DS GAG chains are then acted on by exoenzymes without further processing, whereas HS GAG chains were further reduced in size by an apparent HS endoglycosidase activity. This enzyme activity was unaffected by the lysosomotropic amine chloroquine, which inhibits intracellular lysosomal enzyme activity by raising the pH of the lysosome (14).

The results of the present and previous studies provide the basis for considering a schematic diagram to describe the metabolic fate of the PGs of a cell (fig. 6). Galactosaminoglycan PG are synthesized in the Golgi and then secreted into the medium (pathway 1, fig. 6) Substantial amounts of the GAG chains of these PG become associated with intracellular degradative compartments. Two possible pathways of entry into the degradative compartments are proposed; (a) diversion of the GAGs from the secretory pool into the degradative pool (pathway 2,



Figure 6. Schematic diagram of proposed pathways for the degradation of GAGs in fibroblasts. Both galactosaminoglycan and HS PGs are synthesized in the Golgi (G). The galactosaminoglycan PGs are then secreted from the cell (pathway 1). They reach light lysosomes (LL) by diversion from the secretory pathway (pathway 2) or by receptor mediated endocytosis after secretion (pathway 3). HSPG are transported to the cell surface as membrane components (pathway 4). They reach the LL by internalization from the cell surface (pathway 5). GAGs in the LL exist as free GAG chains. From here, they may be degraded directly by exoenzymes (pathway 6), or they may be transported (or the LL may mature) to dense lysosomes (DL) (pathway 7). Exoenzyme activity in the DL completes the process of degradation, releasing free monomers into the medium (pathway 8). At some point, shortly after internalization, HS GAG chains are cleaved by HS endoglycosidase.

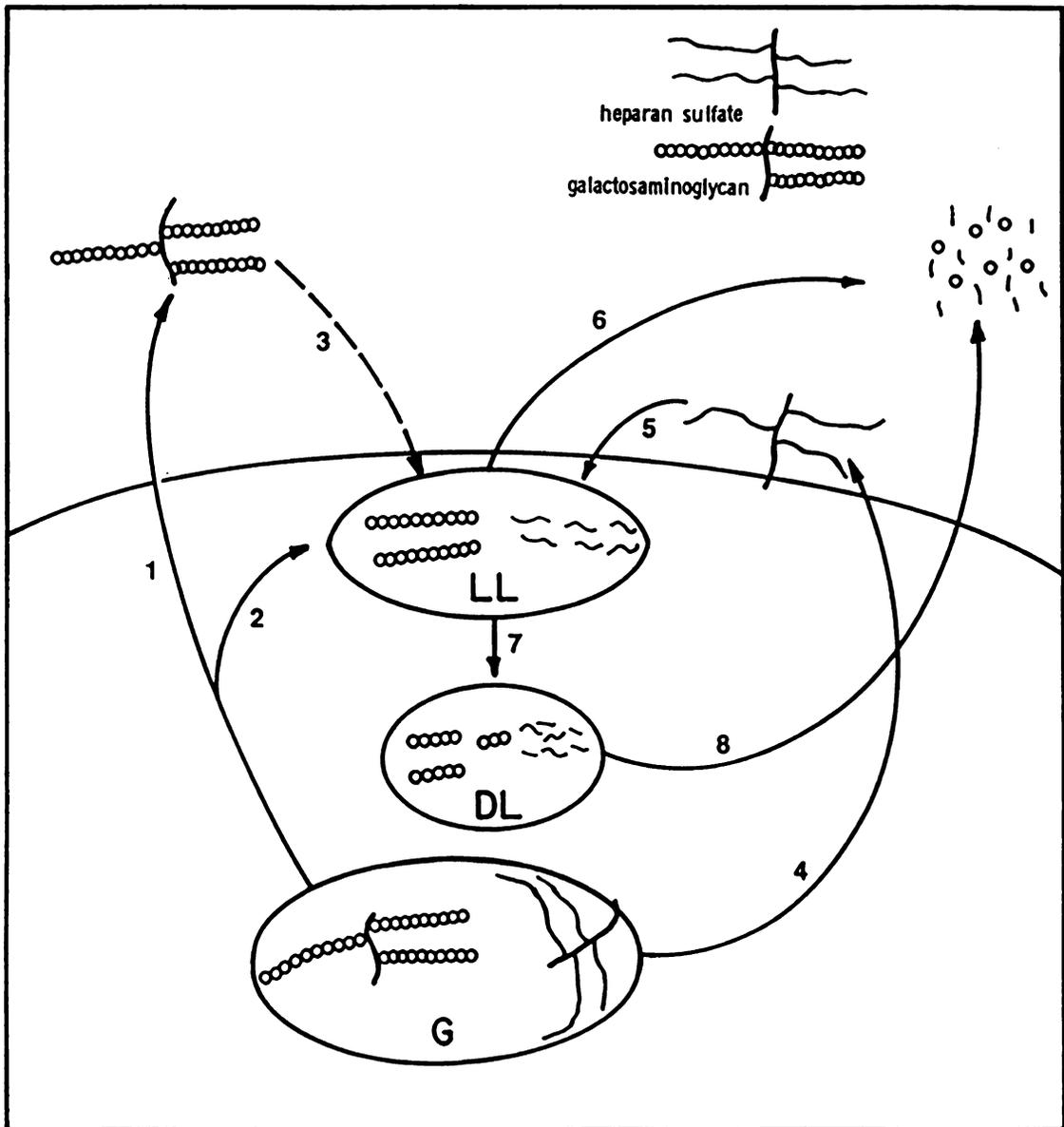


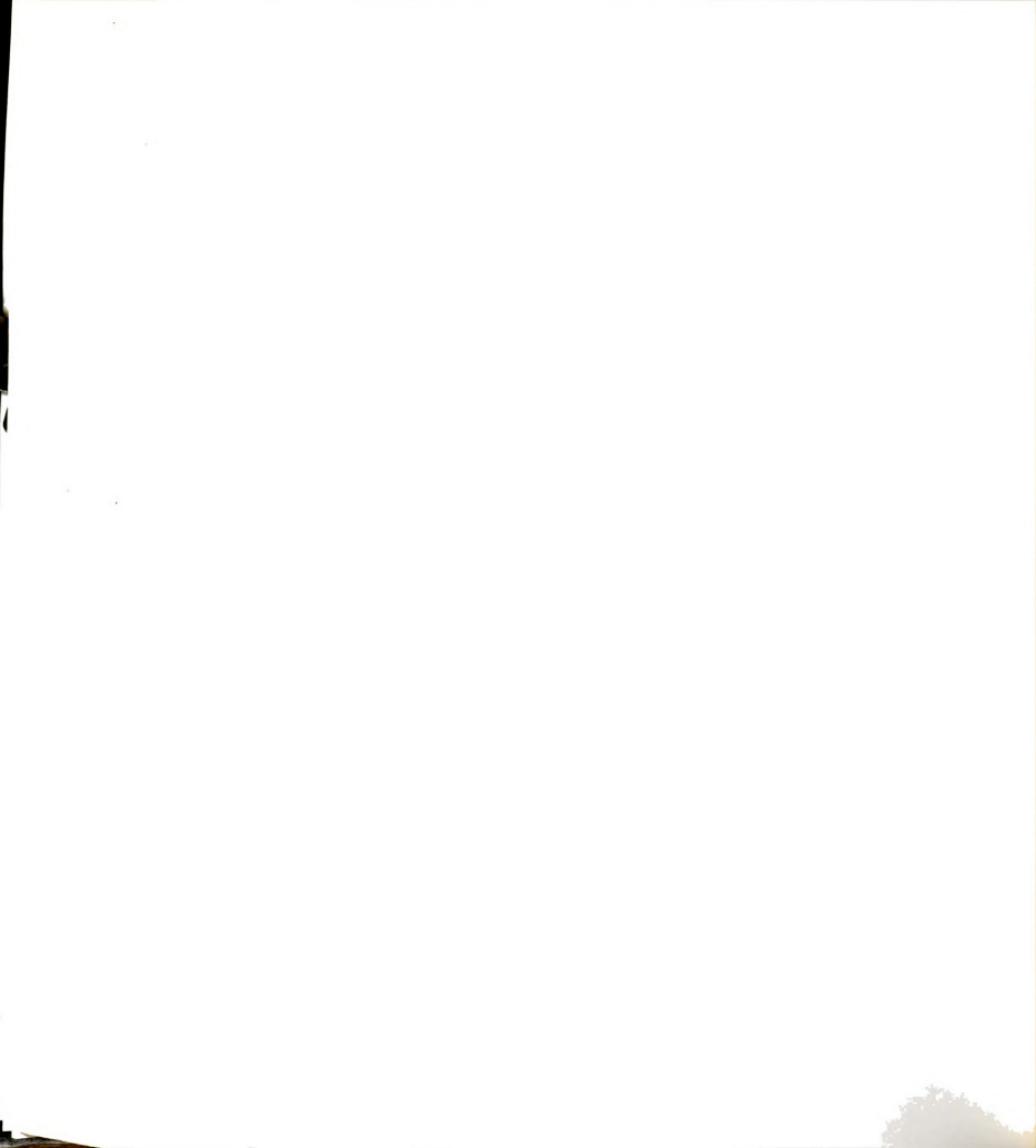
fig. 6) and (b) secretion and recapture via cell surface receptors (pathway 3, fig. 6). In contrast, the HSPG are synthesized mainly as membrane associated components which are transported to the cell surface (pathway 4, fig. 6). These PG must be internalized to reach degradative compartments (pathway 5, fig.6).

The degradative phase of PG metabolism begins in a group of vesicles collectively known as LL (fig. 6). This LL is a light membrane fraction that contains cell membranes, endosomes, multivesicular bodies, and light lysosomes (C.F. Roff, personal communication). Collectively, the contents of LL include both galactosaminoglycans (58%) and HS (42%). The contents of the LL may be degraded directly by exoenzymes (pathway 6, fig. 6) with concomitant release of free sulfate (20), or they may be transported to DL (pathway 7, fig.6) where degradation is completed and free sulfate is released (pathway 8, fig. 6). The DL is thought to represent mature lysosomes (12). The pooled DL fraction yielded 32% HS and 68% galactosaminoglycans. Thus, it appears that both galactosaminoglycans and HS are fairly evenly distributed between the two Percoll gradient fractions.

It is not known whether a single endosome/vesicle contains both types of GAGs. The intriguing possibility exists that LL actually consists of two or more subpopulations of vesicles. One type of vesicle may segregate galactosaminoglycans, either recaptured from the



medium or diverted from the synthetic pool. Another type of vesicle may contain mainly HSPG derived from the cell surface. Implicit in this kind of notion is that galactosaminoglycan PG and HSPG are initially degraded in separate vesicles. Such segregation of PG derived from different sources may account for the phenomenon, observed in our earlier studies, that mannose 6-phosphate prevents degradation of a population of GAGs derived from the extracellular matrix (18). In any case, better fractionation procedures [e.g. 27% Percoll followed by 17% Percoll gradient fractionation (19)] need to be utilized to separate the endosomes, multivesicular bodies, etc., of the LL fraction. With these separation procedures, the idea of separate vesicles for galactosaminoglycan and HS segregation and degradation can be more rigorously tested.



## REFERENCES

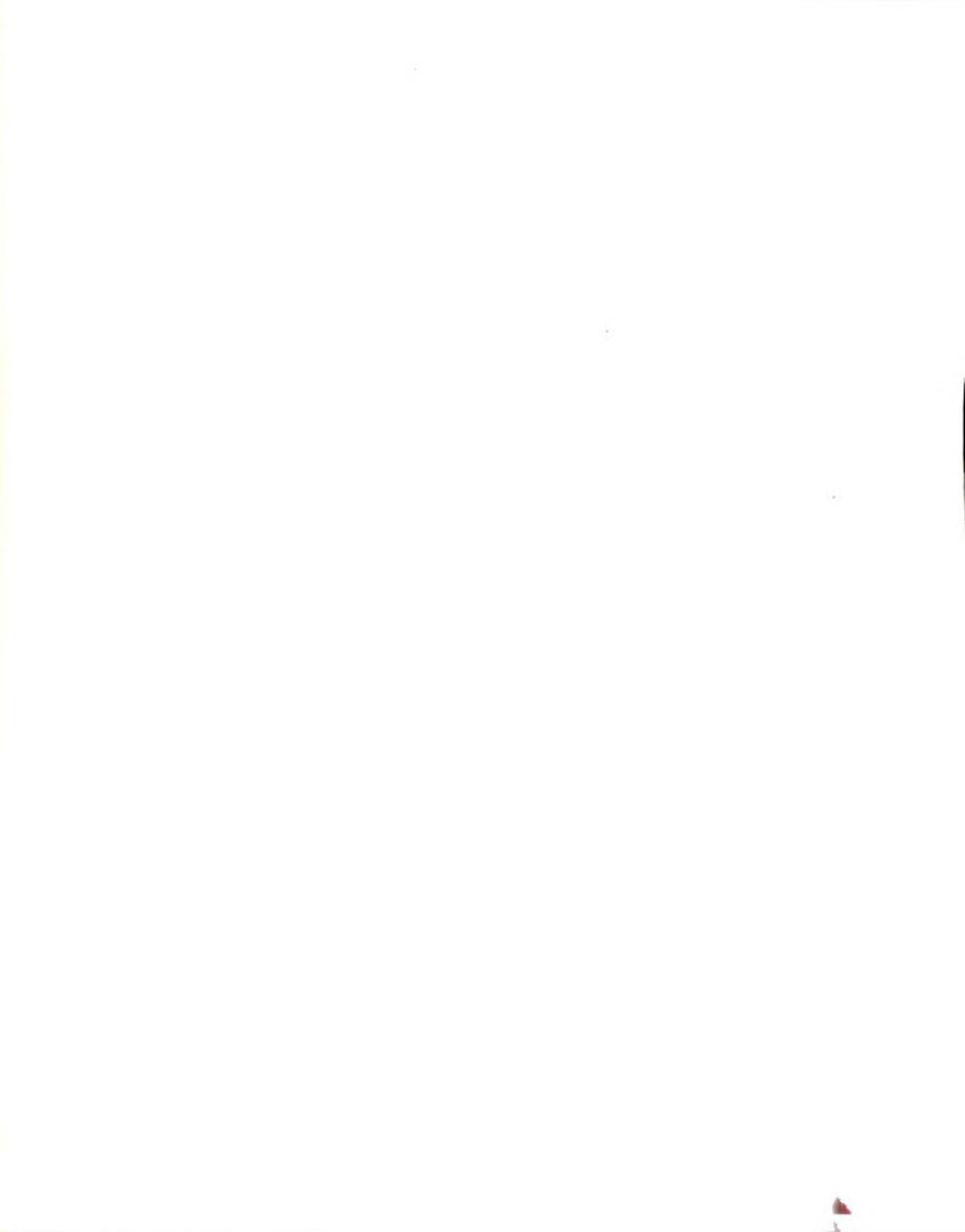
1. Fratantoni, J.C., Hall, C.W., and Neufeld, E.F., (1968) Proc. Natl. Acad. Sci. 60, 699.
2. Vogel, K.G., and Peterson, D.W. (1981) J. Biol. Chem. 256, 13235.
3. Malmstrom, A., Carlstedt, I., Aberg, L., and Fransson, L.A. (1975) Biochem. J. 151, 477-489.
4. Sjoberg, I., Carlstedt, I., Coster, L., Malmstrom, A., and Fransson, L.A. (1979) Biochem. J. 178, 257-270.
5. Kresse, H., von Figura, K., Buddecke, E., and Fromme, H.G. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 929941.
6. Brauker, J.H., and Wang, J.L. (1986) J. Cell. Physiol. (Submitted).
7. Carlstedt, I., Coster, S., and Malmstrom, A. (1981) Biochem. J. 197, 217-225.
8. Neufeld, E.F., and McKusick, V.A. (1983) In The Metabolic Basis of Inherited Disease (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., and Brown, M.S., ed.) McGraw-Hill, New York. pp 778-787.
9. Schmickel, R.D., Distler, J.J., and Jourdian, G.W. (1975) J. Lab. Clin. Med. 86, 672-682.
10. Drinkwater, M.R., Corner, R.C., McCormick, J.J., and Maher, V.M. (1982) Mutat. Res. 106, 277.
11. Roff, C.F., Wozniak, R.W., Blenis, J., and Wang, J.L. (1983) Exp. Cell Res. 144, 333-344.
12. Rome, L.H., Garvin, G.A., Allietta, M.M., and Neufeld, E.F. (1979) Cell 17, 143153.
13. Merion, M., and Poretz, R.D. (1981) J. Supramol. Struct. 17, 337-346.



14. Dean, R.T., Jessup, W., and Roberts, C.R. (1984) Biochem. J. 217, 27-40.
15. Hook, M., Wasteson, A., and Oldberg, A. (1975) Biochem. Biophys. Res. Comm. 67, 1422-1428.
16. Oosta, G.M, Favreau, L.V., Beller, D.L., and Rosenberg, R.D. (1982) J. Biol. Chem. 257, 11248-11255.
17. Yanagishita, M., and Hascall, V.C. (1984) J. Biol. Chem. 259, 10270-10283.
18. Brauker, J.H., Roff, C.F., and Wang, J.L. (1986) Exp. Cell Res. 164, 115-126.
19. Roff C.F., Fuchs, R., Mellman, I., and Robbins, A.R. (1986) J. Cell Biol. (in press).
20. Rome, L.H., and Crain, L.R. (1981) J. Biol. Chem. 256, 10763-10768.









MICHIGAN STATE UNIVERSITY LIBRARIES



3 1293 03196 4947

