UCSF
UC San Francisco Electronic Theses and Dissertations

Title
Pathology of extracellular matrix

Permalink
https://escholarship.org/uc/item/3883n3xg

Author
Lien, Yeong-Hau,

Publication Date
1984

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

PATHOLOGY OF EXTRACELLULAR MATRIX:

MODULATION OF MATRIX INTEGRITY BY INTRINSIC AND EXTRINSIC FACTORS

by

YEONG-HAU LIEN, M.D.

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

EXPERIMENTAL PATHOLOGY

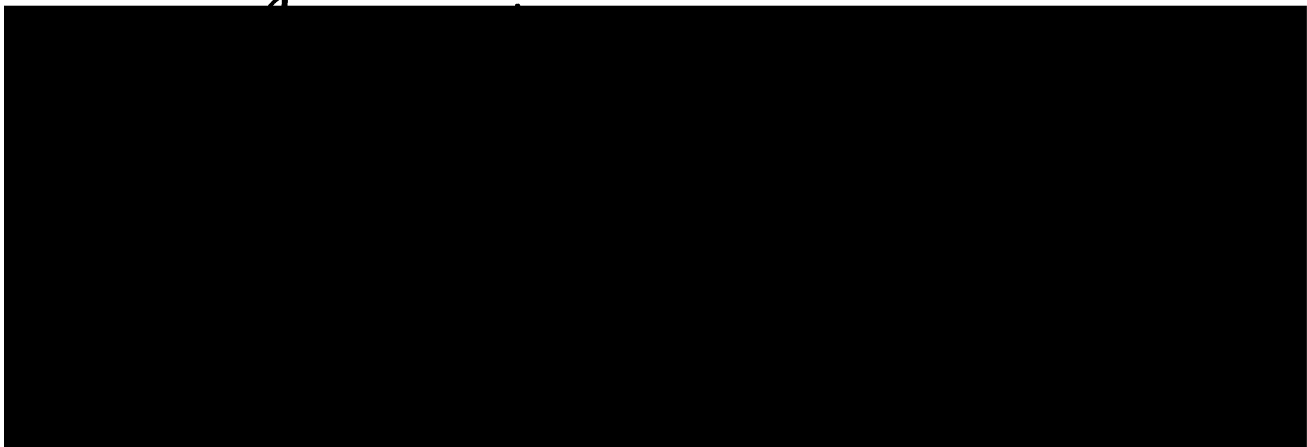
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



Date JUN 17 1984 University Librarian

Degree Conferred: . . . .

TITLE

CANDIDATE

DEGREE

It is the
dissertatio
Microfilms
be obtaine

If it is yo
dissertatio
given the o
yearly peri
microfilm d
upon writte

Unless you s
you indicate
reproduction
two options

1. I hereby
my thesis
thesis or
for reprod

Signature

2. I request th
Unless advis
to distribut
addition, th
University M

Signature

PATHOLOGY OF THE EXTRACELLULAR MATRIX:
MODULATION OF MATRIX INTEGRITY BY INTRINSIC AND EXTRINSIC FACTORS

by

Yeong-Hau Lien, M.D.

Dissertation for the degree of Doctor of Philosophy, The Graduate Program in Experimental Pathology, University of California School of Medicine, San Francisco, California

CONTENTS

Abbreviations... 3

Acknowledgements.. 5

Abstract.. 6

CHAPTER I

Introduction.. 8

Part A: ECM: Structure, Function and Pathology...................... 10

Part B: Abnormalities of ECM in Diabetes mellitis................... 39

Part C: Abnormalities of ECM in Adolescent Idiopathic Scoliosis..... 46

Part D: Working Hypothesis.. 51

CHAPTER II

Modulation of ECM by glucose and its analogs. I Glucose inhibits fibril formation and subsequent cross-linking of collagen *in vitro*.. 54

CHAPTER III

Modulation of ECM by glucose and its analogs. II. The effect of glucose and its analogs on the distribution and degradation of collagen from cells and organs in culture........... 67

CHAPTER IV

The genetic basis of ECM instability in the hereditary scoliotic chick: Properties of collagen in hereditary scoliotic chick skin fibroblasts.. 87

CHAPTER V

The genetic basis of ECM instability in the hereditary scoliotic chick: II. Properties of proteoglycans in hereditary scoliotic chick skin fibroblasts.............................113

CHAPTER VI

Conclusion..138

REFERENCES..142

ABBREVIATIONS

AF	Skin fibroblasts from affected chicks of the scoliotic strain
BAPN	β-Aminopropionitrile fumarate
CM-cellulose	Carboxymethyl-cellulose
CN	Skin fibroblasts from control chicks
CS	Chondroitin sulfate
DEAE-cellulose	Diethylaminoethyl-cellulose
DMEM	Dulbecco's-modified Eagles Minimal Essential Medium
DOG	2-Deoxy-D-glucose
DS	Dermatan sulfate
EB	Epidermolysis bullosa
ECM	Extracellular matrix
EDS	Ehlers-Danlos syndrome
EDTA	Ethylenediamine tetraacetic acid
EHS Tumor	Engelbreth-Holm Swarm Tumor
GAG	Glycosaminoglycan
HA	Hyaluronic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HFBA	n-Heptofluorobutyric acid
HPLC	High Performance Liquid Chromatography
HS	Heparan Sulfate
KS	Keratan sulfate
MEM	Minimum Essential Medium
MPS	Mucopolysaccharidosis
NA	Skin fibroblasts from non-affected chicks of scoliotic strain
NEME	N-Ethylmaleimide

OI

PBS

PCMB

PG

PMSF

SDS

TCA

Tris

UDP

OI	Osteogenesis imperfecta
PBS	Phosphate-buffered saline
PCMB	p-Hydroxymercuribenzoate
PG	Proteoglycan
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
Tris	Tris (Hydroxymethyl) aminomethane
UDP	Uridine diphosphate

ACKN

Stern

thanks

Dr. Cu

valuabl

Dr. Max

tance wi

the elec

gift of t

There

appreciati

Kao, Mr. J

Li-Wen.

Finall

assistance i

ACKNOWLDEGMENTS

This thesis work was performed in the laboratory of Dr. Robert Stern, my Research Advisor. To him I would like to express my deep thanks for his continuous guidance, encouragement, and support. I thank Dr. Curtis Morris, Dr. Dorothy Bainton, and Dr. Ross Beirne for their valuable advice and suggestions. Special thanks to Dr. Robert Siegel, Dr. Max Scheck, Mr. Joseph Fu, and Ms. Joan Gray for thier kind assistance with the lysyl oxidase assay, the proteoglycan analysis, and for the electron microscopic studies. Thanks to Dr. Robert Rucker for the gift of the chicks with hereditary scoliosis.

There are also a number of people to whom I wish to express my appreciation for their supportive efforts. These include Dr. Richard Kao, Mr. Jackson Hall, Ms. Cyndy O'Toole, and most especially my wife, Li-Wen.

Finally, I would like to thank Mr. David Geller for his excellent assistance in the preparation of this thesis.

ABSTRA

In
number
of mole
disease
the cat
It was
cross-li
fibril f
susceptib
collagen
dation co
ture at a
ulatory ef
L-glucose,
added to ch
could be ob
be related t
ing.

Instabi
patients wit
model for AIS
chicks, increa
bone, and cart
fibroblasts fr
crease of coll

ABSTRACT

Instability of the extracellular matrix (ECM) can be invoked in a number of pathological processes. In this thesis, I have probed a number of molecular mechanisms using *in vitro* and culture systems that mimic disease states to determine the basis of that instability. In diabetes, the catabolism of newly synthesized interstitial collagen is enhanced. It was demonstrated that glucose inhibited the lysyl oxidase-mediated cross-linking of collagen *in vitro* through the interference with the fibril formation. The less cross-linked collagen is known to be more susceptible to collagenase digestion. This may underlie the enhanced collagen degradation in diabetes. However, the enhanced collagen degradation could not be demonstrated in fibroblasts and chick calvaria culture at a high concentration of D-glucose. This may be due to the stimulatory effect of D-glucose on collagen synthesis in culture. When L-glucose, an inert sugar which did not affect collagen synthesis, was added to chick calvaria culture, the enhanced collagen degradation then could be observed. The effect of L-glucose on collagen degradation may be related to its inhibition on collagen fibril formation and cross-linking.

Instability of ECM is present in skin and intervertebral disc of patients with adolescent idiopathic scoliosis (AIS). A suitable animal model for AIS in human is the chick with hereditary scoliosis. In those chicks, increased collagen extractability has been demonstrated in skin, bone, and cartilage. The instability of ECM was demonstrated in cultured fibroblasts from scoliotic chicks. The abnormalities included the increase of collagen extractability, the decrease of proteoglycan (PG)

aggre

chondr

tion c

ular c

tractab

The act

and CS,

The liab

ase acti

disorder.

aggregation, and the deposition of collagen, hyaluronic acid (HA), and chondroitin sulfate (CS) in ECM. The structure of collagen and proportion of collagen types, as well as lysyl oxidase (LO) activity and cellular copper (an LO cofactor) were normal. The increased collagen extractability, therefore, may be secondary to the changes in proteoglycan. The activity of hyaluronidase, a lysosomal enzyme that degrades both HA and CS, was found enhanced in skin fibroblasts from scoliotic chicks. The liability of PG's appears to be related to the increased hyaluronidase activity. This may be the basis of the instability of ECM in this disorder.

CHAPTER I

INTRODUCTION

The extracellular matrix (ECM) is the stable structural material that underlies epithelia and surrounds connective tissue cells. Only two decades ago, the ECM was considered an inert supporting material, created by cells as a mere scaffolding on or in which to reside. However, as the structural complexity of the ECM and its components, such as collagen, fibronectin, and proteoglycans, began to be established, more and more functions of the ECM were revealed. Cells not only create their ECM, but also interact with it, and not only with their own ECM, but with those of neighboring cells as well. As Dr. Elizabeth D. Hay (1982) described it:

> at the cell surface, a structural and functional continuum seemingly is formed between the cell interior, the cell membrane, and the molecules of the matrix, so that the metabolism and fate of the cell, its shape and many of its other properties are continuously related and dependent on the composition and organization of the matrix.

The term "dynamic reciprocity" was introduced by Bornstein and colleagues (1981) and was re-emphasized by Bissell et al. (1982) to address the continuity of the interaction between the cell and the ECM: "The ECM affects the cell, which in turn responds by synthetic and degradative processes causing the composition and structure of ECM to change, which in turn influences the cell, and so forth" (Bissell et al., 1982).

Many properties of the cell are influenced by the ECM, including cell shape, cell migration, cell proliferation, and differentiation. Obviously, the ECM also plays an important role in disease conditions. From Marfan's syndrome to cirrhosis of the liver, from rheumatoid

arthritis to tumor invasion, the abnormalities in the synthesis or degradation of the ECM, or a combination of the two have been observed. Much effort has gone into this field in order to elucidate the pathogenesis of diseases and, furthermore, to develop better treatment modalities for such disorders.

This, then is the direction of this dissertation. I have investigated the changes of ECM *in vitro* and have created culture conditions that mimic disease states such as diabetes mellitus (Chapters II and III). Extrinsic factors such as glucose have been added to the cell milieu. I have also investigated the instability of the ECM in skin fibroblasts cultured from chicks with hereditary scoliosis. This disorder may have an intrinsic genetic basis (Chapters IV and V). Before reviewing the scientific literature on the ECM changes associated with these two diseases (diabetes mellitus and idiopathic scoliosis), a general background of ECM structure, function, and pathology will be presented.

PART A

ECM:

Th

three c

1)

2)

3)

Each of t

structure

Collagen

Structure

Collag

molecules o

son, 1982;

brate body c

organisms.

exceptions,

sist of three

another in a

about 1000 am

$(x\text{-}y\text{-}gly)_n$ (n

PART A

ECM: Structure, Function, and Pathology

The basic structural composition of the ECM includes the following three classes of macromolecules:

1) Structural proteins that provide tissue strength, including collagen and elastin
2) Adhesion proteins that provide tissue cohesiveness, including fibronectin, laminin, and chondronectin
3) Proteoglycans that provide tissue resiliency, including chondroitin sulfate, keratin sulfate, dermatan sulfate, and heparan sulfate proteoglycans.

Each of these individual components will be reviewed briefly and their structure related to their function and pathology.

Collagen

Structure and Function

Collagen is the best studied and the best understood of the macromolecules of the ECM (Bornstein and Sage, 1980; Linsenmayer, 1982; Burgeson, 1982; Mayne, 1983). It is the most abundant protein in the vertebrate body constituting more than a third of the total protein in adult organisms. Collagen molecules are rod-like structures and, with a few exceptions, are 300 nm long and 1.5 nm in diameter. The molecules consist of three polypeptide chains, called α chains, coiled around one another in a unique triple helical structure. Each α chain contains about 1000 amino acids with its primary structure in the form of $(x\text{-}y\text{-}gly)_n$ (n = 338 for type I collagen), where the variables in position

x are often proline residues and y often are hydroxylated proline residues. Having a large number of hydroxyproline residues in the y position seems to add stability to the helical structure.

On the basis of peptide analysis, more than eight different collagen isotypes have been identified in vertebrates. The isotypes differ in their primary structure and, therefore, are apparently products of different genes. In the past, collagens have been segregated into two categories: interstitial collagens, including types I, II, and III; and basement membrane collagens, including types IV and V. However, this classification is no longer suitable. First, type V collagen is not restricted to the basement membrane, but rather, is distributed around cells in various tissues (Linsenmayer et al., 1983). Secondly, many new collagen isotypes have been described recently that do not belong to either the interstitial or basement membrane collagen groups. Third, the technique of rotary shadowing makes it possible to visualize the macromolecular organization of various types of collagen using electron microscopy (Furthmayr and Madri, 1982). Therefore, collagens can now be classified according to their macromolecular organization. Table 1 shows the structural characteristics and distribution of collagen isotypes (Mayne, 1983).

TABLE 1

Subtypes of Collagen

	Chain	Chain Organization	Distribution
Fibril-Forming Collagen (Interstitial collagen)			
Type I	$\alpha 1(I)$ $\alpha 2(I)$	$[\alpha 1(I)]_2 \alpha 2(I)$ $[\alpha 1(I)]_3$	Skin, tendon, bone, dentin cornea, fascia
Type II	$\alpha 1(II)$	$[\alpha 1(II)]_3$	Cartilage, vitreous body, notochord, nucleus pulposus
Type III	$\alpha 1(III)$	$[\alpha 1(III)]_3$	Fetal skin, spleen, blood vessel
Network-Forming Collagen (Basement membrane collagen)			
Type IV	$\alpha 1(IV)$ $\alpha 2(IV)$	$[\alpha 2(IV)]_2 \alpha 2(IV)$ $[\alpha 1(IV)]_3$ $[\alpha 2(IV)]_3$	Basement membrane
Collagen with Unknown Macromolecular Organization (Pericellular collagen)			
Type V	$\alpha 1(V), \alpha 2(V)$ $\alpha 3(V), \alpha 4(V)$	$[\alpha 1(V)]_2 \alpha 2(V)$ others	Pericellular location

	Chain	Chain Organization	Distribution
Microfibril-Forming Collagen			
Type VI (intima collagen)	$\alpha1(VI), \alpha2(VI)$	?	Uterus, placenta, skin
Type VII	$\alpha1(VII)$	$[\alpha1(VII)]_3$	Amnion
Miscellaneous Collagens			
Type VIII (EC Collagens)	EC_1, EC_2, EC_3	?	Cultures of some endothelial cells
Minor cartilage collagens	1α, 2α, 3α HMW, LMW, SC	?	Cartilage

Abbreviations used: EC, endothelial cell; HMW, high molecular weight; LMW, low molecular weight; SC, short chain

Types I, II, and III collagens are fibril-forming or interstitial collagens. In electron micrographs, the native fibrils exhibit a characteristic 64 - 67 nm axial periodicity. Because of the unique fibrillar structure, these collagens provide tissue strength. Type I collagen forms large, well-organized fibrils and bundles that are distributed in dense connective tissue and in hard tissue. Type II collagen forms a meshwork of fine fibril within which the proteoglycans are restrained. Therefore, type II collagen is distributed in tissues with a high water content, such as cartilage, the vitreous body, and the nucleus pulposus. Type III collagen is believed to be the collagen of the fine reticular fibrils of classical histology. It nearly always coexists with type I collagen and predominates in tissues that require high levels of compliance, such as blood vessels, and during rapid growth, such as in fetal tissues, and in early wound healing.

Type IV collagen, or basement membrane collagen, has a very unique macromolecular organization that is related to its function. Using rotary shadowing techniques, Timpl et al. (1981) introduced the "spider model" for type IV collagen. The center of the spider is the region where the amino termini of four type IV collagen molecules overlap with each other in an antiparallel manner to form a tetramer. The four legs of the spider represent the rod-like structure of each molecule. At the carboxyl terminus, a large non-collagenous domain is located which interacts with the C-termini of other tetramers, thereby forming a network structure. This unique structure allows type IV collagen to function as a filter in the basement membrane of the vascular wall, especially in renal glomeruli.

Type V collagen is present in all connective tissues with the exception of hyaline cartilage. Both the macromolecular structure and the functions of type V collagen are unknown. The pericellular localization of type V collagen suggests that it can function as an exoskeleton. It may be an anchoring molecule holding type I and type III collagen in position, or it may have a role in mediating the interaction between the cell and other types of collagen.

Other collagen types have recently been discovered. Their structure and function are not well-understood. Interestingly, some of the minor cartilage collagens, such as 1α, 2α, and HMW chains are very similar in their properties (e.g., amino acid composition) to type V collagen, and these may be the cartilage equivalent of type V collagen (Mayne and von der Mark, 1983). Type VI collagen or intima collagen has been isolated from uterus, placenta, and skin (Furthmayr et al., 1983). Type VII collagen, isolated from human amnion, appears to be a collagen with an extended triple-helical structural domain (Bentz et al., 1983). Both types VI and VII collagens form microfibril structures. EC (endothelial cell) collagen has so far been identified in cultures of endothelial cells (Sage et al., 1980). It is unclear how this relates to some of the other collagens described above. This collagen has been more recently termed Type VIII collagen (Sage et al., 1983; 1984).

Pathology

The synthesis and degradation of collagen involves numerous biochemical reactions. The disturbances of any one step of collagen metabolism may manifest itself in a disease process. Table 2 summarizes the major steps in synthesis and degradation of collagen, their functional

significance, and associated clinical or experimental pathological conditions (Uitto and Bauer, 1982). Those lesions involving collagen can be categorized into four classes of mechanisms:

1) Imbalance of synthesis and degradation
2) Imbalance in the relative synthesis of genetically distinct collagen types
3) Structural defects of a particular collagen chain
4) Abnormalities in the packing of collagen molecules into fiber structures.

Some diseases involve a single metabolic defect and are well-documented. For example, Ehlers-Danlos syndrome (EDS) type VI results from lysyl hydroxylase deficiency (Pinnell et al., 1972); some patients with EDS VII have procollagen protease deficiency (Lichtenstein et al., 1973). Menkes syndrome is due to the unavailability of copper, a cofactor of lysyl oxidase (Freiberger and Pinnell, 1979). Lathyrogens, D-penicillamine, and homocystine are inhibitors of collagen cross-linking (Siegel, 1976). The above examples all involve post-translational modifications. Some forms of osteogenesis imperfecta (Williams and Prockop, 1983; Deak et al., 1983) and Marfan's syndrome (Byers et al., 1981) involve a genetic defect in type I collagen, while some forms of EDS IV constitute a genetic defect of type III collagen (Pope et al., 1975). There are some disorders that involve more than one defect simultaneously, such as diabetes, which may involve abnormal glycosylation, fibril formation, cross-linking, and degradation of collagen, etc. (Uitto and Bauer, 1982). The abnormalities of collagen are therefore complicated and difficult to interpret. Still, there are many other pathologic states which have collagen anomalies without a known mechanism, such as the desmoplasia of

breast

type I

types o

quired

be exte

breast cancer. Furthermore, most of the diseases listed above involve type I or III collagen, the best studied collagens. It is likely other types of collagen will be found to be involved in some genetic or acquired diseases as more information accrues. Obviously, this list will be extended greatly in the future.

TABLE 2:

Collagen Metabolism and Associated Pathological Conditions

Step	Functional Significance	Associated Pathology
Synthesis of collagen polypeptides		
1. Gene selection	Determines the collagen type synthesized	Some forms of E-D IV, OI, and the Marfan syndrome, osteoarthrosis
2. Transcription	Formation of mRNA templates	Some of those listed in 1 - 4 may have the defect at this level
3. Translation of mRNAs	Polypeptide assembly	
4. Control of the translational rate	Determines the amount of polypeptides synthesized	Scleroderma, proliferative phase of rheumatoid arthritis, various fibrotic processes, familial cutaneous collagenoma (?), acromegaly, corticosteroid-induced changes
Intracellular Post-transcriptional Modifications		
1. Synthesis of 4-hydroxyproline	Stabilization of triple-helix	Scurvey, tissue anoxia
2. Synthesis of 3-hydroxyproline	Unknown	None known
3. Synthesis of hydroxylysine	Stabilization of cross-links, and attachment sites for glycosylation	E-D VI, alkaptonuria, scurvey, tissue anoxia, some forms of OI, vitamin D-deficiency
4. Synthesis of hydroxylysine-o-glycosides	May influence cross-link formation and determine the fiber diameter	Some forms of dominant EB simplex
5. Glycosylation of extension peptides	Unknown	Some forms of OI
6. Removal of the signal sequence	Unknown	

Step	Functional Significance	Associated Pathology
7. Degradation of nonhelical chains	Removal of defective polypeptides and modulation of collagen production	β-Adrenergic stimulation, scurvy, anoxia, incorporation of proline analogs
8. Chain association and disulfide bonding	Facilitation of triple helix formation	None known
9. Triple-helix formation	Prerequisite for proper secretion	Incorporation of proline analogs, any condition preventing the formation of 4-hydroxyproline
Secretion	Transport of procollagen to the extracellular space	Type III collagen in some forms of E-D IV, presence of inhibitors of microtubular function
Extracellular Modifications		
1. Removal of the propeptides	Necessary for fiber formation	E-D VII, dermatosparaxis in various animal species
2. Nonenzymatic glycosylation	May interfere with fiber formation	Diabetes
3. Deamination of lysine and hydroxylysine	Necessary for cross-link formation	X-linked cutis laxa, homocystinuria, Menkes syndrome, aneurysm-prone mice, lathyrism, copper deficiency, diabetes
Fiber Formation		
1. Alignment of the molecules	Formation of microfibrils	Defective removal of extension peptides in dermatosparaxis, E-D VII
2. Formation of cross-links	Stabilization of fiber structures	D-penicillamine-induced changes and same as in 3 above
3. Supramolecular assembly	Architectural organization of collagen in tissues	E-D I and II (?)

Step	Functional Significance	Associated Pathology
4. Interactions with other extracellular macromolecules	Determines the physiologic properties of tissues	Some forms of spondylo-epiphyseal dysplasia, Marfan's syndrome (?)
Extracellular Degradation		
1. Cleavage by specific collagenases	Rate-limiting step	Rheumatoid arthritis, recessive EB, Paget's disease of bone, hyperparathyroidism, hyperthyroidism, tumor invasion, uremia, inflammatory processes involving leukocyte collagenase, postpartum involution of uterus
2. Further degradation by peptidases and enzymes metabolizing free amino acids	Removal of degradation products	Hydroxyprolinemia, hydroxylysinemia

Abbreviations used: E-D, The Ehlers-Danlos syndrome; OI, osteogenesis imperfecta; EB, epidermolysis bullosa.

Elastin

Structure and Function:

Elastin (for reviews see Sandberg et al., 1981; Franzblau and Faris, 1982) is the functional protein component of the elastic fiber, the component of connective tissue that has an amorphorous, high refractive, generally wavy appearance under the light microscope. Elastin-rich tissues such as ligamentum nuchae, vocal cord, aorta, and lung, have a distinct rubber-like quality. Native elastin is insoluble and resistant to heat and alkaline degradation. This unique property results from the presence of strong tetra-functional lysine-derived cross-links called desmosine and isodesmosine. When cross-linking of elastin is inhibited by either treatment with lathyrogens or by a copper depleted diet, the soluble precursor, tropoelastin, can be obtained. Tropoelastin is composed of a single polypeptide chain (MW 70,000) of about 800 amino acid residues. The nonpolar amino acids: glycine, proline, alanine, valine, phenylalanine, isoleucine, and leucine make up the majority of the residues. These hydrophobic amino acids exist in elastin molecules frequently in the form of the repeating pentapeptides, val-pro-gly-val-gly; or the hexapeptide, pro-gly-val-gly-val-ala. The unique primary structure allows elastin to form a beta spiral structure, which is important in elastin function and in elastic fiber formation. In elastic fibers, another component, microfibrillar protein, which represents 8% of the fiber, functions as a scaffolding for the alignment of the tropoelastin molecule prior to the cross-link formation (Ross and Bornstein, 1969; Robert et al., 1971).

Pathology

Abnormalities of elastin are not as well understood as those of collagen. Table 3 summarizes the major steps of elastin metabolism and associated pathological conditions (Sandberg et al., 1981; Uitto et al., 1982b). Most abnormalities of elastin involve degradation. Two important diseases, atherosclerosis and emphysema, may involve excessive degradation of elastin. Fragmented elastic fibers have been observed in the atherosclerotic aorta and in emphysematous lung tissue. In atherosclerosis, elastolysis is likely to occur after endothelial injury because of the exposure of intima to blood constituents, such as platelets and fatty acid. Once stimulated, platelets can release elastase (Robert et al., 1971). Fatty acids can also stimulate elastase activity (Jordan et al., 1974). Both, therefore, contribute to excessive elastolysis. In emphysema, the excessive elastolysis is likely to result from the imbalance of the elastase and an inhibitor system (α_1-antitrypsin). A well known human form of emphysema is caused by a genetic deficiency in α_1-antitrypsin activity (Laurell and Eriksson, 1963).

The elastin diseases involving the defects of cross-linking of elastin follow the same mechanisms as in the corresponding collagen diseases. Pseudoxanthoma elasticum may involve abnormal interactions between elastin and proteoglycans (Martinez-Hernandez et al., 1976). In some patients with Marfan's syndrome, a decrease of elastin cross-linking may be the molecular basis of their disease (Abraham et al., 1982). A rare autosomal dominant inherited disease, Buschke-Ollendoff syndrome, manifested by yellowish dermal papules (dermatofibrosis lenticularis) and bone dysplasia (osteopoikilosis) may involve excessive synthesis of

TABLE 3:

Elastin Metabolism and Associated Pathologic Conditions

Steps	Functional Significance	Associated Pathology
I. Expression of genes coding for elastin polypeptides		
a. Gene selection	Determines the elastin isotype to be synthesized	Some forms of Marfan's syndrome (?)
b. Transcription	Formation of mRNA precursor	
c. Processing of mRNA precursor	Formation of functional mRNA	
d. Translation	Assemply of polypeptide chains	
e. Control of the rate of transcription, mRNA processing, and translation	Determines the quality of peptide synthesis	Some forms of Marfan's syndrome (?), Buschke-Ollendorff syndrome
II. Intracellular Co-translational and Post-translational Modifications		
a. Removal of signal sequence	May be necessary for secretion	
b. Formation of 4-hydroxyproline	Unknown	
c. Intracellular degradation of some of the polypeptides	Removal of defective polypeptides and modulation of elastin production	

Steps	Functional Significance	Associated Pathology
III. Secretion	Transport through the Golgi apparatus to the extracellular space	
IV. Extracellular Post-Translational Modifications		
a. Oxidative deamination of some lysyl residues	Necessary for cross-link formation	Cutis laxa, Menkes Syndrome, Marfan's syndrome (?), copper deficiency, lathyrism
V. Fiber Formation		
a. Assembly with microfibrillar protein	Formation of elastin fibers	
b. Formation of covalent cross-links	Stabilization of fibers	
c. Interactions with other extracellular macromolecules and cells	Determines the physical chemical properties of tissues	Pseudoxanthoma elasticum
VI. Extracellular Degradation		
a. Cleavage by specific elastases and other proteolytic enzymes	Turnover of extracellular fibers	Emphysema, atherosclerosis

Adhesion Proteins

Structure and Function:

At least three kinds of adhesion proteins are documented. They are fibronectin, laminin, and chondronectin. Table 4 compares the properties of those adhesion proteins. Fibronectin is the best documented of these (Hynes, 1982; Yamada, 1982). There are at least two types of fibronectin, plasma and cellular fibronectin. Plasma fibronectin exists as a soluble protein while cellular fibronectin occurs in fibrillar form on cell surfaces and in the ECM. Although plasma and cellular fibronectin are distinguishable with monoclonal antibodies (Altherton and Hynes, 1981), they are very similar in structure. The fibronectin molecule is asymmetrical and consists of two similar subunits of molecular weight 220,000 daltons held together by disulfide bonds near their carboxyl termini. Physical measurements indicate that fibronectin contains compact globular domains separated by flexible, extendable regions of polypeptide chains. The globular domains are known to bind various materials such as those listed in Table 4. Corresponding to the multiple binding domains of fibronectin, several functions are mediated, including cell adhesion, migration, phagocytosis, and hemostasis, etc. Fibronectin can mediate the adhesion of various mesenchymal cells and some epithelial cells such as hepatocytes to the substrate composed of collagens from type I to V. The universal binding of fibronectin to various types of collagen make it distinct from the specific binding of laminin to type IV collagen (Wicha et al., 1979; Kleinman et al., 1981), and the binding of chondronectin to type II collagen (Hewitt et al., 1980).

Lam

importan

1981; Ya

weight.

220,000

elongatec

component

It mediat

IV collag

action be

receptor,

cancer cel

al., 1983)

muscle cel

because it

be highly s

Chondr

protein fou

It is compos

80,000 dalto

sulfate prot

cytes to typ

The discovery

undescribed m

extracellular

Laminin is a recently discovered glycoprotein that appears to be an important structural component of basement membrane (Kleinman et al., 1981; Yamada, 1982). Laminin is a protein of 800,000 daltons molecular weight. It is composed of two disulfide bonded polypeptide subunits (MW 220,000 and 440,000), that cross each other to form a four-stranded elongated structure. Laminin interacts with other basement membrane components such as type IV collagen and heparan sulfate proteoglycans. It mediates the adhesion of epithelial and epidermal cells to the type IV collagen substratum. Recently, it has been reported that the interaction between the cell and laminin is receptor-mediated. A laminin receptor, molecular weight 67,000, has been identified in human breast cancer cells (Terranova et al., 1983), murine melanoma cells (Rao et al., 1983), mouse fibrosarcoma cells (Malinoff and Wicha, 1983), and in muscle cells (Lesot et al., 1983). This discovery is of great importance because it indicates that the interaction between cells and the ECM can be highly specific.

Chondronectin (Hewitt et al., 1980; 1981) is a 180,000 MW adhesion protein found in plasma, cartilage, and in the vitreous body of the eye. It is composed of two disulfide-bonded polypeptide subunits with a MW of 80,000 daltons. Chondronectin can bind to type II collagen, Chondroitin sulfate proteoglycan, and heparin. It mediates the adhesion of chondrocytes to type II collagen and appears to be specific for chondrocytes. The discovery of chondronectin suggests that there may yet be additional, undescribed molecules that are able to mediate cell adhesion to various extracellular matric moieties.

Pathology

Up to now, very few diseases are known to be caused by the abnormalities of adhesion proteins in the ECM. The only report of a disease associated with a genetic defect of fibronectin is by Arneson et al. (1980). They described a new form of Ehlers-Danlos syndrome in a kindred. Clinical features of this mild, recessively inherited variant of the Ehlers-Danlos syndrome include hypermobility of joints, hyperextensibility of skin, mitral valve prolapse, and a bleeding tendency. The platelet aggregation defect can be completely corrected by the addition of normal human fibronectin. Since the content of fibronectin in plasma and in platelet α-granules appears to be normal, a structural defect of fibronectin has been suggested as the molecular basis of this platelet dysfunction and connective tissue disorder.

Plasma fibronectin levels drop in fulminant hepatic failure (Gonzalez-Calvin et al., 1982), septic shock (Saba et al., 1978), and in disseminated intravascular coagulation (Mosher and Williams, 1978). Fibronectin content increases in keloid or in hypertrophic scar formation of skin (Kischer and Hendrix, 1983), scleroderma (Cooper et al., 1979), and pulmonary fibrosis (Crystal et al., 1983). Since fibronectin is implicated in a wide variety of cellular properties, the increased fibronectin levels may not only be the result, but also one of the causes of pathologic fibrotic processes.

The decreased level of fibronectin in transformed cells is a well-known phenomenon. Recently, it has been reported that the decreased fibronectin level in Rous sarcoma virus-transformed chick embryo fibroblasts is due to decreased transcription of the fibronectin gene (Tyagi

et al., 1983). How the decrease of fibronectin levels relates to the aberrant behavior of transformed cells, however, is still unclear.

TABLE 4

Properties of Adhesion Proteins

Adhesion Protein	MW*	Subunits*	Interactions	Function	Distribution
Fibronectin	440,000	220,000	Collagen I-V proteoglycans fibrin/fibrinogen, bacteria (S. aureus) cells, C_{1q}, Actin, DNA C-reactive protein	Cell adhesion: mesenchymal cells, hepatocytes, etc. Cell migration Hemostasis phagocytosis	Body fluids: plasma, cerebrospinal fluid, amniotic fluid, etc. Basement membrane, Loose connective tissue
Laminin	800,000	220,000 440,000	Collagen IV HSPG, heparin cells	Cell adhesion: Epithelial and epidermal cells	Basement membrane
Chondronectin	180,000	80,000	Collagen II CSPG, Heparin Chondrocytes	Cell adhesion: Chondrocytes	Plasma, cartilage, vitreous body

Abbreviations used: HSPG, heparan sulfate proteoglycan; CSPG, chondroitin sulfate proteoglycan. *MW estimated using SDS-polyacrylamide gel electrophoresis.

Proteoglyc

Structure

Protec
Comper and
1980; Hasca
a core pro
covalently
ized. The
protein wh
chains of
and keratan
PG's are o
molecules,
protein (H
and core p
of link pr
intervals
three mole
40 monomer
have a sim

There
distributi
Hook, 1978
and, excep
core prote
differ in

Proteoglycans

Structure and Function

Proteoglycans (PG) (For reviews see Muir and Hardingham, 1975; Comper and Laurent, 1978; Lindahl and Hook, 1978; Roden, 1980; Muir, 1980; Hascall and Hascall, 1982) are complex macromolecules that contain a core protein to which at least one glycosaminoglycan (GAG) chain is covalently bound. PG's isolated from cartilage have been well-characterized. The cartilage-derived PG monomer is composed of a backbone core protein which has a molecular weight of about 250,000 and several side chains of chondroitin sulfate (CS), each with an average MW of 20,000, and keratan sulfate (KS), with an average MW of 10,000. The monomer PG's are organized in ECM with the involvement of two additional macromolecules, hyaluronic acid (HA) (Hardingham and Muir, 1972) and link protein (Hascall and Sajadera, 1969). The link protein binds to both HA and core protein through a specific site, respectively. In the presence of link protein, PG monomers are locked into place on the HA chain at intervals averaging 30 nm. Therefore, the association between the three molecules yields a stable structure of aggregates which may contain 40 monomers and have a molecular weight approaching 10^8. Other PG's have a similar basic structure, but with a smaller unit size.

There are at least seven GAG types. The structural properties and distribution of each are listed in Table 5 (modified from Lindahl and Hook, 1978). Basically, each GAG is composed of repeating disaccharides and, except for HA, is sulfated to various degrees. Each GAG binds to a core protein through a special linkage region. The sugar components differ in the repeating disaccharide region and is specific for each

class of (
through a
protein th
the cornea

PG's
PG's enable
polyanionic
the polyani
tin, and ot
for the int
glomerular
(HS) PG, fu
1980). The
the resilien
endure high
proteoglycan
in tissues u
into these E
tiate, the p
water. Ther
this way, th
regulated (T

class of GAG. Except for KS, the sulfated GAG links to core protein through a xylose-O-serine linkage. KS from skeleton attaches to core protein through an N-acetylgalactosamine-O-serine linkage, and KS from the cornea through an N-acetylglucosamine-N-Asp linkage.

PG's play an important role in the ECM. Two unique properties of PG's enable them to perform some special functions: 1) they are highly polyanionic; 2) they can occupy large hydrodynamic volumes. Because of the polyanionic charge, PG's have a high affinity to collagen, fibronectin, and other adhesion proteins. These interactions are very important for the integrity of the ECM. Furthermore, the polyanionic sites in glomerular basement membrane, which are now known to be heparan sulfate (HS) PG, function as selective macromolecular filters (Kanwar et al., 1980). The ability of PG's to contain large volumes of water provides the resilience in tissues, such as nucleus pulposus, and enables them to endure high pressures for the entire body. The highly hydrated and proteoglycan-enriched ECM's are observed in some embryonic tissues and in tissues undergoing repair. It is very important for cells to migrate into these ECM-rich areas. Once cells settle down and start to differentiate, the proteoglycans are degraded. This is accompanied by a loss of water. Then, further migration of undifferentiated cells cease. In this way, the development, as well as repair processes, are tightly regulated (Toole, 1982).

TABLE 5

Properties and Distribution of Glycosaminoglycans

Sulfate/	Sugar components

TABLE 5

Properties and Distribution of Glycosaminoglycans

GAG	Mol. Wt. ($x10^3$)	Repeating disaccharides	Sulfate/ disaccharide unit	Sugar components at linkage region	Distribution
Hyaluronate	4-8000	D-glucuronic acid D-glucosamine	0		Various connective tissues, skin, vitreous humor, synovial fluid, umbilical cord, cartilage, inter-vertebral disc
Chondroitin 4- and 6-sulfates	5-50	D-glucuronic acid D-galactosamine	0.1 - 1.3	D-galactose D-xylose	Cartilage, cornea, bone, skin, arterial wall, inter-vertebral disc, kidney
Dermatan sulfate	15-40	D-glucuronic acid L-iduronic acid D-galactosamine	1.0 - 3.0	D-galactose D-xylose	Skin, tendon, arterial wall, heart valve
Heparan sulfate	6-30	D-glucuronic acid L-iduronic acid D-glucosamine	0.4 - 2.0	D-galactose D-xylose	Lung, liver, kid-ney, cell surfaces, basement membrane

GAG	Mol. Wt. $(x10^3)$	Repeating disaccharides	Sulfate/ disaccharide unit	Sugar components at linkage region	Distribution
Heparin	6-25	D-glucoronic acid	1.6 - 3.0	D-galactose	Lung, liver, ski[illegible]

GAG	Mol. Wt. ($x10^3$)	Repeating disaccharides	Sulfate/ disaccharide unit	Sugar components at linkage region	Distribution
Heparin	6-25	D-glucoronic acid L-iduronic acid D-glucosamine	1.6 - 3.0	D-galactose D-xylose	Lung, liver, skin, intestinal mucosa, mast cells
Keratan sulfate	4-19	D-galactose D-glucosamine	0.9 - 1.8	D-galactos-amine D-mannose L-fucose sialic acid	Cartilage, cornea, intervertebral disc

Pathology

Table 6 summarizes the major steps of PG metabolism, their significance, and some associated diseases (Darfman, 1982; Fluharty, 1982). Among these diseases involving PG, mucopolysaccharidoses (MPS) (mucopolysaccharide is the old name of PG) are the best understood (for review see Dorfman and Matalon, 1976; Fluharty, 1982). MPS is a group of genetic disorders that involve PG degradation enzymes, usually lysosomal. Therefore, MPS are now considered to be lysosomal storage diseases. There are at least 7 types of MPS. Table 6 also lists the respective enzyme defect in each type of MPS and the function of each enzyme. I-cell disease, a genetic disease involving sorting of lysosomal enzymes, therefore, involves multiple lysosomal enzyme defects (Hasilik et al., 1981). Because of the defect of the PG degradative enzymes, excessive PG's accumulate in tissues, especially in cartilage and the central nervous system. They are manifested by skeletal deformities, mental retardation, and other anomalies. Decreased PG degradation also occurs in hypothyroidism (deMartino and Goldberg, 1981) and scleroderma (Ninomiya et al., 1982). In the former, the hyaluronidase activity is suppressed, which results in myxedema or the accumulation of HA in skin. In the latter, the mechanism of decreased PG degradation is unknown.

There are some diseases that involve PG synthetic processes. Several of these diseases are better understood and are important as models for studying other, less well understood, disorders.

1) Brachymorphic mice, an animal model for achondroplasia in humans, have a disproportionately short stature characterized by short limbs. The mutant mice have a defect in the sulfation of CS. The undersulfated PG's hold smaller amounts of water and result in the

reduction in the width of cartilage growth plates and, therefore, shortening of the limbs (Orkin et al., 1976).

2) Macular corneal dystrophy, an autosomal recessive inherited disease, manifests itself by cloudiness of the cornea and blindness. It has been reported that the corneal stroma cells from patients with macular corneal dystrophy fail to synthesize a mature KSPG (Hassell et al., 1980). This observation provides direct evidence that KSPG plays an important role in the transparency of the cornea.

3) Diabetic nephropathy is a frequent complication due to thickness of glomerular basement membrane. The level of HSPG in glomerular basement membrane decreases in experimental diabetic rats (Cohen and Surma, 1981). This disease is probably due to the decrease of core protein synthesis but not HS synthesis (Wagner et al., 1983). Since HSPG is the polyanionic site in glomerular basement membrane that functions as a selective molecular filter, the loss of HSPG may cause proteinuria and other complications. In other nephrotic syndromes, such as in lupus nephritis in mice, the decrease of HSPG is also demonstrated (Melnick et al., 1981). However, the mechanism involved is still unclear.

4) In cultures of rat hepatoma cells, simian virus 40-transformed cells, and transformed mouse mammary epithelial cells, the HSPG synthesized is undersulfated (Winterbourne and Mora, 1981; David and van den Berghe, 1983; Robinson et al., 1984). The undersulfated HSPG from hepatoma cells has lower affinity for fibronectin and for hepatoma cells when compared to normal HSPG. The defect in sulfation of PG may have implications in the instability of tumor ECM, as well as in the uncontrolled proliferation of tumor cells.

TABLE 6:

Proteoglycan Metabolism and Associated Pathological Conditions

Steps	Functional Significance	Associated Pathology
A. Synthesis of		
1. core protein	Form template for GAG attachment	Diabetes mellitus (HS core protein)
2. GAG		Macular corneal dystrophy (KS)
a. Chain initiation	Transfer of xylose to OH group of serine in core protein	
b. Formation of galactosyl-xylosyl protein linkage	Transfer of galactose to xylose	
c. Completion of linkage region	Add the 2nd galactose and the 1st glucuronic acid	
d. Chain elongation	Add disaccharide units	
e. Sulfation	Transfer sulfate to GAG acceptor	Brachymorphic mice. Transformed cells
f. Chain termination	Determine the chain length	
3. HA	Formation of PG aggregate	Glucocorticoid treatment
4. Link Protein	Formation of PG aggregate	

Steps	Functional Significance	Associated Conditions
B. Degradation		Scleroderma, I-cell disease
Enzyme	GAG Cleaved	
Endoglycosidases		
Hyaluronidase	HA, C4S, C6S	Hypothyroidism
Exoglycosidases		
β-Glucuronidase	HA, C4S, C6S, Hep/HS	MPS VII
α-Iduronidase	DS, Hep/HS	MPS I, MPS V
β-Galactosidase	KS	MPS IVB
α-N-Acetylhexosaminidase	Hep/HS	MPS IIIB
β-N-Acetylhexosaminidase	HA, C4S, C6S, KS, DS	
Sulfatases		
Arylsulfatase B	C4S, DS	MPS VI
Iduronate 2S Sulfatase	DS, Hep/HS	MPS II
Heparin Sulfamidase	Hep/HS	MPS IIIA
Gal/GalNAc6S Sulfatase	C6S, KS	MPS IVA
GlcNAc6S Sulfatase K	KS	MPS IVC
GlcNAc6S Sulfatase H	Hep/HS	MPS IIID
Other		
Acetyl Transferase	Hep/HS	MPS IIIC

Abbreviations used: HA, hyaluronic acid; C4S, chondroitin 4-sulfate; C6S, chondroitin 6-sulfate; Hep, heparin; HS, heparan sulfate; KS, keratan sulfate; Gal, galactose; GlcNAc6S, N-acetylglucoasamine 6-sulfate; GalNAc6S, N-acetylgalactosamine 6-sulfate; MPS, mucopolysaccharidosis.

PART B

Abnormalities of ECM in Diabetic Mellitus

Diabetic patients are susceptible to a series of complications that lead to considerable morbidity and premature mortality. The average life expectancy of diabetic patients is only two-thirds that of the general population (Crofford, 1975). The chronic complications of diabetes mellitus include blindness, renal failure, neuropathy, hypertension, atherosclerosis, gangrene of the extremities, osteoporosis, and poor wound healing (Brownlee and Cerami, 1981). Most of these complications are secondary to the changes of ECM found in the diabetic state. The thickening of basement membranes may explain the increased permeability of retinal and glomerular capillaries and the poor perfusion of the microvasculature of the extremities. The changes in fibrillogenesis of collagen may play an important role in the pathogenesis of impaired wound healing and the osteoporosis found in diabetic patients. Here, we survey some ECM changes observed in the basement membrane and the interstitial connective tissues of the diabetic.

Basement Membrane

The major ECM macromolecular components in basement membranes are type IV collagen, laminin, fibronectin, and heparin sulfate proteoglycans. In diabetic basement membrane, all four of these components are altered.

1. Collagen:

Quantitatively, the amount of total basement membrane collagen is increased in diabetic basement membrane. Glomeruli isolated from human diabetic kidney are larger and heavier than those from non-diabetic kidney. Diabetic glomeruli have a higher content of collagen (Klein et al., 1975). Isolated glomerular basement membrane also has increased levels of hydroxyproline and hydroxylysine (Beisswenger and Spiro, 1970). Immunohistochemical studies show that the changes of basement membrane collagen are dependent on the duration of diabetes. In early and moderate diabetes, both types IV and V collagen are increased in diabetic glomerular basement membrane; however, in severe diabetes, both types IV and V collagen are decreased. In the mesangium, there is a greater increase of types IV and V collagen in early and moderate phases. In severe diabetes, type IV collagen diminishes but type V persists in the mesangium (Falk et al., 1983).

The increase of collagen content in diabetic basement membrane are caused mainly by increased collagen synthesis. The pieces of evidence that support the increase of collagen synthesis in diabetic tissues are 1) an increase of radioactive proline incorporation into the glomeruli of alloxan-induced diabetic rats (Brownlee and Spiro, 1979); 2) an increase of enzyme activities involved in the post-translational modifications of collagen in diabetic tissues. The elevated enzymes include prolyl hydroxylase (Risteli et al., 1976), lysyl hydroxylase (Cohen and Khalifa, 1975; Risteli et al., 1976), glucosyl transferase (Spiro and Spiro, 1971), and lysyl oxidase (Madia et al., 1979).

Decreased degradation of basement membrane collagen may also occur in diabetic tissues; however, direct evidence for this is currently

lacking. The findings of decreased glomerular β-glycosidase activities (Fushimi and Tarui, 1976; Chang, 1978) and urinary basement membrane-like protein (Weil et al., 1976) in diabetic rats suggest that decreased catabolism may play a role in basement membrane thickening.

Qualitatively, type IV collagen in diabetic tissues is different from that in normal tissue. Like hemoglobulin, albumin, and other proteins, type IV collagen is also glycosylated nonenzymatically in the diabetic (Cohen et al., 1980; LePape et al., 1981a; Uitto et al, 1982). The nonenzymatic glycosylation of type IV collagen involves a ketoamine-linkage between a sugar residue and the -amino group of lysyl or hydroxylysyl residues in collagen (LePape et al., 1981a). The increased carbohydrate content may lead to decreased turnover of basement membrane collagen in diabetic glomeruli; however, direct evidence for this is lacking. Other compositional changes of basement membrane such as increased hydroxylysine and hydroxylysine-linked disaccharide residues (Beisswenger and Spiro, 1973; Canivet et al., 1979) remain controversial (Kefalides, 1974; Uitto et al., 1982). There is a significant decrease in the cystine and sialic acid content of glomerular basement membrane (Westberg, 1976; Kefalides, 1974; Canivet et al., 1979). Since both cystine and sialic acid are found primarily in the terminal peptides rather than in the central helical domains of basement membrane collagen, their decrease implies that terminal peptides are partially removed in diabetic basement membrane (Westberg, 1980). However, whether reduction of cystine and sialic acid actually occurs in purified type IV collagen needs to be confirmed.

2. Laminin and Fibronectin:

Laminin is increased in the EHS (Engelbreth-Holm Swarm) tumor, a basement membrane producing tumor, grown in genetically diabetic mice (Rohrbach et al., 1982). Immunohistochemical studies show that an increase of laminin in the glomerular basement membrane is observed only in early and moderate diabetes. In the late phase, laminin content decreases. Fibronectin is not changed in early and moderate diabetes and is decreased in severe diabetes (Falk et al., 1983).

3. Heparan Sulfate Proteoglycans:

HSPG of basement membrane is reduced in diabetics. ^{35}S-sulfate incorporation into glomerular basement membrane is decreased in streptozotocin-induced diabetic rats (Cohen et al., 1981). HSPG is also decreased in EHS tumor grown in diabetic mice (Rohrbach et al., 1982). The decrease of HSPG is probably due to decreased synthesis of core protein instead of HS. Decreases in HSPG and core protein levels are reversed by insulin administration (Wagner et al., 1983). Based on these observations, a hypothetical mechanism of basement membrane thickening has been invoked. Since HSPG in glomerular basement membrane functions as a selective molecular filter, the loss of HSPG may increase the permeability of glomerular capillaries and lead to proteinuria. To compensate for this leakage, the glomerular epithelial and endothelial cells synthesize more basement membrane collagen and laminin, which are, however, ineffective. Therefore, the basement membrane becomes thicker and thicker. This intriguing mechanism needs to be investigated further.

Interstitial Connective Tissue

Collagen

Schneir et al. (1979) have reported the tissue specific effects on collagen metabolism in streptozotoin-induced diabetic rats. In skin, healing wound, and bone, there is a net loss of collagen mass (Goodson and Hunt, 1977; Levin et al., 1976; Schneir et al., 1979), while in intestine and glomerular basement membrane, a net accumulation of collagen mass occurs (Cohen and Klein, 1979; Schneir et al., 1979). The collagen in the former is fibril-forming collagen (types I and III), while in the latter two, it is in a network form (type IV). The increase of type IV collagen in diabetic tissue has been described previously. It is not clear why in diabetes there is a differential effect in different tissues or in different types of collagen. The mechanisms of decreased collagen in connective tissue may involve both decreased synthesis and increased degradation. The catabolic state of diabetes explains the decrease in general protein synthesis, including collagen. The excessive degradation of collagen in skin has been demonstrated by Schneir et al. (1982). Thirty percent of newly synthesized collagen is degraded in the skin of streptozotocin-induced diabetic rats, while only 13% is degraded in control rats. However, the pre-existing collagen in diabetic skin has a similar turnover rate to that in control rats. The mechanism of enhanced catabolism of newly synthesized collagen is not clear. In Chapters II and III of this dissertation, I shall describe the investigation of this mechanism by using *in vitro*, cell culture, and organ culture systems. The working hypothesis will be presented later in this chapter.

Qualitatively, interstitial collagen from diabetic tissues is distinct from normal collagen: 1) Like type IV collagen, there is an increase of nonenzymatic glycosylation of fibril forming collagen in diabetic tissue (Schnider and Kohn, 1980; La Pape et al., 1981b); 2) It has been implicated that collagen from diabetic tissues is more cross-linked because of the observation that it is more resistant to collagenase and is less extractable with acetic acid (Hamlin et al., 1975; Golub et al., 1978; Chang et al., 1980).

Proteoglycans

Table 7 summarizes the previous studies of GAG's in diabetic tissues, including aorta, skin, and gingiva. In general, the amount of proteoglycans decreases in diabetic tissues. The significance of increased HA in aorta and increased heparin sulfate in skin and gingiva is not clear. Since the changes of GAG's in diabetic skin can be reversed by the administration of insulin, it has been suggested that insulin may play an important role in GAG synthesis (Dorfman et al., 1963). Schiller and Hille (1969) have demonstrated that UDP-glucuronic acid is degraded more rapidly by skin from diabetic rats than by skin from normal animals. Insulin reverses this effect. Since UDP-glucuronic acid is a key compound in the synthesis of GAG's (Table 6), it is possible that the over-degradation of UDP-glucuronic acid might be related to the mechanism by which decreased levels of proteoglycans occurs.

TABLE 7

Changes of Proteoglycans in Tissues of Diabetic Rats

Tissue	Treatment	Duration	HA	GAG CS/DS	HS/Heparin	References
Aorta	Alloxan	5 wk	--	↓	↓	Paterson and Heath, 1967
	Pancreatectomy	3 mo	--	↑	↑	Cohen and Fuglia, 1970
		6 mo	--	↓	↓	
	Alloxan	2 wk	↑	↑	↑	Malathy and Kurup, 1972
		2 mo	↑	↓	↓	
Skin	Alloxan	3 wk	↓	↓	↑	Schiller and Dorfman, 1963
	Alloxan	2 mo	↓	↓	--	Malathy and Kurup, 1972
	Streptozotocin	10 wk	↓	↓	--	Berenson et al., 1972
Gingiva	Alloxan	25 days	↓	↓	↑	Kofoed and Tocci, 1973

Note: ↑ , increase; ↓ decrease; --, no change

PART C:

Changes of the ECM in Adolescent Idiopathic Scoliosis

Scoliosis, or lateral curvature of the spinal column, may arise from a variety of causes, including neuromuscular disorders, skeletal disorders, systemic connective tissue disorders, trauma, and congenital anomalies, etc. However, by far, the most common form is adolescent idiopathic scoliosis, for which the precise etiology has not been established. The population incidence of adolescent idiopathic scoliosis has been variously reported as between 0.2 and 6% (Ponseti et al., 1976). Eighty-two percent of the patients are female. Scoliosis usually develops during the adolescent growth spurt (Harrington, 1977). There are strong genetic factors acting in adolescent idiopathic scoliosis. Among the first degree female relatives of adolescent female patients, 12% are affected (Wynne-Davies, 1968). Either autosomal dominant inheritance or multiple gene inheritance has been suggested.

According to a recent long-term follow-up study (Weinstein et al., 1981), 37% of the patients have thoracic curves; 25% have lumbar curves; 26% have double major curves; and 12% have thoracolumbar curves. Many curves (68%) continue to progress slightly in adults. Curves that measure between 50 and 75 degrees at skeletal maturity, particularly thoracic curves, progress the most (Weinstein and Ponseti, 1983). The mortality rate is 15%, which is no different from the predicted death rate of the age and sex matched group from the general population.

Scoliosis is one of the manifestations of inherited disorders of connective tissues such as Ehlers-Danlos syndrome, osteogenesis

imperfecta, Marfan's syndrome, homocystinuria, etc. (McKusick, 1972). Young animals fed lathyrogens, inhibitors of collagen cross-linking, also develop scoliosis (Geiger et al., 1933). Since collagen is a major structural protein of the body, any disturbance involving the metabolism of collagen may result in some degree of scoliosis. The abnormalities of collagen and other matrix components have been suggested to be related to the pathogenesis of adolescent idiopathic scoliosis. There are some abnormalities of ECM that have been observed in tissues from scoliotic patients, particularly in the intervertebral disc. Here we survey these changes of ECM in adolescent idiopathic scoliosis.

Intervertebral disc

The intervertebral disc is a hydrostatic load bearing structure. It acts as a confined fluid where the nucleus pulposus enclosed by the lamellae of annulus fibrosus converts axial load into tensil strains on the annular fibers and cartilaginous endplates. The water content of the disc, ranging 70 to 90%, is highest at birth and decreases with age (White and Panjabi, 1978). The mechanical behavior of the intervertebral disc is largely dictated by its two major macromolecular components: PG's and collagen. PG's are responsible for the fluid pressure in the nucleus pulposus and the collagen in the annulus is ideally suited to accomodate the complex stresses caused by compression of the nucleus.

Collagen

In the normal disc, type II collagen is the only collagen type in the nucleus pulposus, while both types I and III collagens are in the the annulus fibrosus with a gradient increase of type I collagen outward

(Parsons et al., 1982). In adolescent idiopathic scoliosis, the collagen content in the nucleus is higher than normal. In the annulus, there is a fall in collagen content on the concave side and a rise on the convex side (Bushell et al., 1979). The types of collagen change in the scoliotic annulus, but not in the nucleus. The scoliotic annulus concave to the curve is distinctly different from the normal annulus, with an increased proportion of type I collagen. The scoliotic annulus convex to the curve appears to have a decreased proportion of type I collagen in the outer area and an increase of that in the area next to the nucleus (Parsons et al., 1982). However, similar collagen changes are also observed in scoliosis secondary to myelomeningocele (Zaleske et al., 1980) and in ventrally herniated discs of rabbits (Lipson and Reilly 1982). Therefore, the changes of collagen in the scoliotic disc described above may not be primary changes but may represent a secondary effect.

Proteoglycans

The principal GAG's in the normal intervertebral disc are CS, KS, and HA. The proportion of PG aggregate is about 28% in the nucleus and 50-60% in the annulus (Pedrini-Mille et al., 1983). The content of PG decreases in the scoliotic nucleus but not in the annulus. The decrease of PG content may be contributed to by both a decrease in the number of GAG's and shortening of the chain length of GAG's (Pedrini et al., 1973). However, the proportion of PG aggregates is not changed in either the nucleus or the annulus (Pedrini-Mille et al., 1983). Using zonal methods of dissection, differences can be detected in the level of individual GAG types from the concave and convex sides of the curve. CS is elevated

on the concave side but depressed on the convex side. KS and HA are elevated on the convex side (Taylor et al., 1981). Whether these changes in PG's are primary or secondary ones is still not clear.

Interstitial Connective Tissue

Zorab et al (1971) have reported that the 24 hr urinary hydroxyproline is elevated in patients with adolescent idiopathic scoliosis. The instability of skin collagen tested either by cold alkali treatment or by pronase and thermal denaturation is higher in scoliotic patients. Collagen stability appears to become normal once skeletal system maturation occurs (Francis et al., 1975). However, collagen stability in spinal ligament appears to be similar among normal persons and patients with idiopathic scoliosis or secondary scoliosis (Ponseti et al., 1976; Venn et al., 1983).

The collagen abnormalities have also been demonstrated in a highly inbred strain of chicks with hereditary scoliosis (Riggins et al., 1977; Lin et al., 1980). The scoliotic strains of chicks was established by Taylor (1971). The spinal curvature develops at 4-6 weeks of age when sexual development and the growth spurt of the chick occur. Male chicks have a higher prevalence of scoliosis and develop more severe curvatures than the female. No evidence of growth defects or primary neuromuscular disorders have been observed in these chicks. The sexual development is slightly delayed. The extractability of collagen from skin, vertebral column, bone, and cartilage, and serum levels of hydroxyproline are elevated. The primary structure of type I collagen from skin is normal (Kenney et al., 1982). These chicks appear to be a suitable animal model for human adolescent idiopathic scoliosis. The species difference

may underlie the opposite sexual predominance of scoliosis between the two. In humans, the homogametic sex is female while in birds it is the male. Therefore, the chick model presents an ideal opportunity to study the pathogenesis of the human adolescent idiopathic scoliosis.

In this dissertation I have investigated the properties of collagen and proteoglycans in cultured hereditary scoliotic chick skin fibroblasts that are described in Chapters IV and V.

PART D

Working Hypotheses

In this dissertation I have investigated 1) modulation of the ECM by glucose and its analogs (Chapters II and III), and 2) the genetic basis of ECM instability in hereditary scoliotic chicks (Chapters IV and V). The working hypotheses for these experiments are described below:

Modulation of the ECM by Glucose and its Analogs

As mentioned previously, there is a tissue-specific effect of diabetes on collagen metabolism. In interstitial connective tissues, there is excessive degradation of collagen in the diabetic; while in basement membranes, collagen is synthesized in excess (Schneir et al., 1982). The macromolecular organization of interstitial collagen and basement membrane collagen is different; the former is fibril-forming, while the latter is not (Bornstein and Sage, 1980). Since glucose and other sugars inhibit the fibril formation of collagen (Hayashi and Nagai, 1972), I postulated that the inhibition of fibril formation by the high sugar environment in the diabetic may underlie the enhanced degradation of interstitial collagen. Furthermore, this may explain the differential effect of diabetes on different types of collagen.

The degradation rate of interstitial collagen is known to be related to the level of cross-linking in collagen. Vater et al. (1979) have demonstrated that the presence of as few as 0.1 Schiff-base cross-link per collagen molecule causes increased resistance to collagenase digestion. Cross-linking of collagen is a very important process of

posttranslational modification. The initial reaction in the cross-linking is mediated by the extracellular enzyme, lysyl oxidase. In the presence of cofactors, such as copper, pyridoxine, and O_2, lysysl oxidase catalyzes the oxidation of -amino groups in certain lysyl and hydroxylysyl residues of collagen. -aldehydes are thus formed, which are the precursors of cross-linking (Pinnell and Martin, 1968; Traub and Piez, 1971; Tranzer, 1973). The aldehydes can react to form cross-links spontaneously by either an aldol condensation with a second aldehyde residue, or by Schiff base formation with the -amino group of lysyl or hydroxylysyl residues (Bornstein and Piez, 1966; Bailey et al., 1970; Tanzer and Mechanic, 1970). Siegel (1974) has reported that lysyl oxidase activity is dependent on the physical state of the collagen substrate. There is high activity with precipitated collagen fibrils and much lower activity with soluble collagen. Since glucose inhibits fibril formation, it is likely that glucose also inhibits subsequent cross-linking and further affect the turnover rate of collagen.

To test this hypothesis, I used both *in vitro* and culture systems. In Chapter II, the effect of glucose on collagen fibril formation and the subsequent cross-linking reaction was tested *in vitro*. In Chapter III, the effect of glucose and its analogs on the distribution and degradation of collagen in cultured chick calvaria and in human skin fibroblasts were investigated.

The Genetic Basis of ECM Instability in Hereditary Scoliotic Chicks

The etiology of hereditary scoliosis in chicks is still unknown. Since scoliotic chicks have some systemic derangement of connective tissue (Riggins et al., 1977; Lin et al., 1980; Kenney et al., 1982). I

examined the cultured skin fibroblasts that are known to express the genetic defect of connective tissue such as shown in Ehlers-Danlos syndrome type VI (Pinnell et al., 1972). By metabolic labeling with radiolabeled precursors, it is possible to investigate even low levels of ECM components.

To study the mechanism of collagen instability, I posed the following questions:

1) Are there structural defects in collagen?
2) Are there changes in the proportion of types of collagen?
3) Are there any changes in the collagen cross-linking reaction, such as lysyl oxidase activity or the availability of copper?
4) Are there defects in the packing of collagen into fibrils?
5) Is there an imbalance of synthesis and degradation of collagen?

The investigation of the properties of collagen will be described and discussed in Chapter IV.

PG's play an important role in the integrity of the ECM. In Chapter V, the properties of PG's in cultured skin fibroblasts will be described. The experiments were performed in the following sequence:

1) Aggregation of PG's
2) Quantity and quality of HA and sulfated GAG's
3) Degradation of PG's.

The information derived from these experiments may not only provide a better understanding of the mechanism underlying ECM instability, but also reveal the pathogenesis of hereditary scoliosis in chicks.

CHAPTER II

MODULATION OF THE ECM BY GLUCOSE AND ITS ANALOGUES

Part I:
Glucose Inhibits Collagen Fibril Formation and Subsequent Cross-Linking *In Vitro*

Introduction

Diabetic patients have poor wound healing. A compromised vascular supply because of thickened capillary basement membranes is the mechanism usually invoked. However, in addition there is a lower content of interstitial collagen in both wounds (Goodson and Hunt, 1977) and in skin (Schneir et al., 1979) in diabetics. The tensile strength of wounds and the collagen content of wound chambers is lower in streptozotocin-induced diabetic rats than in control animals (Goodson and Hunt, 1977). The mechanism by which this occurs is unknown. But accelerated catabolism of interstitial collagen, as observed by Schneir *et al*. (1982), in streptozotocin-induced diabetic rats, is probably involved in this process.

The rate of degradation of interstitial collagen is related to the degree of collagen cross-linking. Cross-linked collagen is more resistant to collagenase degradation than the non-cross-linked polymer (Vater et al., 1979). Fibril formation is the critical step in the cross-linking of collagen (Siegel, 1974). It has been demonstrated that glucose and other sugars inhibit collagen fibril formation *in vitro* (Hayashi and

Nagai, 1972). Herein, I test the hypothesis that glucose may decrease cross-linking through the inhibition of fibril formation, and present evidence that decreased cross-linking and enhanced susceptibility to proteolytic attack may be the mechanism of the connective tissue defect in diabetes.

Materials and Methods

Preparation of lysyl oxidase and substrates. The partially purified lysyl oxidase was prepared from the femoral and tibial epiphyseal cartilage of 17-day-old chick embryos as previously described (Siegel and Fu, 1976). Briefly, the enzyme fraction was extracted with 6 M urea 0.05 M Tris pH 7.6 from an epiphyseal cartilage homogenate and partially purified with a DE-52 cellulose column (Whatman Co.) using a linear gradient of NaCl from 0 to 0.5 M. Fractions from the peak of enzyme activity were pooled and dialysed overnight at 4°C against PBS. This partially purified lysyl oxidase preparation was used in all subsequent reactions.

[6-^{3}H]lysine labeled collagen was prepared from 17-day-old chick calvaria (Siegel, 1974). [4,5-^{3}H]lysine-labeled elastin was prepared from 17-day-old chick embryo aortas (Siegel *et al*., 1970; Siegel, 1974).

Lysyl oxidase assay. Assays employing the tritium-labeled collagen and elastin substrates were performed as previously described (Siegel *et al*.,1970; Siegel, 1974), except a one hour preincubation was used in the case of the elastin assay to facilitate examination for a glucose effect. Glucose, 1.25 M, in PBS was prepared as a stock solution. In each assay

tube 0.5 to 0.6 nmole of collagen (specific activity 1.0×10^{15} cpm per mole) or an insoluble elastin suspension (5 to 6×10^5 cpm) was used. Various amounts of glucose stock solution were added to the assay tube to make the final concentrations of glucose 5-500 mM. In control tubes, PBS was added. The final reaction volume was 250 µl. Tubes were incubated at 37°C for 60 min.to permit collagen fibril formation. Enzyme fractions of 0.5 ml were then added and incubations continued for 2 h. After distillation, tritium water was counted in 10 ml of Aquasol (New England Nuclear)in a Beckman liquid scintillation counter with a counting efficiency for tritium of 30%.

In some experiments, glucose was added after collagen fibril formation had occurred to examine for an effect of glucose directly on the lysyl oxidase reaction. In those experiments in which reversibility of the glucose effect on collagen cross-linking was investigated, the collagen substrate and glucose mixture were placed in dialysis tubing and dialysed against the same concentration of glucose in PBS at 37°C to allow fibril formation. However, no increase of the turbidity was observed in the presence of glucose. In control groups, no glucose was added and the volume was adjusted by adding PBS. The collagen substrate was then dialysed against PBS thoroughly at 4°C to remove glucose. The preformed fibril dissolved during dialysis. The collagen substrates were harvested. An aliquot of 50 µl was removed for counting of radiolabel. Collagen substrates with radioactivity of approximately 5×10^5 cpm were used for the assay. Collagen substrates were preincubated for 60 min at 37°C. At that time, the fibril formation occurred to the same extent in control and experimental groups, then enzyme was added as previously described. The lysyl oxidase activity was expressed as cpm tritium water released per 5×10^5 cpm substrate.

Studies of fibril formation: In each microfuge tube 0.5 to 0.6 nmole tritium labeled collagen was used. The glucose stock solution was added and the total volume of mixture was adjusted to 250 ul, and incubated at 37°C with shaking for 60 min. Samples were then centrifuged in a Beckman Microfuge for 5 min. An aliquot of 50 ul was removed from the supernatant of each tube for scintillation counting. The percentage of fibril formation was calculated as follows:

$$\frac{\text{total radioactivity added - radioactivity of supernatant}}{\text{total radioactivity added}} \times 100 = \%\ \text{fibril formation}$$

The assumption was made that the volume of the pellet following centrifugation was negligible.

Results

The effect of glucose on collagen cross-linking was investigated using an *in vitro* model. Radiolabeled native collagen was obtained from cultured embryonic chick calvaria incubated *in vitro* with [6-^{3}H]lysine. The initial reaction in the cross-linking of collagen by the enzyme lysyl oxidase is the formation of -aldehydes from the -amino groups in certain lysyl and hydroxylysyl residues (Pinnell and Martin, 1968; Traub and Piez, 1971; Tanzer, 1973). Formation of aldehydes at the 6 position of lysyl or hydroxylysyl residues of the [6-^{3}H]lysine-labeled collagen substrate results in release of tritium from that position and formation of tritium water. The radioactivity of released tritium water correlates directly with formation of aldehydes which are the actual precursors of the lysine derived cross-links of collagen (Pinnell and Martin, 1968;

Traub and Piez, 1971; Tranzer, 1973). By measuring the released tritium water, the rate of aldehyde formation under our experimental condition could be estimated.

Lysyl oxidase activity is dependent on the physical state of the collagen substrate. There is high activity with precipitated collagen fibrils and much lower activity with soluble collagen (Siegel, 1974). The effect of glucose on fibril formation and the subsequent substrate activity of the test collagen was measured by preincubating the substrate with varying glucose concentrations at 37°C for 1 hr and then incubating with lysyl oxidase. Tritium water released after the lysyl oxidase reaction was suppressed in the presence of glucose (Fig. 1). A linear glucose concentration dose-response relationship was observed up to 100 mM. Glucose inhibited tritium water release at concentrations as low as 5 mM. Glucose at 50 mM was required for half-maximum inhibition. At 200 mM, the tritium water released was less than 10% of the control reaction.

To determine whether glucose affected the cross-linking reaction by acting directly on the inhibiting lysyl oxidase, glucose was added after the collagen had been precipitated and reconstituted as a fibril. The rate of tritium water release was not affected by the presence of glucose, even at a concentration as high as 500 mM (Fig.2). To determine whether the inhibitory effect of glucose was reversible, glucose was added to the radiolabeled collagen substrate and incubated at 37°C for 1 h. The glucose was then removed by dialysis and collagen substrate was allowed to form fibrils by incubation at 37°C for 1 hr prior to performing the lysyl oxidase assay. The tritium water release using the glucose-pretreated substrate was not suppressed (Fig. 2). As noted, the zero

glucose point here showed a lower value than the actual glucose pretreated group. This can be explained by the presence of trace amounts of endogenous lysyl-oxidase, an enzyme known to be tightly bound to its collagenous substrate (Siegel, 1974; Siegel and Fu, 1976). During preliminary incubation at 37°C, the endogenous lysyl-oxidase may be releasing some tritium water as fibril formation occurs. The tritium water was then lost during the subsequent dialysing step. When glucose was present in the preliminary incubation, loss was limited because of the inhibition of fibril formation. Therefore, when the subsequent assay was performed, the tritium water appeared slightly higher in those samples in which glucose pretreatment had occurred.

To test whether glucose inhibits tritium water release by interfering with fibril formation, we chose elastin, an amorphous non-fibrillar substrate for the enzyme. When insoluble elastin was incubated with glucose at 37°C for 1 hr and then assayed, no effect of glucose was observed (Fig. 2). Even at 500 mM of glucose, the level of tritium water released was the same as in controls to which no glucose had been added.

It has been reported, using a spectrophotometric method, that glucose inhibits collagen fibril formation (Hayashi and Nagai, 1972). Both the rate of fibril formation and the final opacity are suppressed by glucose. In order to compare the effect of glucose on tritium water release to that on fibril formation, we used a centrifugation method to examine fibril formation. The collagen substrates were incubated at 37°C for 1 hr., then centrifuged to separate reconstituted fibrils from soluble collagen. Without glucose, the percentage of fibril formation was in the range of 65-75%, compatible with previous reports (Siegel and Fu, 1976). Glucose inhibited fibril formation linearly up to 100 mM

(Fig. 3). The inhibitory effect reached a maximum of 75% at 200 mM. No further inhibition occurred as the glucose concentration increased in the range of up to 500 mM. Glucose at concentrations of 50 mM was required for half-maximum inhibition of fibril formation.

Discussion

We conclude from these studies that of glucose inhibits collagen cross-linking by interfering with fibril formation. The inhibitory effect of glucose on fibril formation and cross-linking was linear up to a concentration of 100 mM (Fig. 1 and 3). The physiological range of blood glucose in humans is 3.3 to 5 mM. The first effect we observed on *in vitro* cross-linking occurred at 5 mM glucose. It is conceivable that there could be a significant decrease of both fibril formation and cross-linking of collagen when blood glucose, which also reflects glucose levels in tissues and in wound fluid (Goodson and Hunt, 1977), is elevated to three to four times the normal level, as observed in some diabetic patients. The nonenzymatic glycosylation of collagen which is increased in both interstitial collagen (Schnider and Kohn, 1980), and basement collagen (Cohen et al., 1980) in diabetes, is not likely to play a role in our *in vitro* model because it is a slow reaction (Higgins and Bunn, 1981).

An explanation for the ability of glucose to inhibit collagen fibrillogenesis comes from a recent model. A hydrophobic cluster in the C-terminal extrahelical peptide of type I collagen directs lateral aggregation of chains during fibrillogenesis (Capaldi and Chapman, 1984). Glucose, a polyol, might interfere with such hydrophobic interactions.

Since fibril formation is an early event in collagen synthesis (Trelstad and Hayashi, 1979), we speculate that the high glucose environment in diabetes may prevent fibril formation and the subsequent cross-linking reaction that occurs soon after synthesis of collagen. Schneir et al. (1982) have demonstrated that 30% of newly synthesized collagen is degraded in the skin of streptozotocin-induced diabetic rats, while only 13% is degraded in control rats. Our observations may explain the enhanced catabolism of newly synthesized collagen in diabetes. It has been implied that collagen from diabetic tissues is more cross-linked because of the observations it is more resistant to collagenase and is less extractable with acetic acid (Hamlin et al., 1975; Golub, et al., 1978). Collagen becomes progressively more cross-linked with age. Since in diabetes newly synthesized collagen is not cross-linked and is less stable than the preexisting collagen, the selective depletion of newly synthesized collagen may leave tissues composed predominantly of preexisting, more cross-linked collagen. The net result then would be an increase of cross-linking and a decrease in the quanitity of interstitial collagen in diabetic tissues.

The specific effect of glucose on fibril formation may also explain the tissue-specific changes of collagen in diabetes. There is a net loss of collagen mass in intact skin (Schneir et al., 1979), wounds (Goodson and Hunt, 1977), and bone (Levin et al., 1976), whereas a net accumulation of collagen mass occurs in the intestine (2) and in glomerular basement membranes (Cohen and Khalifa, 1977). The collagen in the former is interstitial collagen, while in the latter two it is basement membrane collagen. The major difference between these two types of collagen is that interstitial collagen forms fibrils while basement

membrane collagen does not (Bornstein and Sage, 1980). The accumulation of basement membrane collagen in diabetes may be due to enhanced synthesis (Bornstein and Sage, 1980), decreased degradation (Fushimi and Tarui, 1976) and by some additional mechanisms (Vracko, 1974). Thus, glucose specifically stimulates the catabolism of interstitial collagen, that requires cross-linking for persistance and does not affect the catabolism of basement membrane collagen.

Legends to Figures

Figure 1: Inhibitory effect of glucose on tritium water release from [6-^{3}H] lysyl chick calvaria collagen. 500,000 cpm of [6-^{3}H] lysyl-labeled collagen was preincubated with glucose for 60 min. at 37°C without enzyme. Partially purified lysyl oxidase was then added and further incubated for 2 h. Samples were analysed for release of tritium water. The tritium water release of control group was expressed as 100%. Each symbol represents an independent experiment. Four separate experiments were performed.

Figure 2: Three control experiments are shown. The direct effect of glucose on the lysyl oxidase assay was evaluated. The glucose was added after collagen fibril formation had occurred following the preincubation at 37°C for 60 min. The tritium water release was expressed as cpm per $5x10^{5}$ cpm of collagen substrate. No inhibitory effect of glucose was observed (o—o). The reversibility of the glucose effect on the lysyl oxidase catalyzed-tritium water release was evaluated using [6-^{3}H] lysine-labeled collagen. Tritium water release is expressed as cpm per $5x10^{5}$ cpm of collagen substrate (●—●). The glucose effect was measured on the lysyl oxidase reaction using insoluble elastin as a substrate. 5×10^{5} cpm of the [^{3}H] labeled elastin suspension was preincubated with various concentrations of glucose for 60 min at 37°C. Lysyl oxidase was then added and incubation continued for 2 hr. The tritium water release is expressed as cpm/5×10^{5} of elastin substrate. No inhibitory effect of glucose on tritium water release was observed (●---●).

Figure 3: Inhibitory effect of glucose on fibril formation of [^{3}H]collagen. 500,000 cpm of [6-^{3}H] lysine-labeled chick calvaria collagen was incubated with glucose in a microfuge tube in a final volume of 250 µl for 60 min with shaking at 37°C. Samples were then centrifuged in a Beckman Microfuge for 4 min. Radioactivity in a 50 µl aliquot of the supernatant was measured by scintillation counting. The percentage of fibril formation was calculated as described in the text. The extent of fibril formation in control groups was expressed as 100%.

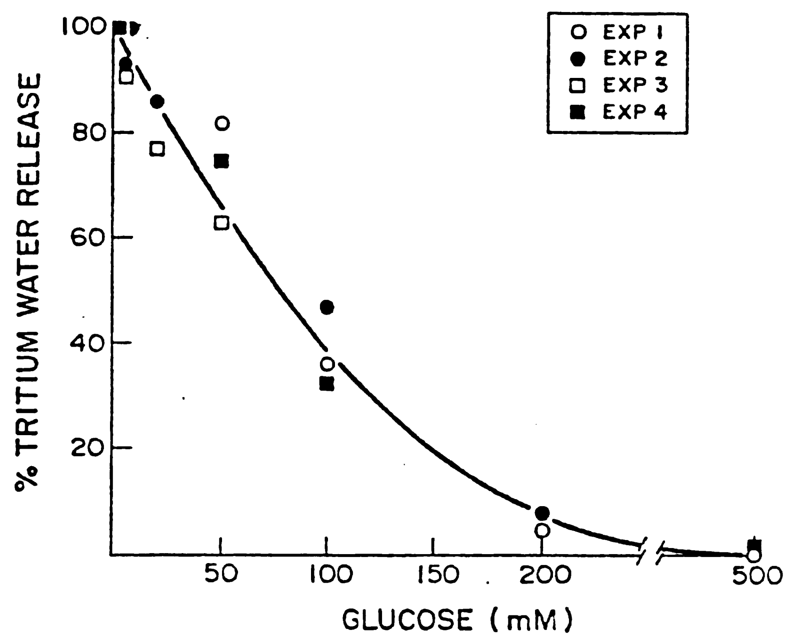


Fig. 1

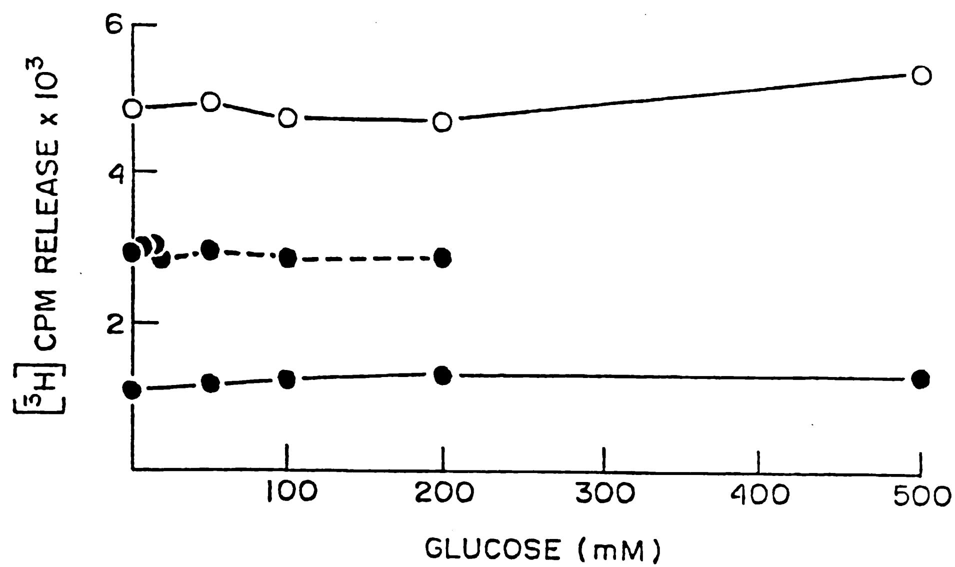


Fig. 2

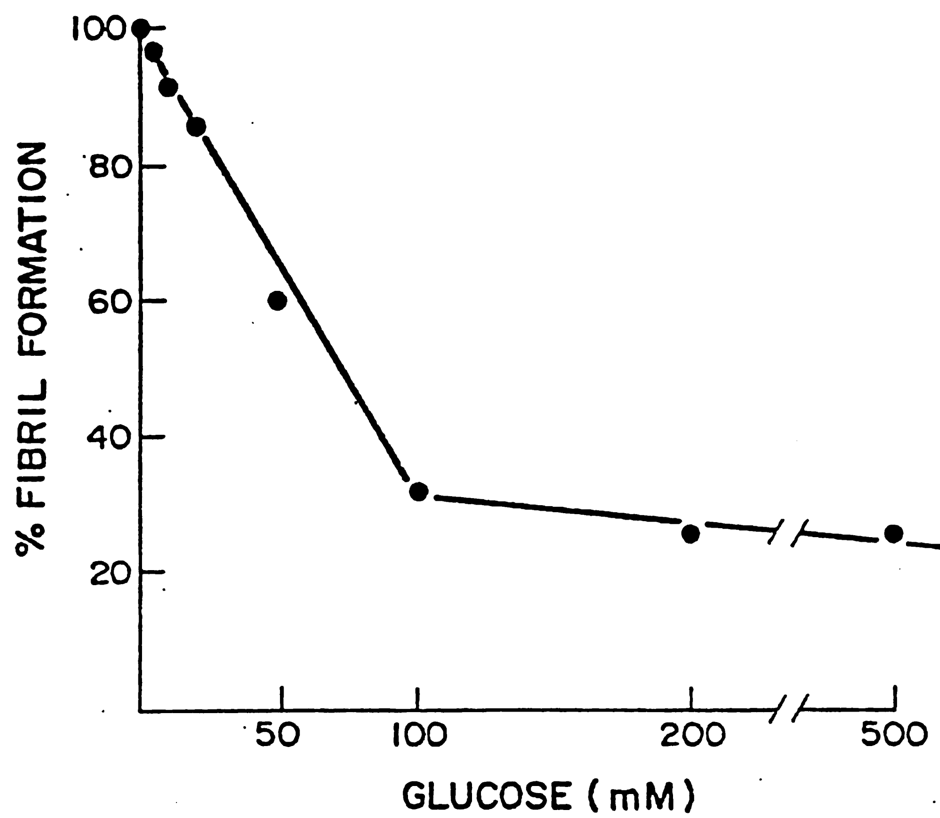


Fig. 3

CHAPTER III

MODULATION OF THE ECM BY GLUCOSE AND ITS ANALOGS

Part II

The Effect of Glucose and its Analogs on the Distribution and Degradation of Collagen from Cells and Organs in Culture

Introduction

In the diabetic, there is a net loss of collagen mass in skin (Schneir et al., 1979) and wounds (Goodson and Hunt, 1977). The loss of interstitial collagen may result from the enhanced catabolism of newly synthesized collagen (Schneir et al., 1982). The mechanism of enhanced collagen degradation in the diabetic is unknown. Newly synthesized collagen may be degraded in the procollagen form intracellularly, or may be degraded extracellularly before maturation and deposition into fibers. The intracellular degradation of collagen is well documented by Bienkowski et al. (1978) and is observed widely in various tissues and cells (for reviews, see Rennard et al., 1982). Whether the high glucose environment in the diabetic can induce the increase of intracellular degradation of collagen has not been tested. On the other hand, glucose is known to inhibit fibril formation and subsequent cross-linking of collagen *in vitro* (Chapter II). It is likely that in a high sugar environment, the collagen synthesized is less cross-linked and therefore more susceptible to collagenase digestion. In this study, I cultured chick calvaria

and human skin fibroblasts in medium containing various sugars at different concentrations and investigated the collagen metabolism in each of these culture conditions.

Materials and Methods

Studies of collagen fibril formation

[^{3}H]lysine-labeled chick calvaria collagen (specific activity, 1 x 10^9 cpm/μM collagen) was used for these studies. D-glucose, L-glucose, and 2-deoxy-D-glucose 200 mM in PBS, were prepared separately as stock solutions. Fibril formation was studied as described in Chapter II.

Chick calvaria organ cultures

Chick calvaria from 17-day-old chick embryos were incubated at 37°C in Eagle's minimal essential medium (MEM, glucose 1.0 mg/ml), 3 pairs/2 ml, without proline and glutamine, and supplemented with ascorbic acid (50 μg/ml), penicillin and streptomycin. After preincubation for 30 min to remove free proline, the medium was changed. D-glucose, L-glucose, or 2-deoxyglucose was added, as indicated, to a final concentration of 25 mM or 50 mM (the background glucose was not included). [5-^{3}H]proline was added to each well at a concentration of 25 μCi/ml and incubated in 5% CO_2 in air at 37°C for 24 hr. The calvaria were removed from the medium and washed with 1 mM PMSF in cold water (4°C). The medium and wash were then pooled.

Human skin fibroblast cultures

Human newborn foreskin fibroblasts were maintained in DMEM media (H 16, glucose 1.0 mg/ml) supplemented with 7% fetal calf serum, penicillin, streptomycin, and fungizone. Cells were incubated in 5% CO_2 in air at 37°C. Confluent cells were exposed to medium containing ascorbic acid (25 μg/ml) for 24 hr. The medium was then changed. D-glucose, L-glucose, and 2-deoxyglucose were added separately to a final concentration of 25 mM (the background glucose was not included). The cells were exposed to [5-^{3}H]proline (25 μCi/ml), and ascorbic acid (25 μg/ml) for 24 hr. For studies of the glycosylation of collagen, cells were labeled with [^{14}C]lysine (2.5 μCi/ml). Conditions were otherwise the same as described for [^{3}H]proline labeling. For the quantitation of labeled hydroxyproline, the medium was harvested and the cell layer was washed with cold water containing 1 mM PMSF. The medium and wash were pooled. The cell layer was scraped with a rubber policeman with cold water containing 1 mM PMSF.

Acid and alkaline hydrolysis

For the quantitation of hydroxyproline, acid hydrolysis was performed. The medium protein were precipitated with 10% TCA at 4°C. Whole medium, TCA precipitable medium proteins, chick calvaria, and fibroblast cell layer were then treated with 6 N HCl separately and placed in a sealed tube, and hydrolyzed in a vacuum oven for 24 hr at 110°C. The solution was then evaporated to remove HCl. The dry material was dissolved in distilled water and passed through a nylon-66 filter (Rainin, Woburn, MA) to remove insoluble substances.

For the quantitation of collagen glycosylation, alkaline hydrolysis was performed. [^{14}C]lysine-labeled culture medium was dialyzed against distilled water at 4°C for 48 hr with three changes to remove unincorporated radioactive isotopes. An equal volume of 4 M KOH was then added to the medium. The cell layer was scraped with a rubber policeman into 2 M KOH. Both medium and cell layer were placed in sealed tubes and hydrolyzed separately at 110°C in a vacuum oven for 18 hr. The solution was then neutralized with perchloric acid, centrifuged at 4°C (1000 xg, 20 min), and the supernatants lyophilized to dryness.

Dowex chromatography

For desalting of samples, DOWEX chromatography was performed as described (Dreze, et al., 1954). A 1 ml aliquot of acid hydrolysate was applied to a column (0.7 x 2 cm) of Dowex 2 x 4 (Cl^- form). After elution with 20 ml of distilled water to remove salts, the bound amino acids were eluted with 5 ml of 1.0 N acetic acid and then lyophylized. The recovery rate on Dowex chromatography was 98%.

High performance liquid chromatography

High performance liquid chromatography (HPLC) was performed for the quantiation of hydroxyproline as described (Muramiro et al., 1983). HPLC was carried out on a 250 x 4.6 mm I.D. partisil PXS 10/25 ODS-2 column (Whatman, Pierce Chemical Co.) using a high pressure Milton Roy minipump (Glenco) and a sample injection valve with a 1 ml loop (Rheodyne Co.). Desalted samples were dissolved in distilled water and centrifuged to remove insoluble substances. The supernatant was injected into a column preequilibrated with 50 mM n-heptofluorobutyric acid (HFBA), pH

3.0, and eluted with the same buffer at a flow rate of 1 ml/min. Fractions of 1 ml were collected, of which an aliquot of 200 µl was removed for counting. The column was then regenerated with 20 ml of 100% propanol and reequilibrated with 20 ml of the 50 mM HFBA for subsequent chromatography. The recovery rate for the HPLC was in the range of 95-98%.

Pepsin digestion

The culture media and cell layers were treated with pepsin separately. Medium fractions were dialyzed against 0.5 N acetic acid (4°C, 48 hr), with three changes to remove unincorporated radioactive isotopes. Cell layers were scraped with a rubber policeman in 0.5 N acetic acid and sonicated for 30 seconds in an ice bath. Predissolved pepsin (125 µg/ml) (Sigma) was then added to both medium and cell layer fractions and incubated in water bath at 15°C for 4 hr. The reaction was stopped by neutralization with NaOH to pH 8.5. The digestants were dialyzed against 0.1 N acetic acid at 4°C, 48 hr, with three changes.

SDS-polyacrylamide gel electrophoresis

Pepsin-solubilized collagen was analyzed using SDS-polyacrylamide gel electrophoresis (Neville, 1971). A 3% stacking gel and 5% separating fel were used. After electrophoresis, collagens and procollagens were identified by Coomassie Blue staining as well as by autoradiography.

Paper electrophoresis

For the quantitation of the glycoslyation of collagen, paper electrophoresis was performed as described (Myllyla et al., 1975). The alkaline hydrosylate of the medium or the cell layer was applied onto 3 M chromatography paper (Whatman, 30 x 15 cm) and electrophoresed in 0.1 M pyridine HCl, pH 3.5 at 300 volts for 3.5 hr using a Deluxe Electrophoresis

Chamber (Gelman). Lysine and hydroxylysine standards and type II collagen hydrolysate were co-electrophoresed with each run. The spots were located with ninhydrin (0.1 ninhydrin in 100% ethanol), periodate (Weiss and Smith, 1967), as well as by autoradiography.

Results

Fibril formation

D-glucose, L-glucose, 2-deoxy-D-glucose were used to investigate the changes of collagen metabolism in skin fibroblasts grown in medium containing high sugar content. All three sugars inhibited collagen fibril formation *in vitro* (Fig. 1). The half maximum inhibition of fibril formation after 37°C incubation for 1 hr was observed at 50 mM, 30 mM, and 25 mM, for D-glucose, L-glucose, and deoxyglucose, respectively.

Chick calvaria organ culture

The collagen synthesized by culture chick calvaria in the presence of various sugars was investigated. Dowex chromatography was employed

to remove salt from the samples. Figure 2 shows DOWEX chromatography of an acid hydrolysate of calvaria. The removal of salt was documented by decreased conductivity. The recovery rate was as high as 98%. The desalting process was critical for the performance of subsequent HPLC, which was used to quantitate [^{3}H]hydroxyproline.

Figure 3 shows profiles of HPLC of acid hydrolysates of whole medium (3A), TCA precipitatable medium proteins (3B), and calvaria (3C) of control groups. There are two peaks: hydroxyproline was eluted first and was followed by a proline peak. The percentage of hydroxyproline calculated from HPLC was identical to that from Dowex chromatography (data not shown), which was routinely used for separation of hydroxyproline and proline (Cutroneo et al., 1972). The proportion of hydroxyproline was in the range of 2-8% in whole medium, 30-37% in TCA precipitable medium proteins, and 23-30% in calvaria. Since collagen is the principal hydroxyproline-containing protein synthesized in calvaria culture, the total [^{3}H] labeled hydroxyproline could be used as an estimate of total collagen synthesis (Table I). In the presence of D-glucose, collagen synthesis was stimulated. Total collagen was 185% and 209% of that of control at 25 mM and 50 mM, respectively. L-glucose suppressed collagen synthesis slightly (12% decrease) at 50 mM. Deoxyglucose suppressed collagen synthesis significantly. Collagen synthesis decreased 46% at 25 mM and 50% at 50 mM. The inhibitory effect of deoxyglucose could be reversed by the addition of D-glucose.

The hydroxyproline distribution in the following three compartments is shown in Fig. 4. Hydroxyproline in TCA soluble medium fraction represented the degraded collagen. Hydroxyproline in TCA precipitable fraction represented the intact soluble collagen in the medium. The

calvaria fraction reflected the incorporation of collagen in tissue. In the control group, 20% of hydroxyproline was in TCA soluble medium fraction, 24% in TCA precipitable medium fraction, and the rest in calvaria. In the presence of D-glucose, the distribution of hydroxyproline was similar to the control group. L-glucose increased the degradation of collagen significantly. Thirty-three and sixty-two percent more collagen were degraded at 25 mM and 50 mM of L-glucose, respectively, when compared to the control group. The collagen deposition in calvaria was also suppressed by L-glucose by 16% and 27% at 25 mM and 50 mM. Deoxyglucose also increased collagen degradation (50% and 58% increase at 25 mM and 50 mM) and decreased collagen deposition (28% and 26% decrease at 25 mM and 50 mM). The addition of deoxyglucose, 25 mM, and D-glucose, 25 mM, did not diminish the deoxyglucose effect. Collagen degradation increased 73% and deposition decreased 20%.

Human newborn foreskin fibroblast cultures

Collagen distribution and degradation in the presence of various sugars were studied in cultured human skin fibroblasts. The results are shown in Table 2 and Fig. 5. Both D-glucose and L-glucose did not affect the collagen synthesis, distribution, and degradation significantly. Deoxyglucose, however, suppressed 46% of collagen synthesis and increased collagen degradation by 62%. The percentage of collagen deposition in cell layers was not affected significantly, but the intact soluble collagen in medium was decreased more than 50%.

Figure 6 shows the SDS polyacrylamide gel electrophoresis pattern of skin fibroblast collagen in medium and cell layer. The major collagen types in the medium were types I and III, and in the cell layer, types

I, III, and V. The profiles of collagens synthesized in the presence of various sugars did not differ. The suppression of collagen by deoxyglucose was confirmed by gel electrophoresis.

Glycosylation

The glycosylation of collagen in the presence of various sugars was examined by paper electrophoresis, shown in Fig. 7. The glucosylgalactosyl hydroxylysine and galactosylhydroxylysine was identified by ninhydrin (for amino acid) and periodate (for sugar) staining (Schofield et al., 1971). D-glucose and L-glucose did not affect glycosylation, while deoxyglucose suppressed the glycosylation of collagen significantly.

Discussion

D-glucose, L-glucose, and 2-deoxy-D-glucose were used to study their effect on collagen metabolism. The three sugars were distinct from each other in terms of transportation and utilization in the cell. D-glucose is transported into the cell through a facilitated diffusion process and is used as an energy source. L-glucose is transported into the cell by simple diffusion and is not utilized by the cell. L-glucose does not inhibit the transport and utilization of D-glucose (Graff et al., 1977). Deoxyglucose enters the cell through the same mechanism as D-glucose. However, deoxyglucose is not metabolized by the cell. Deoxyglucose inhibits the transport of D-glucose and interferes with the normal sugar metabolism (Dolberg et al., 1975). When the cells grew in the medium containing deoxyglucose at 25 mM for 24 hr, there was no

lethal effect observed. However, in long-term cultures with deoxyglucose, e.g., 7 days, cell numbers decreased significantly (data not shown).

All the three sugars inhibited collagen fibril formation in vitro (Fig. 1). However, their effects on collagen metabolism were quite different. In chick calvaria culture, D-glucose stimulated collagen synthesis, deoxyglucose suppressed it, while L-glucose had no effect on collagen synthesis (Table 1). The stimulatory effect of D-glucose on collagen synthesis was not unexpected and has been observed previously in cultured skin fibroblasts (Villee and Powers, 1977). Shaw and Amos (1973) have shown that in cells grown in D-glucose concentrations of more than 1 mg/ml, the transport of D-glucose is gradient-dependent and insulin-independent. Therefore, at high concentrations, more D-glucose could be utilized and general protein synthesis was enhanced. The observations that the hydroxyproline ratio in TCA precipitable medium fractions and in calvaria fractions stayed within a narrow range, suggested the D-glucose effect on collagen synthesis was nonspecific. The effects of L-glucose and deoxyglucose on collagen synthesis were consistent with their properties described previously.

The degradation of collagen in chick calvaria cultured in serum-free medium may reflect both intracellular and extracellular degradation, as observed in the cultured chick tibiae and mandibles (Sakamoto et al., 1979; Howard et al., 1981). The percentage of collagen degradation in the control group (20%) is consistent with previous studies with rats (25-30%) and mouse calvaria (20-30%) (Stern et al., 1965; Kream et al, 1980). While D-glucose increased collagen degradation only slightly, both L-glucose and deoxyglucose increased collagen degradation and decreased collagen deposition significantly. Interestingly, the addition

of D-glucose to deoxyglucose-containing medium could reverse the inhibitory effect of deoxyglucose on collagen synthesis, but did not diminish its effect on collagen degradation and deposition.

In skin fibroblast cultures, D- and L-glucose did not affect total collagen synthesis (Table 2). The observation of the D-glucose effect was different from that in calvaria culture (Table 1) and from observations made in other studies (Villee and Powers, 1977). This difference may be explained by the presence of fetal calf serum, which is known to stimulate collagen synthesis and may, therefore, diminish the stimulatory effect of glucose alone (Narayanan and Page, 1977).

When skin fibroblasts are grown in the presence of fetal calf serum, the degradation of collagen may reflect the intracellular degradation only, because extracellular degradation is inhibited by serum protease inhibitors (Bienkowski et al., 1978). Both D- and L-glucose did not increase intracellular collagen deposition while deoxyglucose increased it significantly (Table 2 and Fig. 5).

The findings of the present study are complex. D-glucose apparently did not have a significant effect on collagen degradation either intracellularly or extracellularly. The effects of D-glucose on collagen degradation may have been diminished by the augmentation effect of D-glucose on collagen synthesis. L-glucose did not increase intracellular degradation in skin fibroblast cultures. The increase of collagen degradation in calvaria culture therefore, was likely caused by the increased extracellular degradation. Since L-glucose is not taken up and utilized by the cell, it may act as an inert bystander. L-glucose did not affect collagen synthesis. It may inhibit collagen fibril formation extracellu-

larly, thus resulting in the instability of collagen. However, direct evidence for such a mechanism is still lacking.

Deoxyglucose can affect collagen metabolism at many points in the metabolic pathway: 1) it interferes with sugar metabolism and general protein synthesis; 2) it inhibits glycosylation of collagen (Fig. 6); 3) it inhibits collagen fibril formation (Fig. 1). Glycosylation of collagen may influence cross-linking formation and thus influence or even determine fiber diameter (Schofield et al., 1971; Pinnell et al., 1972). The increase of collagen degradation, including both intracellular and extracellular degradation, may involve any of the above mechanisms in any of several combinations.

TABLE 1

Total Collagen Synthesis in Chick Calvaria Culture

	$[^3H]$Hydroxyproline cpm 10^{-3}/mg calvaria dry weight	Percent
Control	125 ± 12	100
D-glucose, 25 mM	228 ± 21*	183
D-glucose, 50 mM	260 ± 15*	209
L-glucose, 25 mM	118 ± 7	94
L-glucose, 50 mM	110 ± 9	88
2-Deoxy-D-glucose, 25 mM	79 ± 6**	64
2-Deoxy-D-glucose, 50 mM	63 ± 4*	51
2-Deoxyglucose, 25 mM + D-glucose, 25 mM	193 ± 12*	155

Note: Each experiment was performed in duplicate. Collagen synthesis in the control group is to be assumed 100%. $*p < 0.01$. $**0.01 < p < 0.025$.

TABLE 2

Total Collagen Synthesis in Human Skin Fibroblast Culture

	$[^3H]$Hydroxyproline cpm $10^{-4}/10^6$ cells	Percent
Control	12.8 ± 0.3	100
D-glucose, 25 mM	13.6 ± 0.5	106
L-glucose, 25 mM	13.1 ± 0.7	102
2-Deoxy-D-glucose, 25 mM	6.9 ± 0.3*	54

Note: Each experiment was performed in duplicate. Collagen synthesis in the control group is assumed to be 100%. *$p < 0.005$.

Legends to Figures

Fig. 1. Inhibition curve of collagen fibril formation by D-glucose (o--o), L-glucose (o——o), and 2-deoxy-D-glucose (Δ----Δ).

Fig. 2. DOWEX chromatography of acid hydrolysate of [^{3}H]proline-labeled chick calvaria. The sample was processed and chromatographed as described under Materials and Methods. Conductivity of each fraction was measured at room temperature. Successful removal of salt was demonstrated by the decrease of conductivity. Arrows indicate the start of 1.0 N acetic acid elution.

Fig. 3. HPLC profiles of acid hydrolyzed [^{3}H]proline labeled whole medium (panel A), TCA precipitable medium (B), and calvaria (C). Hydroxyproline and proline peaks are indicated.

Fig. 4. [^{3}H]hydroxyproline distribution in chick calvaria culture. [^{3}H]hydroxyproline in TCA soluble medium fraction, TCA precipitable medium fraction, and calvaria fraction are indicated. * indicates $p < 0.005$. #: $p < 0.01$.

Fig. 5. [^{3}H]hydroxyproline distribution in human newborn foreskin fibroblast culture. [^{3}H]hydroxyproline in TCA soluble medium fraction, TCA precipitable medium fraction, and calvaria fraction are indicated. * $p < 0.005$. #: $p < 0.01$.

Fig. 6. Autoradiograms of SDS polyacrylamide gel electrophoresis under non-reducing (A) and reducing (B) conditions. Lanes 1, 2, 3, and 4 are pepsin-treated medium proteins. Lanes 5, 6, 7, and 8 are pepsin-treated cell layer-associated proteins. Lanes 1 and 5 are control cultures; lanes 2 and 6 are cultures with 25 mM D-glucose; lanes 3 and 7, 25 mM L-glucose; lanes 4 and 8, 25 mM 2-deoxyglucose. Collagen types are indicated.

Fig. 7. Paper electrophoresis of acid hydrolysate of [^{14}C]lysine-labeled medium proteins. Lane 1) periodate staining; Lanes 2-6) Ninhydrin staining; Lanes 7-10) Autoradiogram. Lanes 1 and 2 are hydrolysates of type II collagen. Lanes 3, 4, 5, and 6 are hydrolysates of [^{14}C]lysine-labeled medium proteins which were precipitated with 30% ammonium sulfate (cold type I collagen was added as a carrier). Lanes 7, 8, 9, and 10 are the same as lanes 3, 4, 5, and 6, repectively. Galactosyl-OH-lysine and glucogalactosyl-OH-lysine are indicated. Note the decrease of galactosyl-OH-lysine in lane 10.

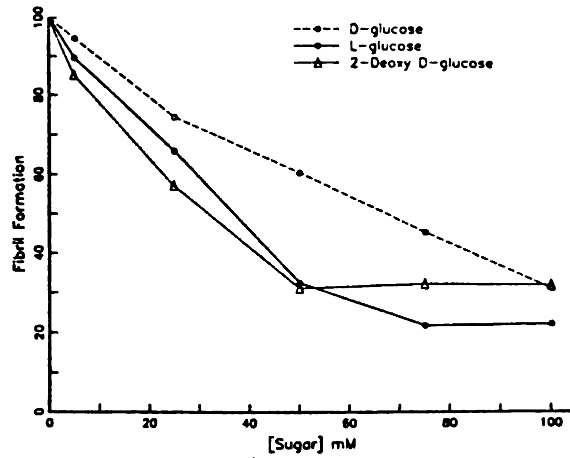


Fig. 1

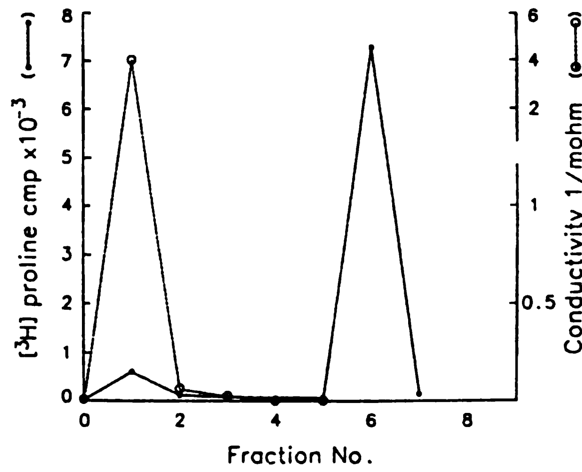


Fig. 2

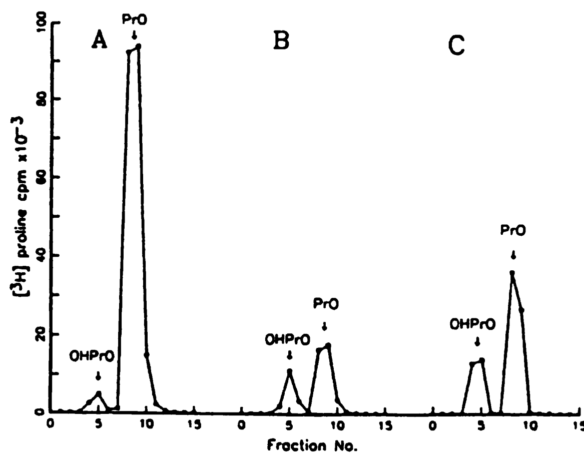


Fig. 3

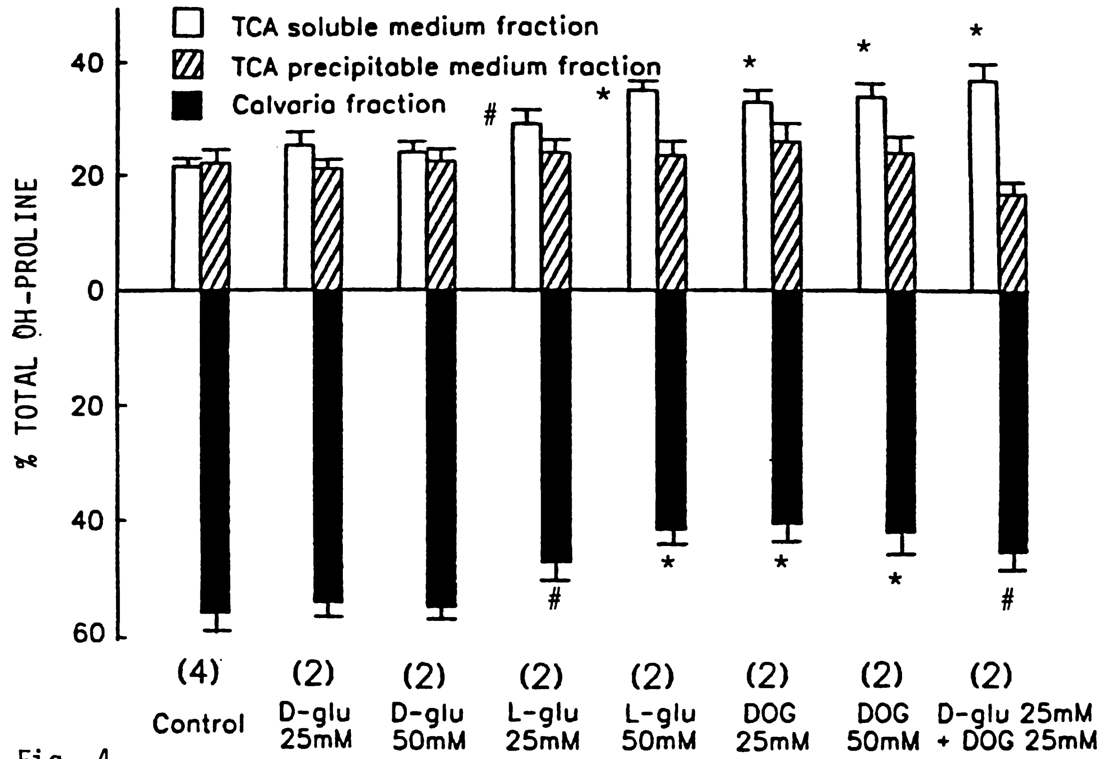


Fig. 4

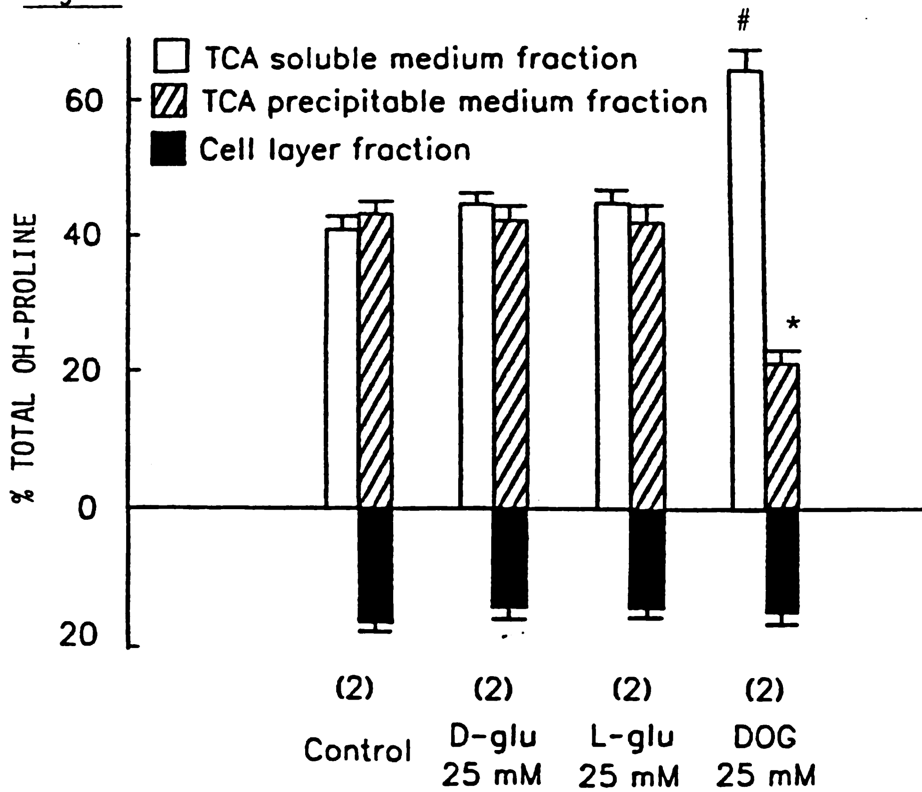
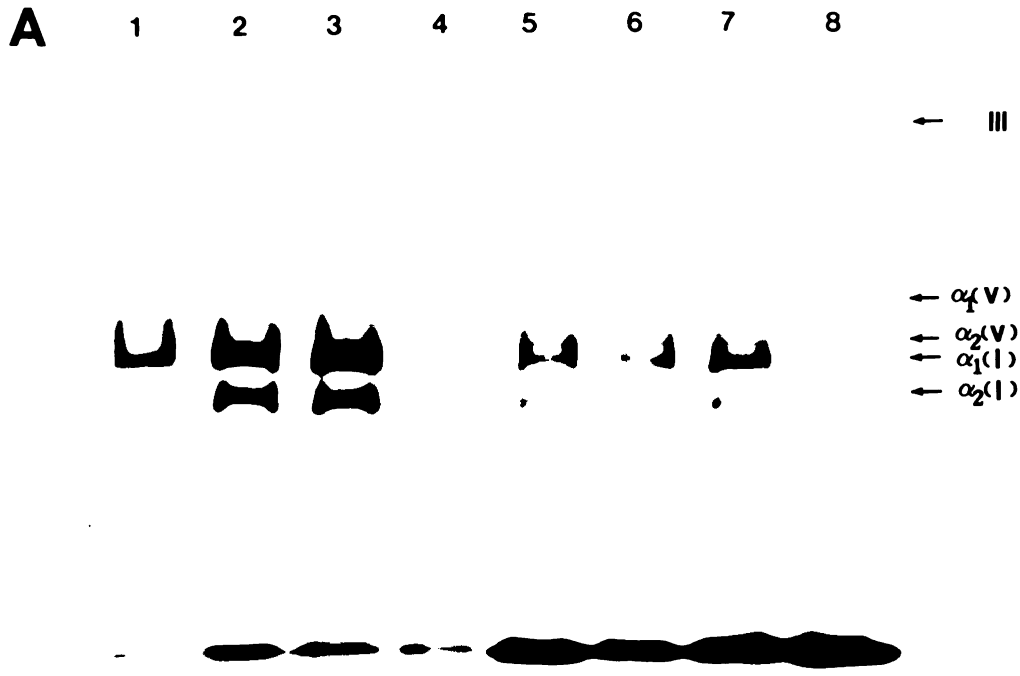


Fig. 5



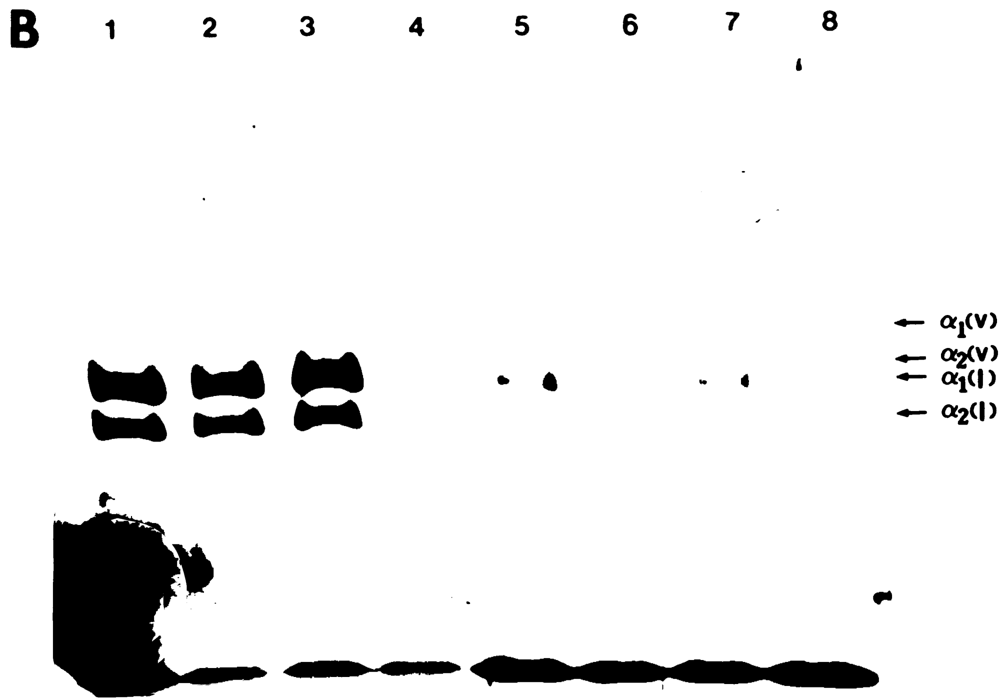


Fig 6

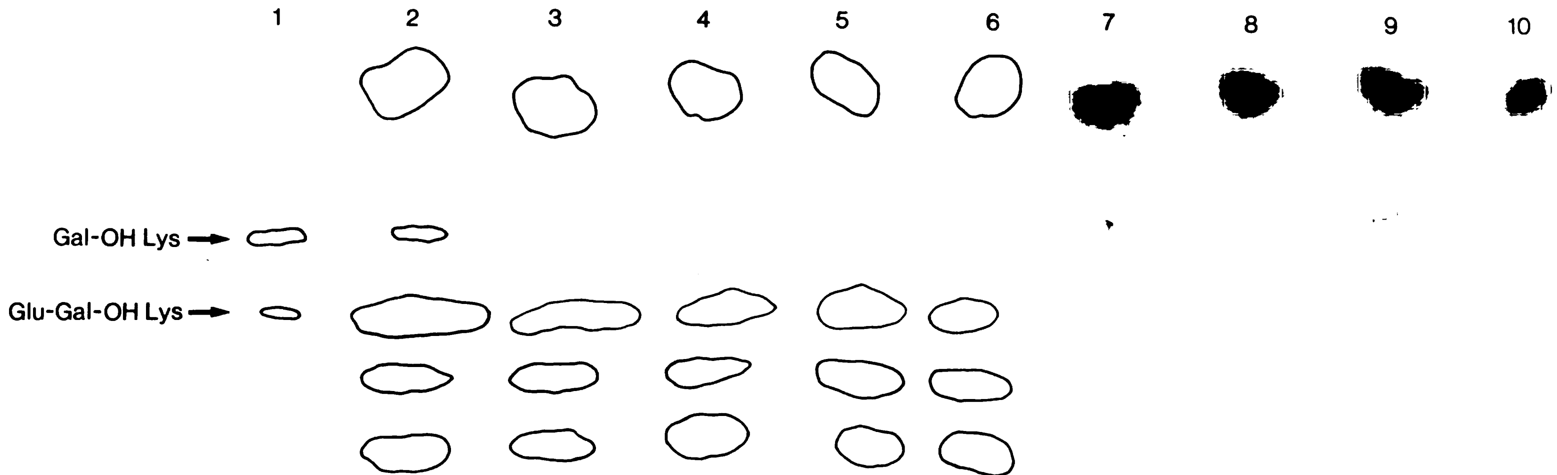


Fig 7

CHAPTER IV

THE GENETIC BASIS OF ECM INSTABILITY IN THE HEREDITARY SCOLIOTIC CHICK

Part I: Properties of Collagen in Hereditary Scoliotic Chick Skin Fibroblasts

Introduction

Scoliosis is a common lesion of the spine in humans, causing deformity and in some cases even disability. More than 50 disease entities are known to cause scoliosis, however 75%-80% of scoliotic cases in the United States are categorized as idiopathic (Harrington, 1977). The patients with idiopathic scoliosis have characteristic clinical features with onset in adolescense and with a predominance of females (80%). There is a familial tendency and the expression is consistent with either a dominant or multiple genic pattern of inheritance (Wynne-Davies, 1968).

Patients with systemic collagen disorders such as Marfan's syndrome, osteogenesis imperfecta, homocystinuria, and Ehlers-Danlos syndrome often manifest scoliosis (McKusick, 1972). Young animals fed lathyrogens, inhibitors of collagen cross-linking, also develop scoliosis (Geiger et al., 1933). Any disturbance involving collagen, particularly the type I collagen molecule, the major structural protein of the body, may cause some degree of scoliosis. Transient problems with collagen

metabolism have also been invoked in adolescent idiopathic scoliosis. An elevated total urinary hydroxyproline and an instability of skin collagen are both demonstrable in many patients with adolescent idiopathic scoliosis (Zorab et al., 1971, Francis et al., 1975). Collagen stability returns to normal however, once skeletal system maturation occurs (Francis et al., 1975). The actual mechanism of the increased collagen instability during the adolescent growth spurt remains unknown, but is probably related to some aspect of the increased turnover rates.

A highly inbred strain of chicks with hereditary scoliosis has been developed by Taylor (1971). This appears to be a suitable animal model for human adolescent idiopathic scoliosis, and provides an opportunity to study the pathogenesis of the disease. The chick model has several parallels with the human disease. First, the spinal curvature in the chick develops at four to six weeks of age, when sexual development and the growth spurt of the chick begins (Lin et al., 1980). Secondly, human scoliosis occurs more often in females, the homogametic sex in mammals, while the male chick, the homogametic sex in birds, has the higher prevalence of scoliosis. The male chick invariably develop more severe curvature than the female (Riggins _et al_, 1977). Thirdly, in scoliotic chicks there is increased extractability of collagen from skin, the vertebral column, cartilage and long bone (Riggins et al., 1977; Kenney et al., 1982) and the serum hydroxyproline level (Lin et al., 1980) is elevated. Apparently, scoliotic chicks also have some systemic derangement of connective tissue that underlies the scoliosis.

In order to explore the molecular mechanisms of the connective tissue instability, skin fibroblasts were cultured from the scoliotic chicks. Using radiolabeled metabolic precursors, we examined the colla-

gens synthesized by those fibroblasts. I demonstrated that the instability in extracellular matrix was also expressed in cultured skin fibroblasts from scoliotic chicks. A molecular basis for the instability was explored in this chapter.

Materials and Methods

Cell cultures

Chicken skin fibroblasts were cultured from the biopsies of four week old normal (CN as control) white Leghorn chicks and chicks with hereditary scoliosis. The latter were an inbred strain developed by Taylor (1971), and maintained at UC Davis. Some of these chicks had frank scoliosis (AF, affected); while others did not (NA, unaffected). Fibroblasts were maintained in DMEM-H 21, supplemented with 7% fetal calf serum, penicillin, streptomycin, and fungizone and incubated in 5% CO_2 in air at 37°C. Cells from the fourth to fifteenth passages were used routinely for this study.

Metabolic labeling

Subconfluent cultures of chick skin fibroblasts were exposed to L-[2,3,4,5-^{3}H] proline, 35 μci/ml, as described in Chapter II. In experiments for the characterization of procollagen, BAPN (50 μg/ml) was added during the labeling period to prevent collagen cross-linking. After 24 h, the medium was removed and the cell layer was washed three times with cold (4°C) PBS containing 1 mM PMSF. The plates were then stored at -70°C until used. Culture medium and the first wash were combined. To inhibit further proteolysis, proteinase inhibitors includ-

ing PMSF, benzamidine, PCMB, NEME, and EDTA (Sigma St. Louis, MO) were added at final concentrations of 1,1,1,5 and 20 mM, respectively. After mixing, the medium was centrifuged at 1,000 xg for 5 min to remove cell debris, and the supernatant was stored at -70°C. For the pepsin digestion studies, the medium was first dialyzed against distilled water at 4°C 24 h to remove protease inhibitors, and then dialyzed against 0.5 M acetic acid at 4°C for 48 h with three changes.

DEAE-cellulose chromatography:

To separate and quantitate the procollagens in the media, DEAE-cellulose chromatography was performed as described (Smith et al., 1972). In brief, saturated ammonium sulfate was added to the medium to a final concentration of 30% (v/v). The resulting suspension was stirred gently overnight at 4°C in the presence of 3 mg of carrier collagen (Vitrogen, Flow Labs. McClean, Virginia). Precipitates were collected by centrifugation (7,000 xg 30 min., 4°C), dissolved in the initial buffer, 2 M urea, 50 mM Tris-HCl pH 7.5, and dialyzed overnight at 4°C against the same buffer. Samples were applied to a column of DEAE cellulose (1.5 X 7 cm) and washed with 25 ml of the initial buffer. Bound proteins were eluted using a linear gradient (0 to 0.2 m) of NaCl (total volumn 175 ml, flow rate 7.5 ml/h). Fractions of 2.5 ml were collected, of which an aliquot of 0.1 ml was removed for counting of radiolabeled materials.

CM cellulose chromatography

CM cellulose chromatography was performed as described previously (Epstein et al., 1971). The peak 1 fraction of DEAE cellulose chromatography was dialyzed against 40 mM sodium acetate, 2 M urea, pH 4.8, the

initial buffer, at 4°C overnight. Prior to chromatography, samples were denatured by heat treatment (42°C, 30 min), and the insoluble material was removed by centrifugation (1000 xg, 10 min) at room temperature. Samples were then applied to a jacketed CM-cellulose column (0.7 x 10 cm), pre-equilibrated with the deareated initial buffer at 42°C, and washed with 25 ml of the initial buffer. Bound proteins were eluted using a linear gradient (0 to 0.1 M) of NaCl (total volume 150 ml, flow rate 37 ml/h). Fractions of 2.5 ml were collected, of which an aliquot of 300 µl was removed for radiolabeled counting. Type I collagen extracted for ^{14}C-proline labeled chick embryo calvaria was also applied to the CM cellulose chromatography as a reference standard.

Pepsin digestion

The culture media and cell layers were treated with pepsin as described in Chapter III.

Bacterial collagenase digestion

For the quantitation of collagen, pepsin-treated medium and cell layer fractions were digested with bacterial collagenase, separately, as described (Peterkofsky, 1982). Aliquots of pepsin digestants were lyophilized and then redissolved in 0.5 ml of 0.1 M HEPES buffer, PH 7.2, containing 0.5 mM $CaCl_2$, and 2.5 mM NEME. Bacterial collagenase (Advance Biofactures, Lynbrook, NY), was added to a final concentration of 100 units/ml and then incubated at 37°C for 90 min. The levels of bacterial collagenase-degraded products were estimated by using the cold TCA (5%) and tannic acid (0.25%) precipitation technique.

SDS-polyacrylamide gel electrophoresis

Peaks from DEAE-cellulose chromatography, pepsin-solubilized collagen and bacterial collagenase digestants were analyzed using SDS-polyacrylamide gel electrophoresis as described in Chapter III.

Sequential extraction of collagen

For the study of collagen extractability, BAPN was not added during the period of metabolic labeling. Collagen associated with the cell layers was extracted sequentially with 1 M NaCl, 0.5 N acetic acid and 8 M urea as described (Peltonen et al., 1983). The cell layer in a 25 cm^2 flask was scraped with a rubber policeman in 1 ml of 1 M NaCl and sonicated for 30 seconds in an ice bath. The suspension was rotated continuously at 4°C for 24 h and then centrifuged at 1,000 xg for 15 min. The pellet was extracted sequentially with 1 ml of 0.5 N acetic acid and then 8 M urea in the same manner. The supernatants obtained from each extraction were dialyzed against HEPES, 0.1 M, $CaCl_2$, 0.1 mM, and NEME, 2.5 mM, pH 7.2, at 4°C overnight. The collagen content was then assayed using the bacterial collagenase digestion technique.

Lysyl oxidase assay

Lysyl oxidase activity released into the medium was assayed according to the method described by Siegel (1974). Confluent cells were cultured in serum-free medium for 24 h. The culture medium was then harvested and assayed directly. After preincubation of the collagen substrate at 37°C for 1 h, the culture medium was added and further incubated at 37°C for 10 h. Controls, without addition of enzyme were routinely included to correct for background levels. The tritium water released by culture medium from [6-^{3}H] lysine-labeled chick calvaria

collagen reflected lysyl-oxidase activity and was expressed as cpm/mg of cellular protein. Cellular protein was determined by the method of Lowry (1951).

Cellular copper content

The cellular copper content was determined by atomic absorption spectrophotometry (Goka et al., 1976). After cells reached confluency, the medium was replaced with fresh complete medium with or without a supplement of exogenous $CuSO_4$ 5μg per ml, 24 h prior to harvest. The medium was then removed. The cell layer was rinsed three times with PBS, then scraped with a rubber policeman into distilled water and sonicated for 15 seconds. The cellular copper content was measured on a Perkin-Elmer model 403 atomic absorption spectrophotometer equipped with a graphite furnace. The absorbance at 324.7 nm of 20μl of samples were equated to copper concentrations of aqueous standards (0-300 ng of Cu per ml). For samples with exogenous copper supplement, a 3-5x dilution was made to allow the values to fall within the linear limitation of the standard curve. The cellular copper was expressed in ng per mg cellular protein.

Electron microscopic study

Cell layer-associated collagen and proteoglycan were examined by electron microscopy. Cuprolinic Blue staining for proteoglycans was performed according to Scott et al., (1981). After cells reached confluence, the medium was replaced with fresh complete medium with a supplement of ascorbic acid, 25μg per ml, 48 h prior to harvest. The medium was then removed. The cell layer was rinsed with PBS and fixed with 1% paraformaldehyde and 3% glutaldehyde in 0.1 M cacodylate pH 7.4 with 7%

sucrose for 30 min at room temperature. The fixed cell layer was rinsed three times with 0.1 M cacodylate with 7% sucrose, then scraped with a rubber policeman into the same buffer. Cell pellets were spun down (1000 xg, 5 min) and stained with 0.05% Cuprolinic Blue in 25 mM sodium acetate, pH 5.7, containing 0.1 M $MgCl_2$ and 2.5% glutaldehyde at room temperature overnight. Specimens were then rinsed three times with the same buffer without Cuprolinic Blue, and three times with 0.5% sodium tungstate. After dehydration, infiltration, embedding, and thin sectioning, the specimens were examined in a Zeiss EM 9A electron microscope.

Results

Cell morphology

Chick skin fibroblasts from normal and scoliotic chicks were similar in appearance. As shown in Figure 1, fibroblasts both in sparse and dense cultures had a similar morphology under phase contrast microscopy. In dense cultures, both the normal and the scoliotic chicken fibroblasts grew into multilayers as is typical of such cells. The growth rate of these cells were similar to each other (data not shown).

DEAE cellulose chromatography

The procollagens synthesized by chick skin fibroblasts were examined by DEAE cellulose chromatography. Figure 2 shows the profile of control (panel A) and affected fibroblasts (panel B). The majority of radiolabeled materials eluted before initiation of the salt gradient and represented processed collagen that had already been converted from procollagen.

Since all samples were processed at 4°C in the presence of protease inhibitors, the removal of propeptides from procollagen was likely to have occurred during the period of cell culture. There were no differences observed between the control and affected fibroblasts. The profile of unaffected fibroblasts was similar and is therefore not shown. Peak 1 and 2 as indicated by the bars in Fig 1, were pooled respectively for CM cellulose chromatography and SDS polyacrylamide gel electrophoresis.

CM cellulose chromatography

Peak 1 of DEAE cellulose chromatography was subjected to CM cellulose chromatography to identify the type-specific collagens. Figure 3 shows the CM cellulose chromatography profile of peak 1 from control fibroblasts. Only two peaks, representing collagen $\alpha1$ and $\alpha2$ chains respectively of type I collagen, could be seen. The profiles of CM cellulose chromatography of unaffected and affected fibroblasts were similar to those in Figure 2 and are not shown.

SDS polyacrylamide gel electrophoresis

Peak 1 and 2 from DEAE cellulose chromagraphy were subjected to SDS polyacrylamide gel electrophoresis. The autoradiogram is shown in Figure 4. Peak 1 contained primarily collagen $\alpha1$ (I), $\alpha2$ (I) and some type I pc collagen. Peak 2 was composed of procollagen, $p_C$collagen, and $p_n$collagen of $\alpha1$(I) and $\alpha2$(I). The migration of collagen and procollagen from affected and control fibroblasts on SDS polyacrylamide did not differ.

The pepsin treated medium and cell layer fractions were also subjected to SDS polyacrylamide gel electrophoresis and are shown in Figure

5. Medium fractions contained mainly type I collagen, while cell layer fractions contained both type I and type V collagen. Since equal proportions of total labeled material in each fraction were applied to the gel, we were able to compare directly the relative amounts of collagen in the medium and the cell layer in each of the cell lines. Apparently in affected and unaffected fibroblasts there was less collagen deposited into the cell layer, compared to the control.

Sequential extraction of cell layer associated collagen

The stability of collagen in the extracellular matrix was investigated by sequential extraction of collagen using a series of solvents. The soluble collagen in each solvent was quantitated using the bacterial collagenase digestion technique. Figure 6 shows the results of such sequential extraction. The extractability of collagen, especially in 1M NaCl and 0.5N acetic acid was significantly higher in unaffected and in affected fibroblasts. The residue was 70% total cell layer-associated collagen in control fibroblasts, and 52% in the affected fibroblasts.

Distribution of collagen

Collagen distribution between medium and cell layer was determined using the bacterial collagenase digestion technique. Figure 7A shows the collagen level expressed per mg cellular protein in the three cell lines. The collagen level in the medium was similar among all three cells; however in the cell layer, the collagen level was much lower in the affected (65% decrease) and unaffected fibroblasts (40% decrease). When the collagen distributions were expressed as the percentage of total collagen synthesized (Figure 7B), more than 40% was deposited in

the cell layer in control fibroblasts while only 20% in affected fibroblasts.

Lysyl oxidase activity and cellular copper content

Lysyl oxidase activity in the medium was determined and is shown in Table 1. No significant difference were observed among the three cell lines. Cellular copper content was determined using atomic absorption spectrophotometer. Without exogenous copper, the cellular copper was in the range of 5-6 ng per mg cellular protein (Table 1) and again no significant differences were observed. The addition of 5 μg copper per ml to the culture medium increased the cellular copper content 8-10 folds. In the presence of this exogenous copper the cellular copper in the affected and unaffected fibroblasts was slightly lower than that of the control.

Electron microscopic studies

The collagen fibrils and proteoglycans in the cell layer were examined by using electron microscopy. Proteoglycans were identified by Cuprolinic Blue staining (Scott et al., 1981). Fig 8 shows electron micrograms of control (Fig 8A and Fig 8B) and affected (Fig 8C and 8D) fibroblasts. Much of the cell layer was composed of actual cell bodies. Some extracellular matrix could be visualized in the intercellular space. The extracellular matrix was comprised of filamentous, fibrillar and granular material. The former two appeared to be immature and mature collagen fibrils that were parallel to the cell surface. The mature collagen fibrils were 40-100 nm in diameter and of various lengths. The granules were 30-50 nm in size. They were Cuprolinic Blue-stained

proteoglycans. The proteoglycans granules always accompanied the filamentous material, and were evenly spaced in the ECM. In both control (Fig 8B) and affected (Fig 8D) fibroblasts, there were some vacuoles containing both filamentous and granular material. These vacuoles were similar to secretary vacuoles in chondrocytes reported previously (Revel and Hay, 1963; Hascall, 1980). The presence of both filamentous and granular material in those vacuoles suggested these components were packaged together for secretion.

The major difference between control and affected fibroblasts was that the ECM components were significantly reduced in affected fibroblasts. Both filamentous and granular material were decreased. This difference could be appreciated at lower magnitude (Fig. 8A,C; X 6080). Because during preparation the cell layer was scraped off after fixation and then spun-down, the ECM was not evently distributed intercellularly. This uneven distribution makes the quantitation of proteoglycan granules difficult. Proteoglycan levels were therefore determined by using biochemical analyses, which will be described in the next chapter.

Discussion

The collagen extractability and plasma hydroxyproline level are elevated in hereditary scoliotic chicks (Riggins et al., 1977; Lin et al., 1980). We examined the collagen synthesized by cultured skin fibroblasts from hereditary scoliotic chicks to determine whether the derangement of connective tissue *in vivo* was also expressed in cell cultures. Chick skin fibroblasts from normal and from scoliotic chicks secreted primarily type I collagen into the medium and deposited type I

and type V collagen into the cell layer (Fig 2 to 5). The migration of each collagen type from scoliotic chick skin fibroblasts on SDS polyacrylamide gel electrophoresis was not different from that from normal chick fibroblasts. The ratio of type V to type I collagen did not differ between scoliotic and normal chick skin fibroblasts. Kenney et al., (1982) have reported that the primary structure of type I collagen from skin is normal in scoliotic chicks. Our finding were consistent with their observations.

The major differences between normal and scoliotic chick skin fibroblasts were the distribution of collagen between medium and cell layer (Fig 6 and 7) and the extractability of cell layer-associated collagen (Fig 5). In control fibroblasts, 42% of total collagen was distributed in the cell layer, while only 20% in affected fibroblasts. There was a two-fold difference between the two. The percentage of cell layer-associated collagen in unaffected fibroblasts (32%) was intermediate between control and affected fibroblasts. The extractability of cell layer-associated collagen was elevated in scoliotic chick skin fibroblasts. The percentage of total extractable collagen was 30% in control fibroblasts, 48% in affected fibroblasts and 46% in unaffected fibroblasts. Therefore we concluded that the instability of extracellular matrix was expressed in cultured skin fibroblasts from scoliotic chicks.

To examine whether a defect in cross-linking might underlie the increased extractability of collagen, we assayed lysyl oxidase activity in the cultured medium. Lysyl oxidase is the enzyme that catalyzes the cross-linking reaction of collagen. Its deficiency has been observed in several inherited disorders of connective tissue including x-linked cutis laxis, Ehlers-Danlos syndrome type IX and Menkes' kinky hair

syndrome etc. (Byers et al., 1980; Kuivaniemi et al., 1982; Royce et al., 1980). In the latter two disease states, the reduced lysyl oxidase activity is related to the abnormal accumulation of intracellular copper that appears not to be functionally available (Goka et al., 1976; Horn 1976; Peltonen et al., 1983). Therefore we determined the level of cellular copper. As shown in Table 1, in scoliotic chick skin fibroblasts, both lysyl oxidase activity and cellular copper content were within the normal range. We concluded that the mechanism of Menkes' syndrome was not applicable to hereditary scoliosis in chicks.

Two possible mechanisms for the instability of collagen were considered, a defect in collagen fibril formation and an abnormality in the interaction between collagen and proteoglycans. Fibril formation of collagen is critical for the proper cross-linking of collagen. The native fibril of collagen, but not the soluble form is the appropriate substrate for the lysyl oxidase reaction (Siegel 1974). Proteoglycan-collagen interaction may influence the deposition of collagen fibrils (Lindahl and Hook 1978). Glycosaminoglycans appear to be a stabilizing factor for collagen fibril formation _in vitro_ (Lilja and Barrach 1981). In tissues, proteoglycans play an important role in the size determination and organization of the collagen fiber (Scott et al., 1981; Borcherding et al., 1975). Therefore, we examined the collagen fibril and the matrix proteoglycans by electromicroscopy. As expected, the content of extracellular matrix was reduced significantly in the fibroblasts from scoliotic chick skin (Fig 8). The collagen appeared to exist in both filamentous and fibrillar forms in both control and affected fibroblasts. The proteoglycans granules, stained with Cuprolinic Blue, in the affected fibroblasts were decreased. Since proteoglycans are a critical compon-

ent of the extracellular matrix, we postulated decreased proteoglycans was the mechanism for the instability of the collagen in affected fibroblasts. Alternatively, the changes of proteoglycan could be secondary to the decreased deposition of collagen in the cell layer. David and Bernfield (1979) have reported that collagen reduces glycosaminoglycan degradation in cultued mammary epithelial cells. Therefore it was possible that in affected fibroblasts, proteoglycans were being degraded more rapidly.

In conclusion, we demonstrated the instability of collagen in cultured skin fibroblasts derived from hereditary scoliotic chicks. The increased extractability of collagen was not because of decreaed lysyl oxidase activity or decreased availablity of copper. The diminished levels of proteoglycans in the cell layer may underlie the observed abnormalities of collagen. The importance of proteoglycan-collagen interactions has been demonstrated _in vitro_ and is well established. However, this is the first report of decreased collagen stability associated with a decreased proteoglycan content in a cell culture system. Whether the same observation can be made _in situ_ in actual tissues of chicks with hereditary scoliosis remains to be determined.

Legends of Figures

Fig 1 Phase contrast micrographs of skin fibroblasts in sparse (A,B,C) and dense (D,E,F) cultures. A and D are control fibroblasts, B and E are unaffected fibroblasts, C and F affected fibroblasts. Note in dense cultures, fibroblasts formed multiple layers.

Fig 2 DEAE-cellulose chromatograms of [^{3}H] proline-labeled medium pro teins of (A) control fibroblasts and (B) affected fibroblasts. Samples were processed and chromatographed as described under "Materials and Methods". Arrows indicate start of the NaCl gradient. Peak 1 represents unabsorbed fractions containing primarily type I collagen. Peak 2 is procollagen type I. Fractions of each peak as indicated by the bar were pooled for subsequent studies.

Fig 3 CM-cellulose chromatography of [^{3}H] proline labeled medium proteins unabsorbed by DEAE-cellulose. Samples were processed and chromatographed as described under "Materials and Methods". Arrow indicates start of the NaCl gradient. Peaks 1 and 2 were α1 and α2 chains of type I collagen respectively. Since cells were treated with BAPN, no β chains could be detected.

Fig 4 Autoradiograms of SDS polyacrylamide gel electrophoresis under non-reducing (A) and reducing (B) conditions. Lane 1, 2, and 3 were ammonium sulfate precipitated medium proteins from control,

affected and unaffected fibroblasts. Lane 4 and 6 were peak 1 of DEAE chromatography of medium proteins from control and affected fibroblasts. Lanes 5 and 7 were peak 2 of control and affected fibroblasts.

Fig 5 Autoradiograms of SDS polyacrylamide gel electrophoresis under non-reducing (A) and reducing (B) conditions. Lanes 1, 2, and 3 were pepsin-treated medium proteins from control, affected and unaffected fibroblasts. Lane 4, 5, 6 were pepsin-treated cell-layer associated proteins in the same sequence. Lane 7 was pepsintreated cell layer associated proteins from human newborn foreskin fibroblasts.

Fig 6 Sequential extraction of cell layer-associated collagen. [^{3}H] proline-labeled cell layer associated collagen from control (CN), unaffected (NA) and affected (AF) fibroblasts was extracted sequentially with 1 M NaCl (1), 0.5 N acetic acid (2) and 8M urea (3) at 4°C for 24 h. Each fraction as well as the residue (4) was then treated with bacterial collagenase to quantitate collagen content. The collagen level in each fraction was expressed as percentage of total cell layer associated collagen.

Fig 7 Collagen distribution between medium and cell layer. [^{3}H] proline labeled collagen in medium and cell layer from control (CN), unaffected (NA) and affected (AF) cultures was quantitated by the bacterial collagenase digestion methods. Collagen

content is expressed as cpm per mg cellular protein (panel A) and as percentage of total [^{3}H] proline labeled collagen (panel B).

Fig 8 Electron micrographs of skin fibroblasts and surrounding ECM from normal (A and B) and affected scoliotic (C and D) chicks. Fig. 8A and C are X 6080, B and D are X 19,200. Solid and open arrows indicate fibile and filamentous collagenous structures, respectively. Arrow heads indicate proteoglycan granules stained with Cuproline Blue. Note: More ECM can be visualized in control fibroblasts (A) than in affected fibroblasts (C). There are vacuoles containing granular and filamentous materials in B and D. Bar = 2.0 μm in A and C. Bar = 0.6 μm in B and D.

TABLE I

Lysyl Oxidase Activity and Cellular Copper Content

	Control	Unaffected	Affected
Lysyl Oxidase Activity 10^{-3} cpm/mg cellular protein	13.7 ± 1.2	14.3 ± 1.4	15.3 ± 2.0
Cellular Copper Content ng/mg cellular protein without exogenous copper	5.04 ± 1.04	4.77 ± 0.48	6.15 ± 1.00
With exogenous copper	57.43 ± 5.86	42.68 ± 4.52	47.82 ± 4.64

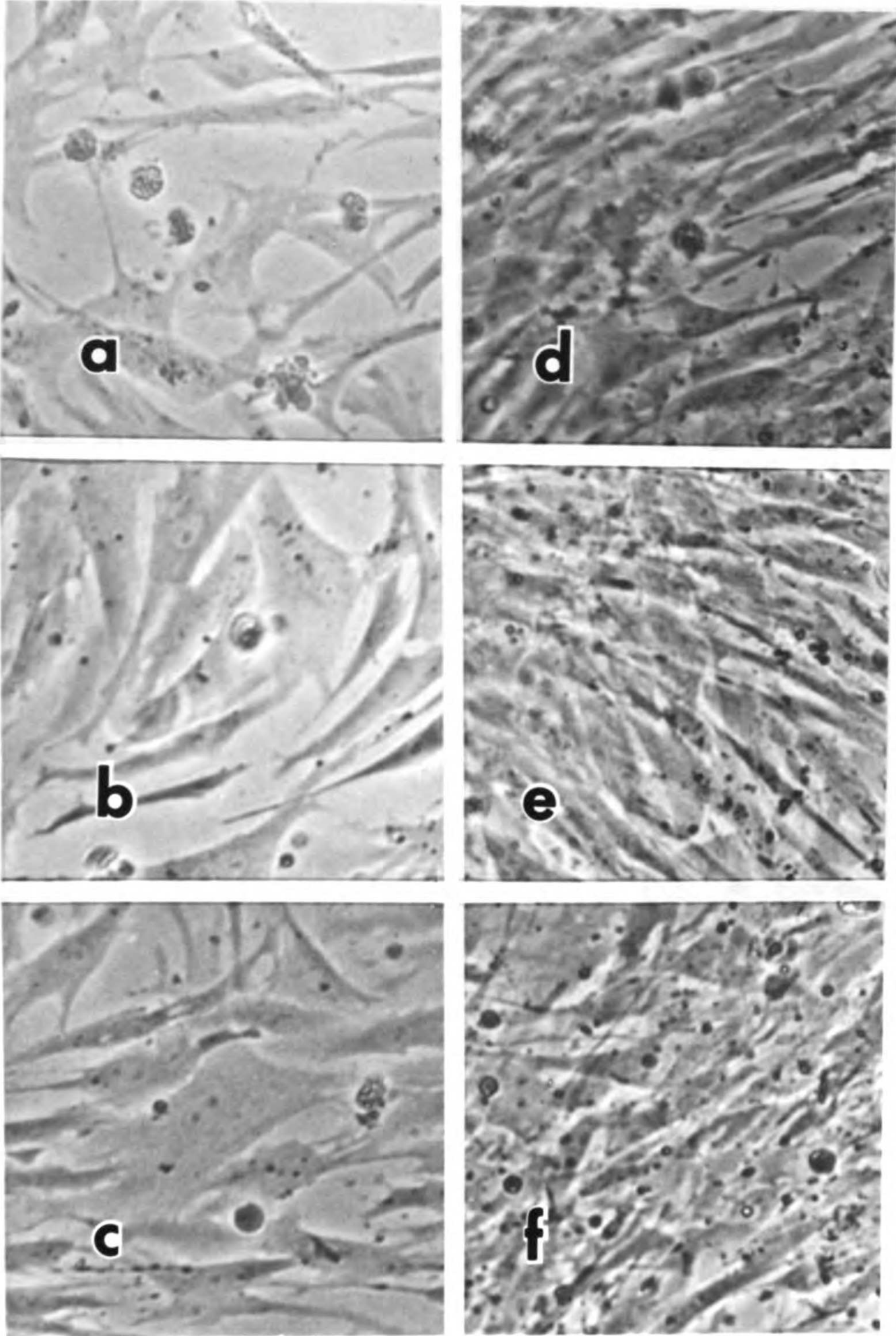


Fig 1

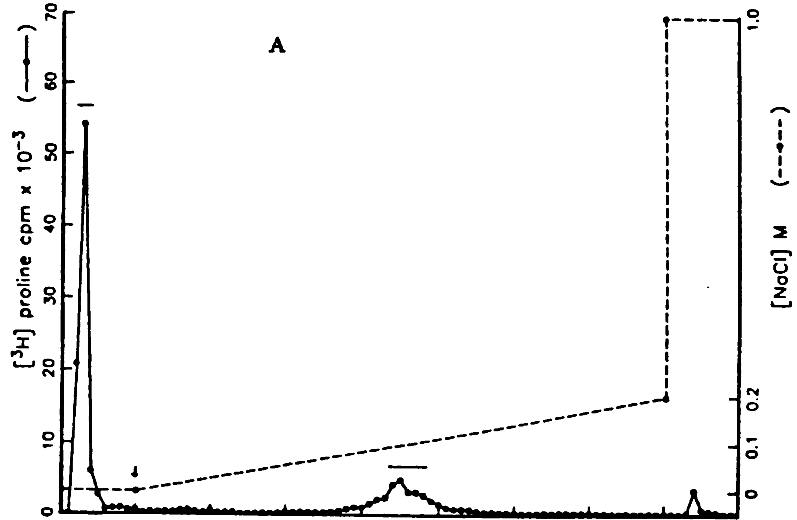


Fig. 2A

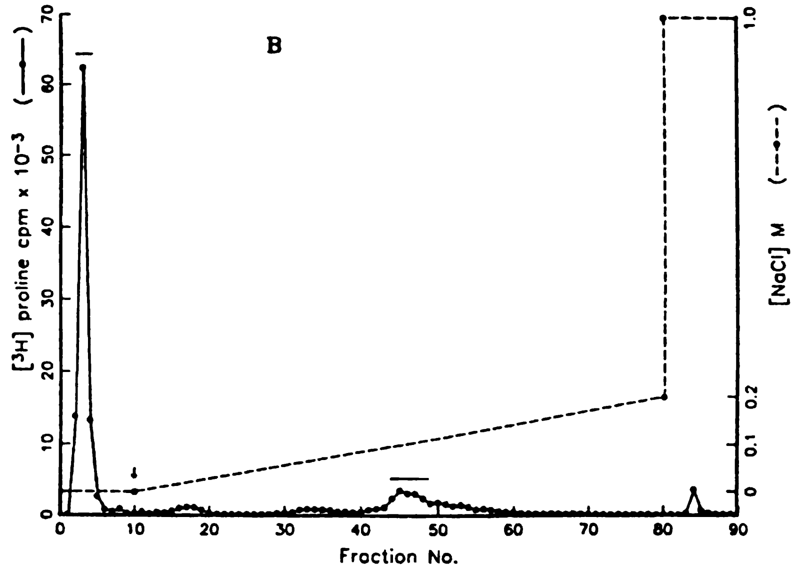


Fig. 2B

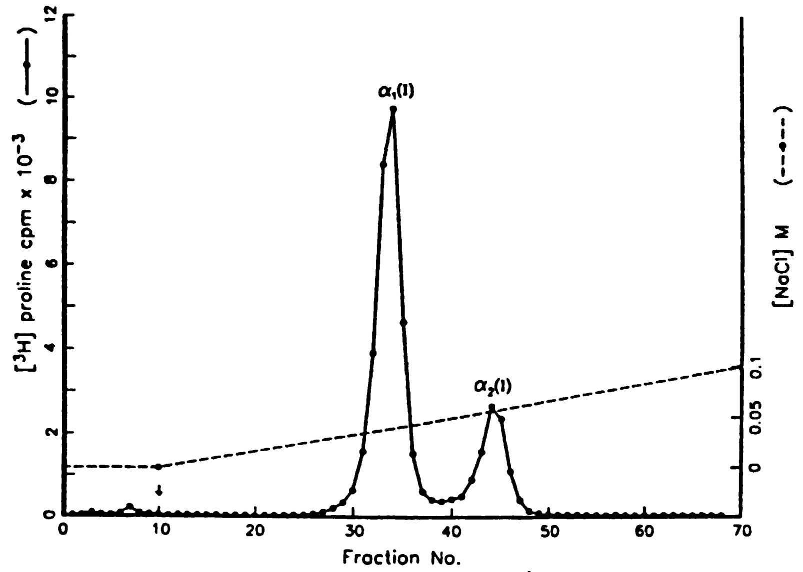
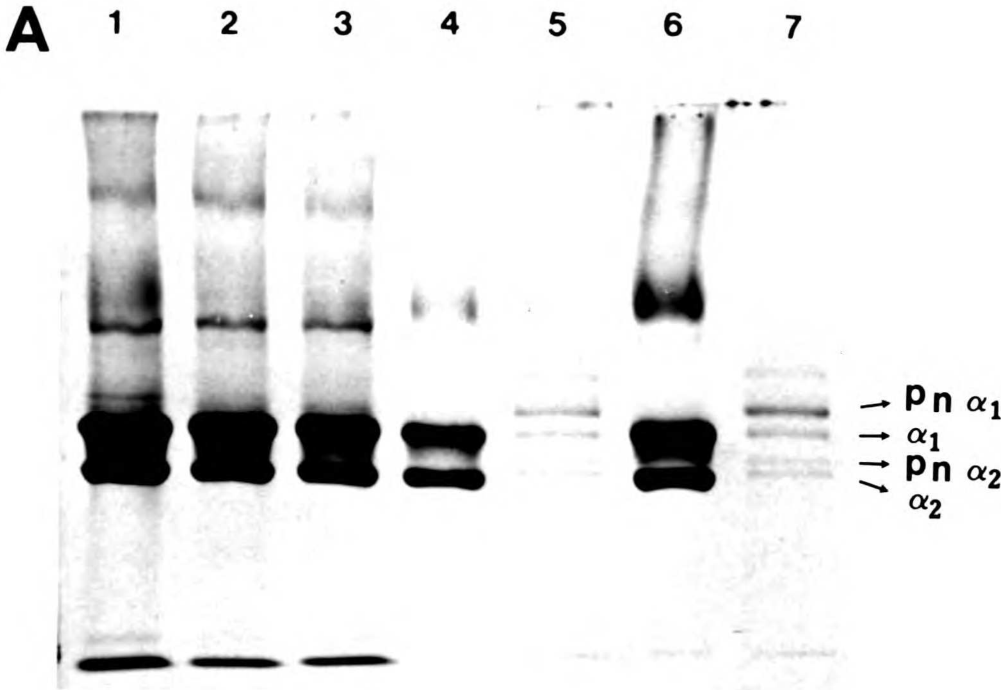


Fig. 3



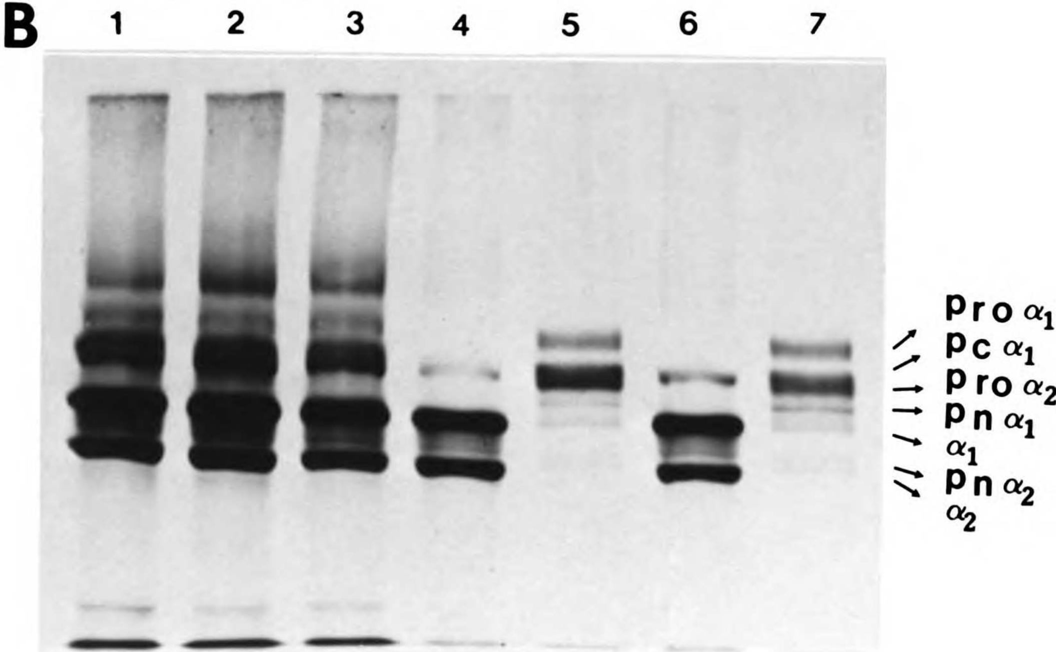


Fig 4

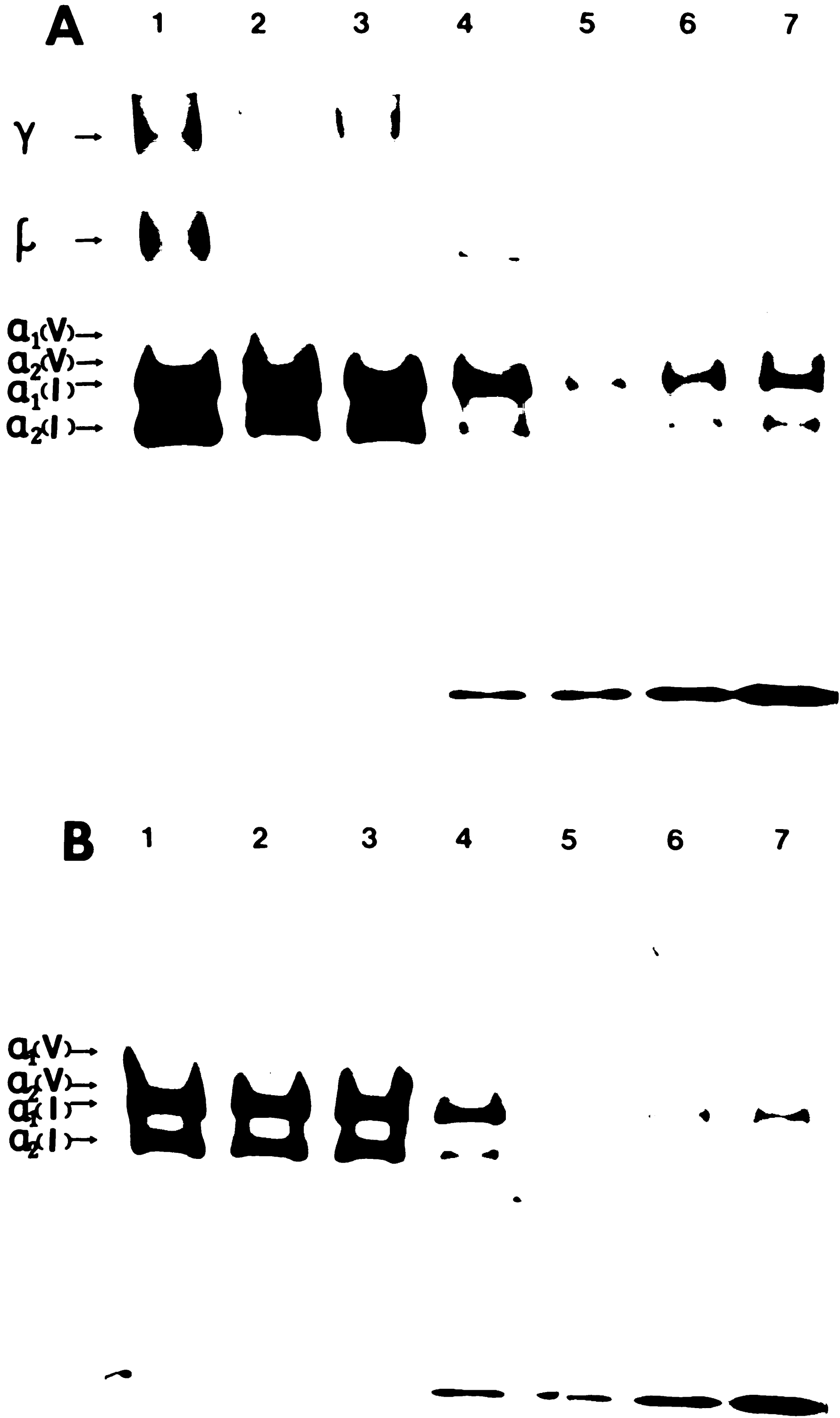


Fig 5

Fig. 6

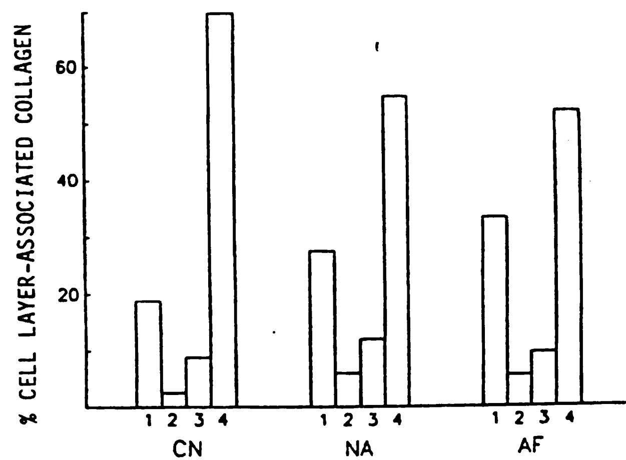


Fig. 7A

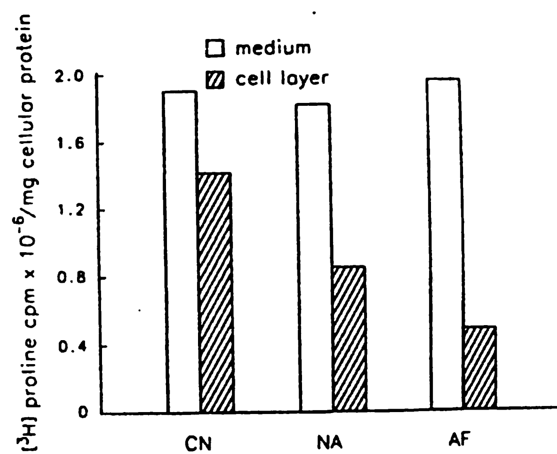
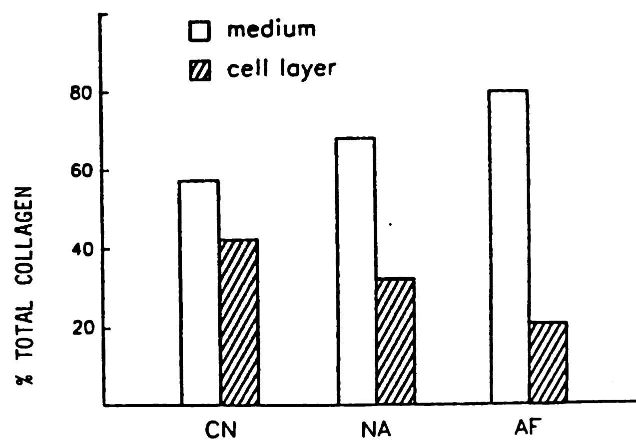


Fig. 7B



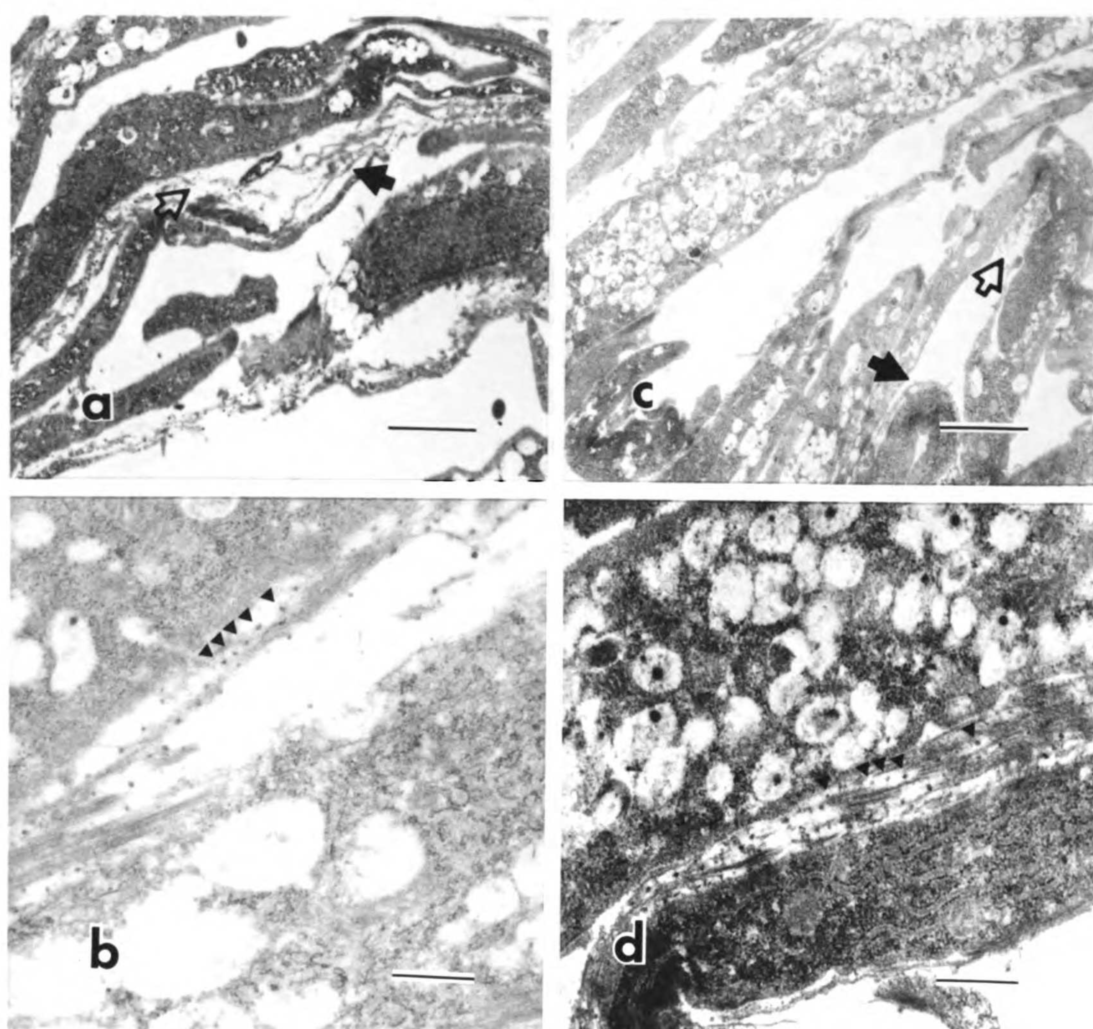


Fig 8

CHAPTER V:

THE GENETIC BASIS OF ECM INSTABILITY OF HEREDITARY SCOLIOTIC CHICK

Part II:

Properties of Proteoglycans in Hereditary Scoliotic Chick Skin Fibroblasts

Introduction

The pathogenetic mechanism of adolescent idiopathic scoliosis in humans is unknown. A biochemical basis for this disorder has long been postulated. Pedrini et al. (1973), demonstrated a decrease of proteoglycans and an in crease of collagen in the nucleus pulposus from patients with idiopathic scoliosis. They proposed that excessive degradation of proteoglycans may be the cause of this disorder. More recently, similar biochemical findings were documented in scoliosis secondary to cerebral palsy (Hunter et al., 1978, Pedrini-Mille et al., 1983) and to myelomeningocele (Zaleske et al, 1980). The increased degradation of proteoglycans is thus more likely to be secondary to the mechanic factors in scoliosis *per se*.

Hereditary scoliosis in a highly inbred strain of chicks appears to be a suitable model for human adolescent idiopathic scoliosis (Taylor, 1971; Riggins, et al., 1977). In scoliotic chicks, the extractability of collagen and levels of serum hydroxyproline are increased (Riggins et al., 1977; Lin et al., 1980). In the previous chapter, I demonstrated

that cultured skin fibroblasts from scoliotic chicks express the same instability of extracellular matrix as demonstrated in whole tissue extracts (Lien et al., 1984). This includes decreased collagen deposition and increased extractability of cell layer-associated collagen. Electron microscopic studies demonstrate the decreased collagen and proteoglycan content in these diseased fibroblasts. A mechanism of extracellular matrix instability secondary to decreased levels of proteoglycans has therefore been invoked. In the present study, I have examined the proteoglycans and glycosaminoglycans synthesized by those fibroblasts. I observed that the proportion of proteoglycan aggregation as well as the levels of hyaluronic acid and chondroitin sulfate were decreased in scoliotic chick skin fibroblasts. Increased hyaluronidase activity was also documented in these cells. This may be the single metabolic lesion that underlies the connective tissue instability, and may explain the several observed biochemical changes.

Materials and Methods

Cell Culture:

Chick skin fibroblasts were cultured as described in Chapter IV.

For metabolic labeling, confluent cells in 75 cm^2 flasks were incubated for 48 h in 6 ml of fresh sulfate-deficient media. The MgS04 was replaced with $MgCl_2$. Radiolabeled [^{35}S] sulfate, 25 uCi/ml or [^5H] glucosamine, 12.5 uCi/ml (New England Nuclear, Boston MA) were used. The culture media were harvested and the cell layers washed three times with 1 ml PBS containing 1 mM PMSF (Sigma, St. Louis, MO). The first wash

was pooled with the medium. Protease inhibitors including PMSF, benzamidine, PCMB, NEME, and EDTA, all products of Sigma, at final concentrations of 1, 1, 1, 5 and 20 mM, respectively, were added. After mixing, the medium was centrifuged for 5 min at 1,000 xg to remove cellular material. Cell layers were scraped with a rubber policeman in PBS with 1 mM PMSF and were sonicated for 30 seconds in an ice bath. The media and cell layer fractions were then dialyzed against distilled water thoroughly at 4°C to remove unincorporated radioactive isotopes, then lyophilyzed.

For the hyaluronidase assay, complete medium was replaced with serum-free medium 48 h prior to harvest. As described by Orkin and Toole (1980), incubation in serum-free medium did not significantly alter levels of enzyme activity in cultured fibroblasts; however it facilitated analysis of medium-associated hyaluronidase by eliminating serum proteins from the preparations. Serum-free medium was collected from confluent cultures and processed separately from the cells, as described below.

Isolation of proteoglycans:

Lyophylized materials from the media and cell layer were dissolved in 1 ml 4 M guanidine hydrochloride containing PMSF, benzamidine, PCMB, NEME, and EDTA at 1, 1, 1, 5 and 20 mM respectively. There were no pellets observed after centrifugation, therefore this step was omitted in subsequent experiments. The samples were then dialyzed against associative buffer containing 50 mM sodium acetate pH 6.8 with EDTA 10 mM, -aminocaproic acid (Sigma), 100 mM, benzamidine, 1 mM, and PMSF, 1 mM, at 4°C overnight. The retentants were recovered and centrifuged (1000 xg, 5 min.) to remove the precipitate.

Sepharose CL-2B chromatography:

The media and cell layer fractions prepared above were subjected to Sepharose CL-2B chromatography (1.5 x 170 cm) and eluted with the associative buffer at 4°C. The flow rate was 9.6 ml per h. Fractions of 4 ml were collected of which 1 ml aliquots were removed for counting. In order to study total aggregatable PG, exogenous HA (Sigma, Grade IIIS), 1 mg, dissolved in 1 ml of 4 M guanidine hydrochloride was added to the lyophilized media and cell layer fractions and dialyzed against associate buffer. The HA-supplemented samples were then subjected to Sepharose CL-2B chromatography.

Isolation of glycosaminoglycans:

Lyophylized media and cell layer fractions were treated with papain as described previously for the isolation of GAG's (Antonopoulos et al., 1964). Briefly, 0.5 ml papain stock solution (1 mg/ml) in 0.1 M phosphate buffer, pH 7.6 with 5 mM EDTA, and 10 mM L-cystine HCl was added to each sample, and then incubated at 65°C for 24 h. Another 0.5 ml of papain stock solution was added and incubated for an additional 24 h. After digestion, samples were deproteinized with 10% cold TCA and centrifuged. The supernatants were dialyzed against distilled water at 4°C with three changes.

Study of hyaluronic acid synthesis:

[^{3}H]-glucosamine labeled preparations were digested with streptomyces hyaluronidase (Miles Lab, Eckhardt, IN) at 40°C for 4 h as described (Roden et al, 1974). The digestants were then subjected to gel chromatography on Sephadex G-50 and eluted with ammonium acetate, 0.2 M, pH 6.5.

The flow rate was 15.5 ml per hour. Fractions of 1.5 ml were collected, of which aliquots of 300 ul were removed for counting.

Cellulose acetate electrophoresis

[^{35}S]-sulfate labeled GAG preparations were lyophilized and redissolved in 20 ul distilled water. An aliquot of the isolated GAG's was subjected to electrophoresis on a cellulose acetate membrane, Sepraphore III (Gelman Sciences). Electrophoresis was performed in 0.1 M barium acetate, pH 8.0, for 4.5 h at a constant current of 1.0 mA/cm strip (Hata and Nagai, 1972). Preparations of standard GAG's, including C6S, C4S, DS, HA, and HS were co-electrophoresed. The GAGs positions were identified by alcian blue staining (1% in 1% acetic acid) as well as by autoradiography. The gel patterns were further quanititated by gel densitometry (LKB 2202 Ultroscan Laser Densitometer, Bromma, Sweden).

Hyaluronidase assay:

The levels of medium-associated and cell layer associated hyaluronidase activity were assayed as previously described (Orkin and Toole, 1980). The cell layer was rinsed with Hank's solution, then scraped into the formate extraction buffer (0.1 M sodium formate, 0.5 M NaCl, and 0.1% Triton X 100, pH 3.7) at 4°C and sonicated for 30 seconds in an ice bath. The serum-free medium was cooled on ice, and then precipitated with ammonium sulfate at 65% saturation. The precipitates were collected by ultracentrifugation at 65,000 xg for 45 min. at 4°C, then redispersed in small volumes of the formate extraction buffer, and dialysed against the formate buffer (0.1 M sodium formate, and 0.15 M NaCl, pH 3.7) at 4°C to remove residual ammonium sulfate. Insoluble materials in both

medium and cell layer fractions were removed by brief centrifugation (1,000 xg, 5 min) at 4°C, before assay.

Hyaluronidase activity present in the medium and cell layer-derived samples was determined by incubation at 37°C for 16 h with an exogenous HA substrate (100 ug/250 ul of assay mixture). The newly formed terminal N-acetylglucosamine was then measured by the colorimetric assay of Reissig et al., (1955). 1.5 mM sacchrolactone (Sigma), an inhibitors of β-glucuronidase, was routinely included in the incubation mixtures. Controls, boiled for 3 min. prior to incubation, were routinely included to correct for background levels. Hyaluronidase activity was expressed as units/mg cellular protein assayed by the method of Lowry et al., (1951). Units of hyaluronidase were defined as micrograms of terminal N-acetylglycosamine released per h at 37°C (Orkin and Toole, 1980).

Substrate specificity for cell layer-derived hyaluronidase was tested by using various GAG preparations including C4S, C6S, DS and HS (100 μg/250 ml of assay mixture). Hyaluronidase was assayed as described above. The release of N-acetylglucosamine from HA was expressed as 100%.

Results

Proteoglycan aggregation:

Proteoglycan aggregation was studied using molecular sieve chromatography under associative conditions. The profiles of Sepharose CL-2B chromatography are shown in Fig 1. The medium PG's from control fibroblasts (panel A1, dashed line) had a small asymmetric peak that eluted at the void volume. The excluded peak represents aggregate forms of

PG's, while the shoulder may represent partially aggregated or monomer forms of PG's. The addition of exogenous HA (panel A1, solid line) caused PG's to become aggregated as shown by the increase of the excluded peak. Therefore this peak represents total potentially aggregatable PG's. The broad peak eluted before the total volume may represent smaller PG's and sulfated proteins that were not affected by the addition of HA, and not potentially aggregatable. The profile of medium PG's from affected fibroblasts is shown in Panel C1. Without addition of HA (dashed line), the excluded peak was not prominent. However, the addition of HA enhanced aggregation of PG's from affected fibroblasts to an extent similar to that of control fibroblasts. The profile of PG's from the culture medium of unaffected fibroblasts (panel B1) was similar to that from the control fibroblasts.

The Sepharose CL-2B chromatography profiles of cell layer associated PG's are shown in panel A2 (control), B2 (unaffected), and C2 (affected). All three cell lines had similar profiles characterized by a small excluded peak (dashed line). The addition of HA did not change profiles significantly (solid line). Most sulfated material from the cell layer existed in a form of small PG's that were not aggregatable.

The proportion of aggregate form of PG's in the various cultures measured in Fig 2 is summarized in Table 1. The proportion of PG aggregation in the medium was 23% in control fibroblasts and 11% in affected fibroblasts. There was thus a 50% decrease in affected fibroblasts compared to the control. The proportion of PG aggregation from unaffected fibroblasts was intermediate (19%) between the values of control and affected fibroblasts. Total aggregatable PG's in medium as estimated by the addition of exogenous HA, was 49% in control, 44% in unaffected, and

40% in affected fibroblast cultures. In the cell layer fraction, the proportion of aggregation was 6.6% in controls, 5.2% in unaffected, and 4.0% in affected fibroblasts. The changes observed in the cell layer were not as marked as those in the medium. The addition of HA increased the proportion of aggregation slightly in the PG's of the cell layers; 9.7% in controls, 6.5% in unaffected, and 6.9% in affected fibroblasts.

Studies of hyaluronic acid:

From the preceding observations, we speculated there might be a defect in the metabolism of HA in the fibroblasts from scoliotic chicks. Therefore, we examined the accumulation of HA in 48 h cultures using ^{3}H-glucosamine labeling. The amount of HA was assessed by the measurement of differences in the chromatographic elution profiles of GAG's before and after digestion with streptomyces hyaluronidase, an enzyme that specifically degrades HA but does not degrade other GAG's. Fig 2 demonstrates elution profiles from columns of Sephadex G 50 of ^{3}H-labeled GAG fractions directly (solid line) or following digestion with streptomyces hyaluronidase (dashed line). Panels A,B,C represent the profiles of medium GAG's from control, unaffected, and affected fibroblasts respectively. The decrease in the size of the excluded peak after hyaluronidase digestion reflects the amount of HA synthesized and secreted into the medium. The proportion of HA among total ^{3}H-labeled medium GAG's was calculated and is summarized in Table 2. As expected, the HA accumulation in the medium of affected fibroblats (2.7%) was 50% lower than that of control fibroblasts (6.0%). When HA levels were expressed as ^{3}H-cpm per mg cellular protein, there was a three fold difference in the media between control and affected fibroblasts. The cell layer

associated HA was also decreased in affected fibroblasts (Table 2). There was a ten-fold difference between control (3.2%) and affected fibroblasts (0.3%). The cell layer associated HA was also decreased significantly in unaffected fibroblasts (0.9%).

Sulfated GAG

Fig 3 shows the patterns of cellulose acetate electrophoresis visualized by alcian blue staining. Lane 1, 2, and 3 represent medium associated GAG's from control, affected, and unaffected fibroblasts. The major component was CS, which comigrated with C6S. Lane 4,5, and 6 represent cell layer-associated GAG's from control, affected and unaffected fibroblasts. The major components were CS, which comigrated with C4S, and HS. A faint band between CS and HS could be detected in control and unaffected fibroblasts that comigrated with DS. This band was however not visualized in affected fibroblasts.

Fig 4 shows the autoradiogram from the same cellulose acetate electrophoresis. Lane 1, 2, and 3 represent medium associated GAG's and 4, 5, and 6 represent cell layer associated GAG's from control, affected, and unaffected fibroblasts. Lane 7 and 8 were C4S from chick sternum labeled with [^{35}S] sulfate. . Again there were no differences in medium GAG's from the three cell lines. It is worthwhile to note however that in cell layer fractions, the CS band of affected cells was lighter when compared to that of control cells. This difference was further demonstrated by densitometry, as shown in Fig 5. The first peak reflects HS and the second CS. Apparently, CS peak was higher than HS peak in control fibroblasts; however they are equal in affected fibroblasts. Table 3 summarized the findings from cellulose acetate electrophoresis

experiments. In the medium, neither total GAG's nor CS was significantly different among the three cell lines. However in the cell layer, total GAG was 30% lower in the affected compared to the control. This decreases was mainly due to the decrease in CS level in the affected cells, a 44% decrease. The CS level in unaffected fibroblasts was also shown to have a 30% decrease. The HS levels of the three cell lines were all similar.

Hyaluronidase assay:

Since decreases in both HA and CS were observed in these experiments, we postulated that an increase of hyaluronidase activity might be the mechanism underlying the defect in the fibroblasts from scoliotic chicks. The lysosomal enzyme is known to degrade both of these GAG's. I assayed hyaluronidase activity in medium and cell layer fractions from the three cell lines. Both medium and cell layer-associated hyaluronidase activity in affected fibroblasts were two fold higher than values in the control cell cultures (Fig 6). The hyaluronidase activity in the unaffected fibroblasts was also significantly higher than that of the control.

The substrate specificity of cell layer-associated hyaluronidase was examined. The results are shown in Table 4. When the susceptibility of HA to this hyaluronidase was expressed as 100%, that of C4S, C6S, and DS were 38%, 37%, and 6% respectively, while HS was not degraded by the hyaluronidase.

Discussion

In the present study, we have characterized the PG's and GAG's in cultured chick skin fibroblasts from normal and from scoliotic chicks, both affected and unaffected. The normal chick skin fibroblasts secreted PG's into the medium, 23% of which was in an aggregated form (Fig 1 A1). Like cartilage-derived CS PG's, the unaggregated PG's could be aggregated by the addition of exogenous HA, and were detected by their shift from the included to the excluded elution position on a Sepharose CL-2B column (Fig 1 A2). This phenomenon has also been observed for PG's in other cultured cells, in human glial cells (Norling et al, 1978), human fetal skin fibroblasts (Coster et al, 1979), monkey arterial smooth muscle cells (Wright and Hascall 1983), and dedifferentiated human chondrocytes (Oegema et al, 1981). However the aggregation of PG's by HA may not be applicable to all cell systems. Recently, Schafer et al., (1984) demonstrated no such increase in aggregation of PG's from human neonatal skin fibroblasts by the addition of exogenous HA. In comparing Schafer's work with the present study, certain experimental differences are noted, however, that may underlie the differences in the ability to aggregate with added HA. They added HA to the A_1D_1 fraction, utilizing the nomenclature suggested by Heinegard (1972), from cesium chloride gradient centrifugation. A_1D_1 contains only PG monomers, while link protein, another essential component for aggregation, is present only in the lowest density fraction (Hascall and Sajdera 1969). The lack of link protein in the Schafer system may explain those results. In separate experiments, they added bovine nasal septum PG A_1 fraction to the A_1D_1 fraction from human skin fibroblasts, which increases the proportion

of aggregation from 3% to 10-14%. This is still much less than the proportion of aggregation in A_1, fraction (47%). We suspect that the fibroblast-derived link protein may be quite different from the cartilage derived-link protein. In our system, we did not separate link protein from PG's, therefore we were able to demonstrate that HA was the critical factor for PG aggregation. The cell layer associated proteoglycans from chick skin fibroblasts contained a small fraction of aggregate forms and the addition of HA increased the proportion of aggregation only slightly (Fig 1, A2).

The HA level in normal chick skin fibroblasts was low (6.6% of total ^{3}H-glucosamine labeled GAG's in medium, Table 2) when compared to the 50-60% observed in human skin fibroblasts (Matalon and Dorfman, 1969). The different HA levels are compatible with the different proportion of PG aggregation in the two species of fibroblasts (23% in chick skin fibroblast, versus 47% in human skin fibroblasts). More interestingly, the different HA levels also parallel the different levels of hyaluronidase activity in chick and human skin fibroblasts. The hyaluronidase activity in chick embryo skin fibroblasts is about 0.23 and 0.45 units/mg cellular protein in the cell layer and medium fraction respectively, while it is not detectable in human neonatal skin fibroblasts (Orkin and Toole 1980). In the present study, the hyaluronidase activity in 4-week-old chick skin fibroblasts was 0.23 units/mg cellular protein in the cell layer, and 0.04 in the medium.

Fibroblasts in culture synthesize HS, CS, and DS (Matalon and Dorfman 1969; Malmstrom et al., 1975). HS is a cell surface constituent among many cell types (Kraemer 1971; Sjoberg and Fransson 1977), while DS and CS are present in both the medium and as components of the extra-

cellular matrix (Malmstrom et al., 1975). In chick skin fibroblasts, the principal sulfated GAG's components are CS (80%) in the medium, and CS (60%) and HS (30%) in the cell layer (Fig 3, 4). DS in trace amounts was detected in the cell layer fraction (Fig. 3). The low content of DS in chick skin fibroblast compared to human skin fibroblasts (Malmstrom et al., 1975) may represent a species difference.

The major differences among PG's and GAG's, comparing scoliotic chick skin fibroblasts to the normal, were: 1) The proportion of PG aggregation in the medium decreased significantly while the total potentially aggregatable PG's in the medium decreased only slightly (Table 1); 2) The HA accumulation in both medium and cell layer decreased (Table 2); and 3) The CS level in the cell layer decreased while HS remained the same (Table 3). It has been well documented that HA is a _sine qua non_ component of PG aggregation (Hardinghaim and Muir 1972). Recently, Lohmander et al. (1983) demonstrated that the concentration of HA in the medium is the limiting factor for PG aggregation in rat chondrosarcoma chondrocyte cultures. In the present study, we observed the same phenomenon, that HA is the limiting factor in PG aggregation in chick skin fibroblasts. The proportion of PG aggregation in the three cell lines paralleled the level of HA (Table 1 and 2).

Because of the low levels of HA, it was difficult to perform pulse-chase studies to elucidate the mechanism of decreased HA in the affected scoliotic chick fibroblasts. Instead, I assayed hyaluronidase activity in both the medium and cell layer of the chick fibroblasts. I demonstrated a two fold increase of hyaluronidase activity in the affected fibroblasts compared with the control (Fig 9). Orkin et al., (1982) demonstrated elevated hyaluronidase activity in simian virus-transformed

3T3 cells. The elevated enzyme level correlates well with the decrease of HA level in both the medium and the cell surface of the transformed cells (Underhill and Toole 1982). In the present studies I demonstrated similar correlations, with one exception. There was a relatively high level of hyaluronidase activity in the medium of unaffected fibroblasts. However such hyaluronidase in the medium may be relatively ineffective because the optimal pH for such enzymatic activity is 3.7 (Orkin and Toole 1980). Furthermore, lysosomal hyaluronidase digests both C4S and C6S, but not HS (Meyer 1971), as was demonstrated in Table 4. The elevated level of hyaluronidase in affected cells therefore could explain both the decreased CS and unchanged HS levels observed in the cell layer.

The elevated hyaluronidase activity in scoliotic chick skin fibroblasts may play an important role in the pathogenesis of adolescent idiopathic scoliosis. First it has been reported that the serum level of hyaluronidase activity is elevated in scoliotic patients (Balaba 1972). Excessive degradation of PG's in the nucleus pulposus from scoliotic patients has been invoked previously as the mechanism of scoliosis development (Pedrini et al., 1973). On the other hand, an elevated lysosomal enzyme activity in intervertebral discs, acid phosphatase, is found in both idiopathic scoliosis, in scoliosis secondary to myelomeningocele (Zoleske et al., 1980), and in ventral herniation of the intervertebral discs in rabbits (Lipson and Reilly, 1982). It is thus considered to be secondary to scoliosis *per se*. Such indirect evidence does not eliminate the possibility of excessive destruction of PG's as the primary cause of scoliosis.

Secondly, Francis et al., (1975) has reported reduced stability of skin collagen in idiopathic scoliotic patients, especially in the younger age groups (12-13 yr). This suggests a temporal instability of collagen during the rapid growth phase, and may be critical to the development of scoliosis. Recently, the finding of that laboratory have been challenged by Venn et al., (1983) who could not demonstrate such differences in the stability of collagen in spinal ligaments from patients with idiopathic and secondary scoliosis. However, patient selection and tissue difference may affect such results. In Venn's study, fewer patients were examined and the age factor was not emphasized. In hereditary scoliotic chicks, collagen extractability is found increased in tissues (Riggens et al., 1977) and in cultured fibroblasts. Proteoglycans play an important role in the size, stability, and organization of the collagen fiber (Scott et al., 1981; Lilja and Barrah 1981; Weinstein 1975). It is likely that excessive degradation of PG's results in an overall instability of the extracellular matrix that is manifest in part as increased collagen extractability. This has the net effect of weakening the intervertebral disc, as well as the supporting structures of the spine.

Finally, during the adolescent growth spurt, there is a rapid turnover of extracellular matrix following the onset of hormonal changes. The metabolism of HA is regulated by various hormones such as sex hormones, glucocorticoids, thyroid hormone, and insulin (for a review see Silbert, 1973). Therefore, the imbalance of synthesis and degradation of PG's during this critical stage, caused by an elevated hyaluronidase, may accelerate dramatically and be the basis of scoliosis.

Whether our _in vitro_ observations are applicable to the _in vivo_ situation or even has clinical significance cannot be assessed. The

mechanism of the elevated hyaluronidase activity also requires further investigation. However the studies presented here, using scoliotic chick fibroblasts, may provide new directions for exploring the pathogenesis of adolescent idiopathic scoliosis in humans.

FOOTNOTES

Table 1 The proportion of PG aggregation was calculated from Sepharose CL-2B chromatography. The percentage of radioactivity in the excluded peak of total eluted radioactivity was defined as the proportion of PG aggregation.

Table 2 The percentage of HA was calculated from the Sephadex G50 chromatography. The percent HA was calculated from the proportion of the excluded peak before and after hyaluronidase digestion of total eluted radioactivity.

Table 3 The percentage in parentheses represents the percentage of individual GAG's in total GAG's. The percentage of GAG's was obtained from densitometry following cellulose acetate electrophoresis.

Table 4 GAG substrate at concentration of 100 μg/250μl was used. An aliquot of 200 μl sodium formate extract of unaffected fibroblast cell layer was added as a source of hyaluronidase. Boiled cell layer extract was used for the correction of background levels. The hyaluronidase assay was performed as described in Materials and Methods. N-acetylglucosamine release is expressed in nmoles. The relative susceptibility of each GAG to the hyaluronidase is expressed in percent, assuming that of HA is 100%.

TABLE I

Proportion of PG Aggregation

		CONTROL	UNAFFECTED	AFFECTED
MEDIA	-HA	23%	19%	11%
	+HA	49%	44%	40%
CELL LAYER	-HA	6.6%	5.2%	4.0%
	+HA	9.7%	6.5%	6.9%

TABLE 2

Hyaluronic Acid Accumulation in 48 Hr

		CONTROL	UNAFFECTED	AFFECTED
% of HA in [^{3}H] Glucosamine-Labeled GAG	Medium	6.0	4.4	2.7
	Cell Layer	3.2	0.9	0.3
[^{3}H] Glucosamine-Labeled HA (cpm/mg Protein x 10^{3})	Medium	77.8	33.4	25.2
	Cell Layer	83.4	9.6	5.5

TABLE 3

Sulfated GAG Accumulation in 48 Hr

	^{35}S CPM/10^3 CELLS	CONTROL	UNAFFECTED	AFFECTED
Medium	Total GAG	51.9 (100%)	57.8 (100%)	53.5 (100%)
	CS	41.5 (80%)	45.9 (86%)	46.1 (80%)
Cell Layer	Total GAG	74.1 (100%)	60.9 (100%)	51.4 (100%)
	CS	44.2 (60%)	31.6 (52%)	24.9 (49%)
	HS	23.5 (32%)	25.0 (41%)	24.2 (47%)

TABLE 4

Substrate Specificity of Chick Skin Fibroblast Hyaluronidase

GAG	N-ACETYLGLUCOSAMINE RELEASE (nmole)	PERCENT
HA	31.6 ± 0.4	100%
C4S	12.1 ± 0.7	38.0
C6S	11.8 ± 1.0	37.3
DS	1.9 ± 0.3	6.0
HS	0	0

LEGENDS

Fig. 1 Sepharose CL-2B chromatography of [^{35}S] sulfate-labeled medium and cell layer-associated PG's under associative condition. Samples with (●——●) and without (○---○) addition of exogenous HA (1 mg/ml) were applied to a column (1.5 x 170 cm) of Sepharose CL-2B equilibrated with 0.5 M sodium acetate pH 6.8 containing protease inhibitors, as described. The column was eluted with the same buffer at a flow rate of 15 ml/h. Fractions of 4 ml were collected of which a 1 ml aliquot was removed for [^{35}S] radiolabeled counting. Panels A1, B1, and C1 represent medium-associated PG's from control, unaffected and affected fibroblasts. Panels A2, B2, and C2 represent cell-layer-associated PG's in the same sequence.

Fig. 2 Sephadex G50 chromatography of [^{3}H] glucosamine labeled medium GAG's before (●——●) and after (○---○) streptomyces hyaluronidase digestion. Samples were applied to a column (1.0 x 100 cm) of Sephadex G50 equilibrated with 0.2 M ammonium acetate, pH 6.5. The column was eluted with the same buffer at a flow rate of 15 ml/h. Fractions of 1.5 ml were collected of which a 300 μl aliquot was removed for [^{3}H] radiolabeled counting. Panels A, B, and C represent medium-associated GAG's from control, unaffected and affected fibroblasts, respectively.

Fig. 3 Cellulose acetate electrophoresis of medium and cell layer-associated GAG's visualized by alcian blue staining. The origin is indicated by the arrow: Lane 1, 2, and 3 represent cell layer-associated GAG's from control, affected and unaffected fibroblasts. Migration of GAG standards of C6S, DS, HS and HA are indicated.

Fig. 4 Autoradiogram of cellulose acetate electrophoresis. The origin is indicated by the arrow. Lanes 1, 2, and 3 represent medium-associated GAG's from control, affected and unaffected fibroblasts respectively. Lane 4, 5, and 6 represent cell layer associated GAG's in the same sequence. Lane 7, 8 are [^{35}S] sulfate labeled C4S isolated from chick sternum.

Fig. 5 Densitometry of autoradiogram of cell layer-associated GAG's following cellulose acetate electrophoresis. The region containing the bands was scanned from bottom to top. The first peak in each panel represent HS, the second represents CS. CN, NA and AF refer to control, unaffected and affected fibroblasts.

Fig. 6 Hyaluronidase activity of skin fibroblasts from control (CN), unafffected (NA), and affected (AF) fibroblasts. Medium and cell layer-associated hyaluronidase activity is indicated. Units of hyaluronidase activity were defined as micrograms of terminal Nacetylglucosamine released her hr at 37°C.

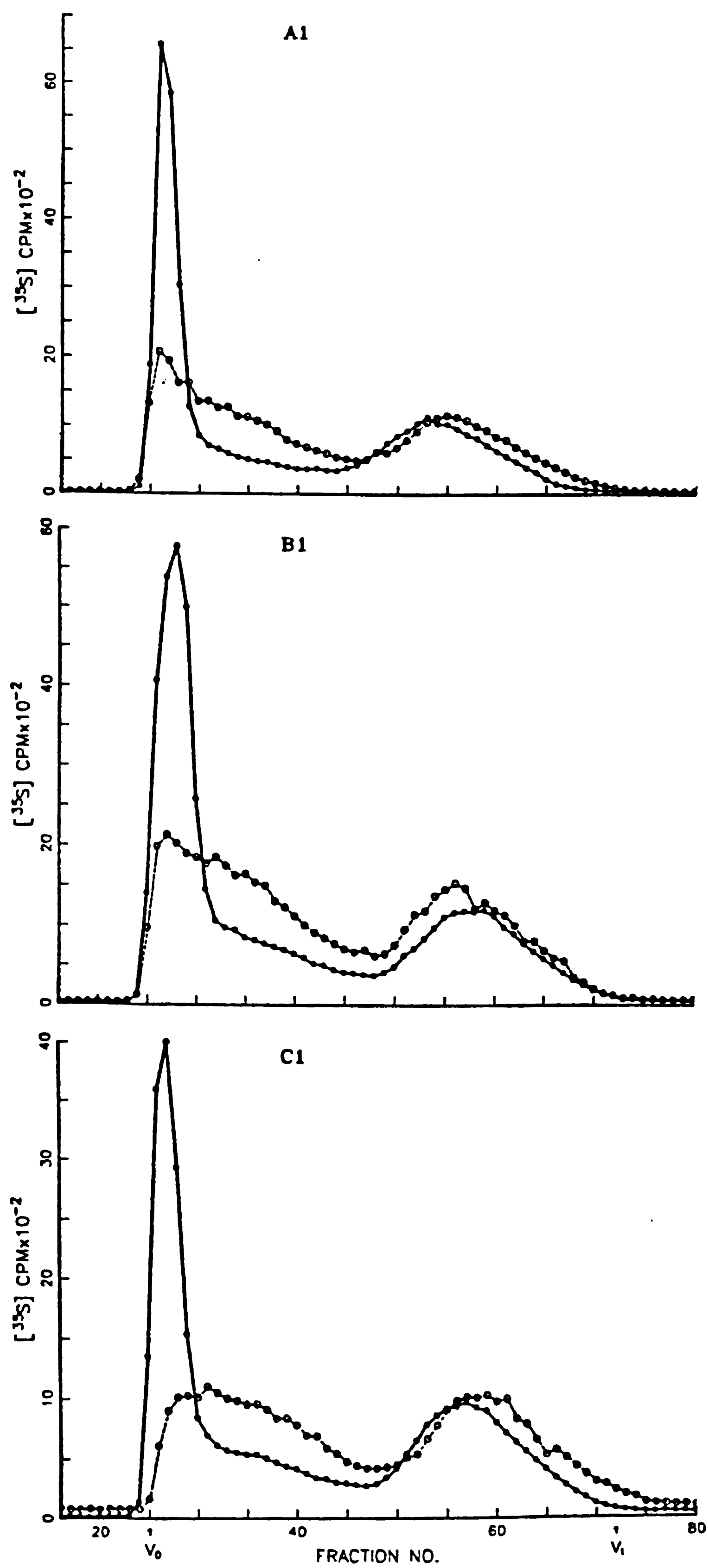


Fig. 1

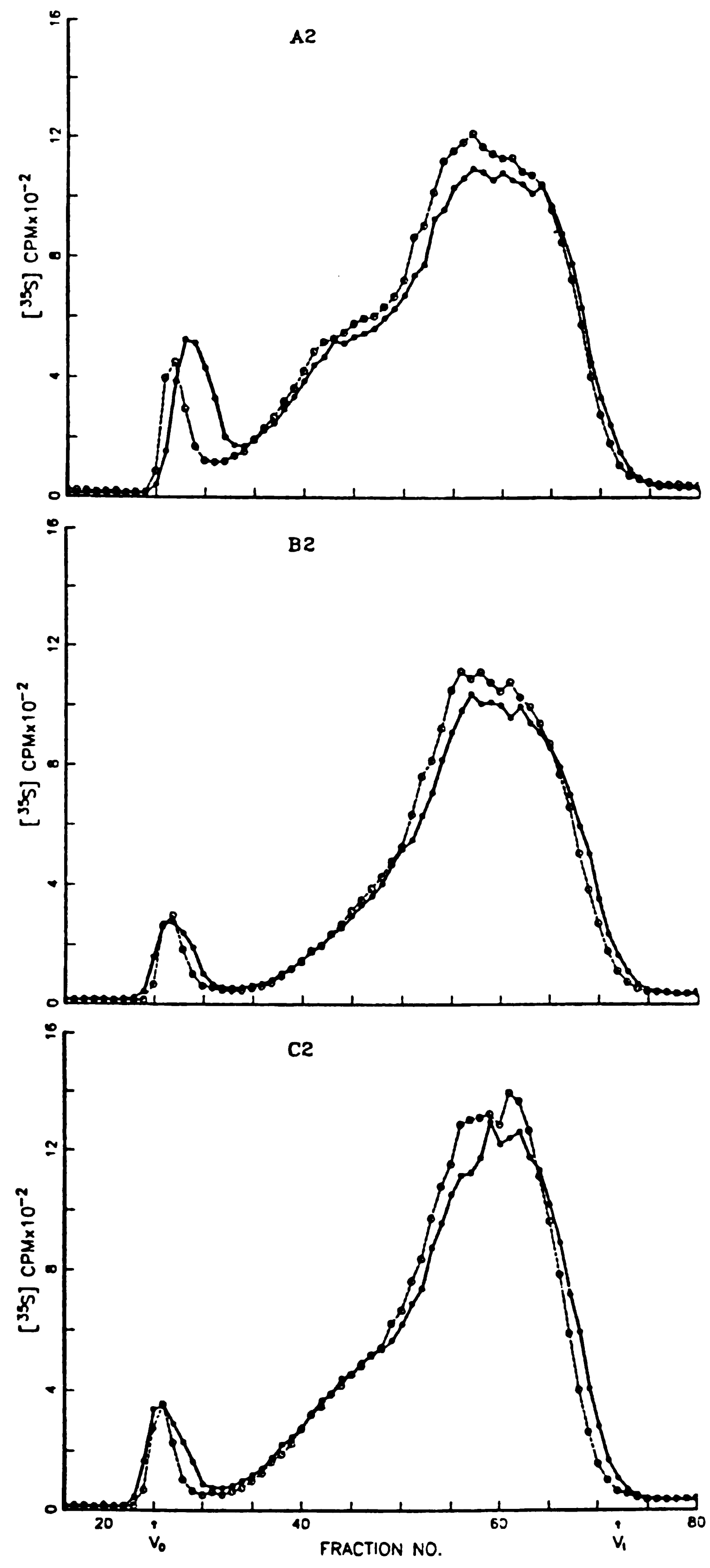


Fig. 1

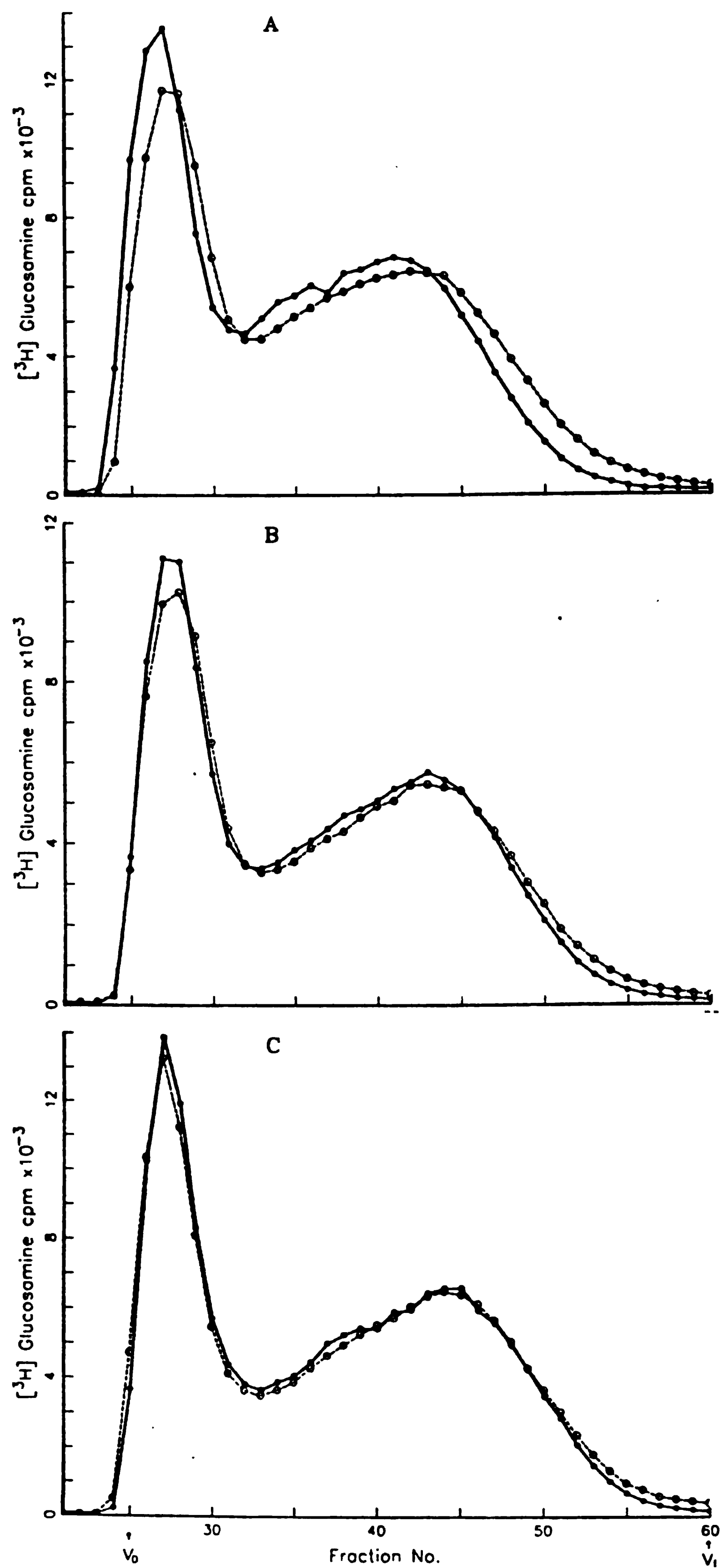


Fig. 2

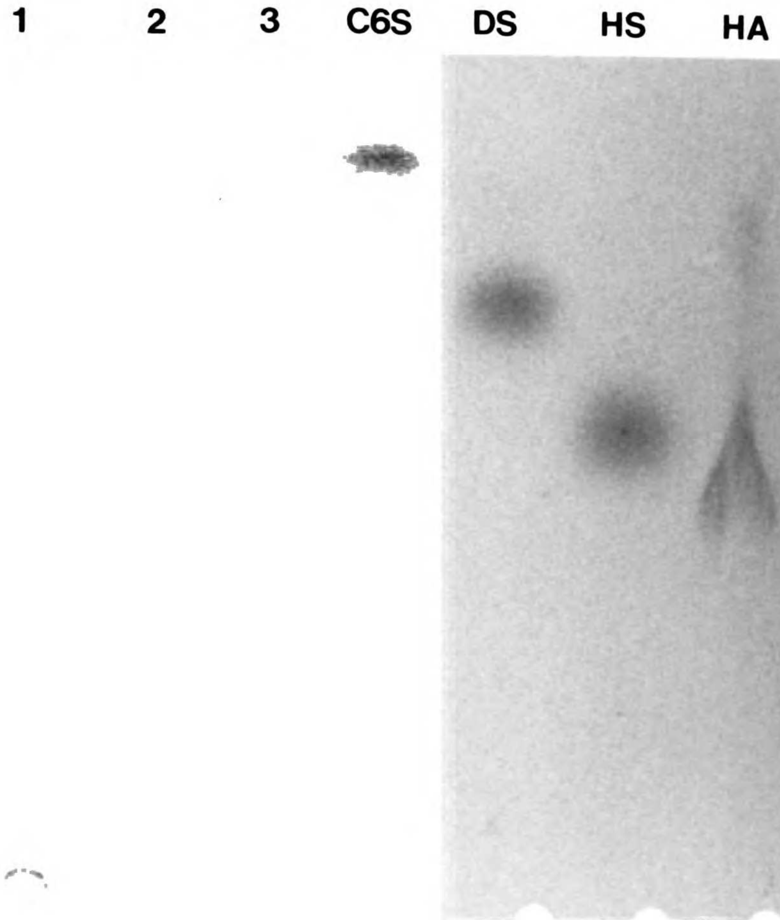


Fig 3

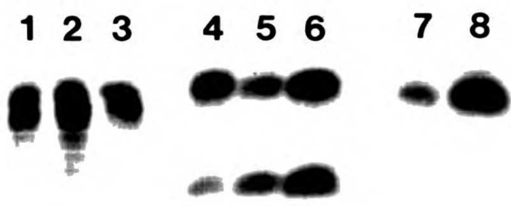


Fig 4

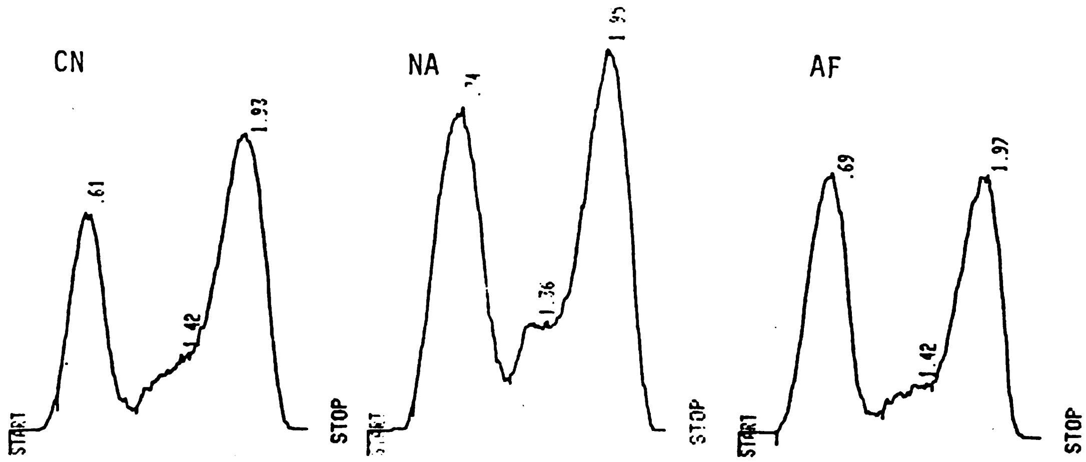


Fig. 5

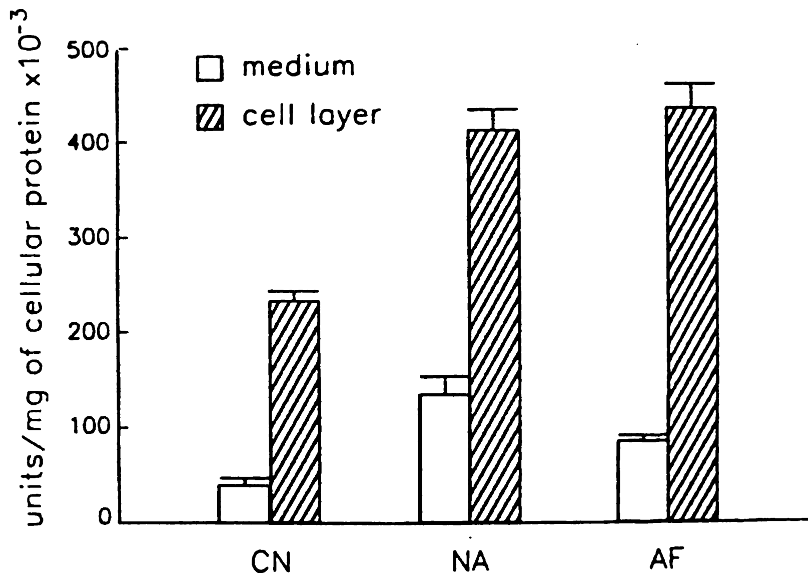


Fig. 6

CHAPTER VI

CONCLUSION

In this dissertation, the mechanisms which underlie the abnormalities of the ECM in two disease states: diabetes mellitus (Chapters II and III) and adolescent idiopathic scoliosis (Chapters IV and V) have been explored.

A working hypothesis has been made (Chapter I, Part D) that hyperglycemia in diabetes may affect fibril formation and subsequent cross-linking of collagen, thus rendering the collagen more susceptible to degradation. This has the effect of enhancing the catabolism of interstitial collagen in diabetic tissue. Using an *in vitro* assay system, definite evidence was provided that glucose decreased the cross-linking of collagen by interfering with fibril formation. The inhibitory effect of glucose on both fibril formation and cross-linking is dose-dependent. The physiological and pathological ranges of sugar concentration fall within the linear limitations of both inhibition curves. Therefore, such inhibitions are likely to occur *in vivo*.

Whether the enhanced degradation of collagen occurs in cultures exposed to high sugar levels is addressed in Chapter III. In human skin fibroblast cultures, both D- and L-glucose did not cause increases in the intracellular degradation of collagen. 2-deoxy-D-glucose, a non-metabolized glucose analog, however, increased the intracellular degradation of collagen. Deoxyglucose may exert its effect by disturbing sugar metabolism as well as the glycosylation of collagen. In chick calvaria cultures, D-glucose did not increase collagen degradation, but stimulated

collagen synthesis. This observation in cultures did not correspond to its _in vivo_ counterpart, in which an exhaustive state of connective tissue is usually the rule. The increase of collagen degradation and decrease of collagen deposition in the presence of L-glucose indicates that the sugar may have affected collagen metabolism by interfering with collagen fibril formation. L-glucose is an inert sugar moiety that is transported into the cell through simple diffusion, not through a facilitated diffusion process. It is not utilized by the cell. Therefore, in terms of cell metabolism, it may act as an inert bystander that does not affect collagen synthesis. It may interfere with collagen fibril formation extracellularly and result in increased collagen degradation. Because of the complexity of the sugar effect in the culture systems, a definitive conclusion cannot be drawn, nor can the observations in culture systems be extrapolated to the _in vivo_ situation.

In Chapters IV and V, the ECM synthesized by cultured skin fibroblasts from normal chicks and chicks with hereditary scoliosis were investigated. The major findings are listed here:

1. Collagen
    a) Qualitative primary structure and ratios of collagen types appeared to be normal
    b) Deposition of collagen into the cell layer decreased.
    c) Extractability of cell layer-associated collagen increased.
    d) Lysyl oxidase activity and cellular copper content were normal.
    3) Microfibrillar structure of collagen appeared to be normal by electron microscopy.

2. Proteoglycans

   a) The proportion of PG aggregation decreased.

   b) HA accumulation in medium and cell layer decreased.

   c) CS in cell layer decreased but HS did not change.

   d) Hyaluronidase activity increased.

With a broad picture of the ECM changes in scoliotic chick skin fibroblasts in mind, a molecular basis can be invoked. The enhanced hyaluronidase activity seems to degrade HA and CS excessively. The level of PG, as well as the proportion of PG aggregation, therefore, decreases. Since PG's can stabilize collagen *in vitro*, the decrease in PG's may result in the instability of collagen, which is observed both in cultured fibroblasts and in tissue from scoliotic chicks. This mechanism is likely to be applicable to adolescent idiopathic scoliosis in humans. Balaba (1972) has reported increases in hyaluronidase activity and decreased hyaluronic acid levels in serum from patients with adolescent idiopathic scoliosis. Such observations in the chick model will provide future directions for re-investigation of the disorder in humans. Furthermore, this represents the first report in a culture system that increased collagen extractability is accompanied by decreased levels of PG's. The importance of collagen-proteoglycan interactions in the maintenance of the integrity of the ECM is therefore confirmed.

In this dissertation, two important biochemical processes in the metabolism of ECM, e.g., collagen fibril formation and collagen-proteoglycan interaction are addressed. The significance of these two processes thus far has been appreciated only by biochemists and cell biologists. The observations of their roles in the pathological conditions diabetes

mellitus and adolescent idiopathic scoliosis hopefully may arouse attention among pathologists.

REFERENCES

1. Abraham PA, Perejda AJ, Carnes WH, and Uitto J. (1982). Marfan syndrome: Demonstration of abnormal elastin in aorta. J Clin Invest 70:1245-1252.

2. Altherton BT, Hynes RO. (1981). A difference between plasma and cellular fibronectins located with monoclonal antibodies. Cell 25:133-141.

3. Antonopoulos CA, Gardell S, and Szirmay JA. (1964). Determination of glycosaminoglycans (mucopolysaccharides) from tissue on the microgram scale. Biochim Biophys Acta 83:1-9.

4. Arneson MA, Hammerschmidt DE, Furcht LT, and King RA. (1980). A new form of Ehlers-Danlos syndrome: Fibronectin corrects defective platelet function. J Am Med Assoc 244:144-147.

5. Bailey AJ, Peach CW, and Fowler LJ. (1970). The chemistry of the collagen cross-links: Isolation and characterization of two intermediate cross-links in collagen. Biochem J 117:819-831.

6. Balaba TY. (1972). Some biochemical aspects of scoliosis and their pathogenetic significance. Reconstr Surg Traumat 13:191-209.

7. Beisswenger PJ, and Spiro RG. (1972). Human glomerular basement membrane: Chemical alteration in diabetes mellitus. Science 168:596.

8. Beisswenger PJ, and Spiro RG. (1973). Studies on the human glomerular basement membrane. Composition, nature of carbohydrate units and chemical changes in diabetes mellitus. Diabetes 22:180-193.

9. Bentz H, Morris NP, Murray LW, Sakai LY, Hollister DW, Burgeson RE. (1983). Isolation and partial characterization of a new human collagen with an extended triple-helical structural domain. Proc Natl Acad Sci (USA) 80:3168-3172.

10. Berenson GS, Radhakrishnamurthy B, Delferes E-R, Ruiz H, Srinivasan SR, Plavidal F, and Brickman F. (1972). Connective tissue macromolecular changes in rats with experimentally induced diabetes and hyperinsulinemia. Diabetes 21:733-743.

11. Bienkowski RS, Baum BJ, and Crystal RG. (1978). Fibroblasts degrade newly synthesized collagen within the cell before secretion. Nature 276:413-416.

12. Bissell MJ, Hall HG, and Parry G. (1982). How does the extracellular matrix direct gene expression? J Theoret Biol 99:31-68.

13. Borcherding MS, Blacik LJ, Sitting RA, Bizzell JW, Breen M, and Weinstein HG. (1975). Proteoglycans and collagen fibre organization in human corneoscleral tissue. Exp Eye Res 21:59-70.

14. Bornstein P, and Piez KA. (1966). The nature of the intramolecular cross-linking in collagen. Biochemistry 5:3460-3473.

15. Bornstein P, Sage H. (1980). Structurally distinct collagen types. Ann Rev Biochem 49:957-1003.

16. Bornstein P, McPherson J, and Sage H. (1982). Synthesis and secretion of structural macromolecules by endothelial cells in culture. P and S Biomed Sci Symposia 6:215-228.

17. Brownlee M, and Cerami A. (1981). The biochemistry of the complications of diabetes mellitus. Ann Rev Biochem 50:385-432.

18. Brownlee M, and Spiro RG. (1979). Glomerular basement membrane metabolism in the diabetic rat. Diabetes 28:121-125.

19. Burgeson RE. (1982). Genetic heterogeneity of collagens. J Invest Dermat 79:25s-30s.

20. Bushell GR, Ghosh P, Taylor TKF, and Sutherland JM. (1979). The collagen of the intervertebral disc in adolescent idiopathic scoliosis. J Bone Joint Surg 61B:501-508.

21. Byers PH, Siegel RC, Holbrook KA, Narayanan AS, Bornstein P, and Hall JG. (1980). X-linked cutis laxa: Defective cross-link formation in collagen due to decreased lysyl oxidase activity. New Eng J Med 303:61-65.

22. Byers PH, Siegel RC, Peterson KE, Rowe DW, Holbrook KA, Smith LT, Chang Y, and Fu JC. (1981). Marfan syndrome: Abnormal α2 chain in type I collagen. Proc Natl Acad Sci (USA) 78:7745-7749.

23. Canivet J, Cruz A, and Mortan-Lalande H. (1979). Biochemical abnormalities of the human diabetic glomerular basement membrane. Metabolism 28:1206-1210.

24. Capaldi MJ and Chapman JA. (1984). The c-terminal extrahelical peptide of type I collagen and its role in fibrillogenesis in vitro: Effect of ethylurea. Biopolymers 23:313-323.

25. Chang AY. (1978). Acid glycohydrolase in Chinese hamster with spontaneous diabetes. I. Depressed levels of renal α-galactosidase and β-galactosidase. Biochem Biophys Acta 522:491-502.

26. Chang K, Uitto J, Rowold EA, Grant GA, Kilo C, and Williamson JR. (1980). Increased collagen cross-linkages in experimental diabetes: Reversal by β-aminopropionitrile and D-penicillamine. Diabetes 20:778-781.

27. Cohen MP and Fuglia VA. (1970). Aortic mucopolysaccharides in experimental diabetes. Diabetes 19:639-643.

28. Cohen MP and Khalifa A. (1977). Renal glomerular collagen synthesis in streptozotocin diabetes: Reversal of increased basement membrane synthesis with insulin therapy. Biochim Biophys Acta 500:395-404.

29. Cohen MP and Klein CV. (1979). Glomerulopathy in rats with streptozotocin diabetes: Accumulation of glomerular basement membrane analogs to human diabetic nephropathy. J Exp Med 149:623-631.

30. Cohen MP and Surma ML. (1981). [^{35}S]sulfate incorporation into glomerular basment membrane glycosaminoglycans is decreased in experimental diabetes. J Lab Clin Med 98:715-722.

31. Cohen MP, Urdanivia E, Surma M, and Wu V. (1980). Increased glucosylation of glomerular basement membrane collagen in diabetes. Biochem Biophys Res Commun 95:765-768.

32. Cohen MP, Urdanivia E, Surma M, and Librowski CJ. (1981). Nonenzymatic glycosylation of basement membranes: In vitro study. Diabetes 30:367-371.

33. Comper WD and Laurent TC. (1978). Physiological function of connective tissue polysaccharides. Physiol Rev 58:255-315.

34. Cooper SM, Keyser AJ, Beaulieu AD, Ruoslahti E, Nimni ME, and Quismoro FP. (1979). Increase in fibronectin in the deep dermis of involved skin in progressive systemic sclerosis. Arthritis Rheum 22:983-987.

35. Coster L, Carlstedt I, and Malmstrom A. (1979). Isolation of [^{35}S] and [^{3}H]-labeled proteoglycans from culture of human embryonic slin fibroblasts. Biochem J 183:669-681.

36. Crofford O. (1975). Report of the national commission on diabetes. DHEW Publ. No. (NIH) 76-1018.

37. Crystal RG, Bitterman PB, Rennard SI, Hance AJ, and Keogh BA. (1984). Interstitial lung diseases of unknown cause: Disorders characterized by chronic inflammation of the lower respiratory tract. New Eng J Med 310:154-166, 235-244.

38. Cutroneo KR, Guzman NA, and Liebelt AG. (1972). Elevation of peptidylproline hydroxylase activity and collagen synthesis in spontaneous primary mammary cancers of inbred mice. Cancer Res 32:2828-2833.

39. David G and Bernfield MR. (1979). Collagen reduces glycosaminoglycan degradation by cultured mammary epithelial cells: Possible mechanism for basal lamina formation. Proc Natl Acad Sci (USA) 76:786-790.

40. David G and von den Berghe. (1983). Transformed mouse mammary epithelial cells synthesize under-sulfated basement membrane proteoglycan. J Biol Chem 258:7338-7344.

41. Deak SB, Nichollas A, Pope FM, and Prockop DJ. (1983). The molecular defect in a nonlethal variant of osteogenesis imperfecta. J Biol. Chem 258:15192-15197.

42. DeMartino GN and Goldberg AL. (1981). A possible explanation of myxedema and hypercholesterolemia in hypothydroidism: Control of lysosomal hyaluronidase and cholesterol esterase by thyroid hormones. Enzyme 26:1-7.

43. Dorfman A. (1982). Proteoglycan Biosynthesis. In Cell Biology of Extracellular Matrix, Ed. Hay ED, Plenum Press, New York and London. pp. 115-138.

44. Dorfman A and Matalon R. (1976). The mucopolysaccharidoses (a review). Proc Natl Acad Sci (USA) 73:630-637.

45. Dolberg D, Bassham JA, and Bissell MJ. (1975). Selective inhibition of the facilitated mode of sugar uptake by cytochalasin B in cultured chick fibroblasts. Exp Cell Res 96:129-137.

46. Dreze A, Moore S, and Bigwood EJ. (1954). On the desalting of solutions of amino acids by ion exchange. Anal Chim Acta II:554-558.

47. Epstein EH, Scott RD, Miller EJ, and Piez KA. (1971). Isolation and characterization of the peptides derived from soluble human and baboon skin collagen after cyanogen bromide cleavage. J Biol Chem 246:1718-1724.

48. Falk RJ, Scheinman JI, Mauer SM, and Michael AF. (1983). Polyantigenetic expansion of basement membrane constituents in diabetic nephropathy. Diabetes 32: (Suppl 2) 34-39.

49. Fluharty AL. (1982). The mucopolysaccharidoses: A synergism between clinical and basic investigation. J Invest Dermatol 79:383-443.

50. Francis MJO. Sanderson MC, and Smith R. (1975). Skin collagen in idiopathic adolescent scoliosis and Marfan's syndrome. Clin Sci Mol Med 51:467-474.

51. Franzblau C and Farris B. (1982). Elastin. In Cell Biology of Extracellular Matrix, Ed. Hay ED. Plenum Press, New York and London. pp. 65-94.

52. Freiberger HF and Pinnell SR. (1979). Heritable disorders of connective tissue. In Dermatology Update, Ed. Moschella SL. Elsevier, New York. p. 221.

53. Furthmayr H and Madri JA. (1982). Rotary shadowing of connective tissue macromolecules. Collagen Rel Res 2:349-363.

54. Furthmayr H, Widemann H, Timpl R, Odermatt E, Engel J. (1983). Electron microscopical approach to a structural model of intima collagen. Biochem J 211:303.

55. Fushimi H and Tarui S. (1976). β-glycosidases and diabetic microangiopathy. II. An insulin-dependent isozyme of β-N-acetylglucosaminidase. J Biochem (Tokyo) 79:265-270.

56. Geiger BJ, Steenbock H, and Parsons HT. (1933). Lathyrism in the rat. J Nutr 6:427-442.

57. Goka TJ, Stevenson RE, Hefferan PM, and Howell RR. (1976). Menkes disease: A biochemical abnormality in cultured human fibroblasts. Proc Natl Acad Sci (USA) 73:604-606.

58. Golub LM, Grennwald RA, Zebrowski EJ, and Ramamurthy NS. (1978). The effect of experimental diabetes on the molecular characteristics of soluble rat-tail tendon collagen. Biochim Biophys Acta 534:73-81.

59. Goodson WH III, and Hunt TK. (1977). Studies of wound healing in experimental diabetes mellitus. J Surg Res 22:221-227.

60. Graff JC, Wohlhueter RM, and Plagemann PG. (1977). Effect of temperature and cytochalasin B and Persantin on the non-mediated permeation of non-electrolytes into cultured Novikoff rat hepatoma cell. J Biol Chem 252:4185-4190.

61. Gross J, Highberger JH, and Schmitt FO. (1954). Collagen structure considered as states of aggregation of a kinetic unit: The tropocollagen particle. Proc Natl Acad Sci (USA) 40:688-697.

62. Hamlin CR, Kohn RR, and Muschin JM. (1975). Apparent accelerated aging of human collagen in diabetes mellitus. Diabetes 24:902-904.

63. Harrington PR. (1977). The etiology of idiopathic scoliosis. Clin Orthop 126:17-25.

64. Hardingham TE and Muir H. (1972). The specific interaction of hyaluronic acid with cartilage proteoglycan. Biochim Biophys Acta 279:401-405.

65. Hascall VC and Hascall GK. (1982). Proteoglycans. In Cell Biology of Extracellular Matrix, Ed. Hay ED. Plenum Press, New York and London. pp. 39-64.

66. Hascall VC and Sajadera SW. (1969). Protein-polysaccharide complex from bovine nasal cartilage: The function of glycoproteins in the formation of aggregates. J Biol Chem 244:2384.

67. Hasilik A, Waheed A, and Figura K. (1981). Enzymatic phosphorylation of lysosomal enzymes in the presence of UDP-N-acetylglucosamine. Absence of the activity in I-cell fibroblasts. Biochem Biophys Res Commun 98:761-767.

68. Hassell JR, Newsome DA, Krachmer J, and Rodriguez M. (1980). Corneal macular dystrophy: A possible inborn error in corneal proteoglycan maturation. Proc Natl Acad Sci (USA) 77:3705-3709.

69. Hata R and Nagai Y. (1972). A rapid and micro method of acidic glycosaminoglycans by two-dimensional electrophoresis. Anal Biochem 45:462-468.

70. Hay ED. (1982). Introductory remarks in Cell Biology of Extracellular Matrix. Ed. Hay ED. Plenum Press, New York and London. pp. 1-4.

71. Hayashi T and Nagai Y. (1972). Factors affecting the interaction of collagen molecules as observed by in vitro fibril formation: Effects of small molecules, especially saccharides. J Biochem 72:749-758.

72. Heinegard D. (1972). Extraction, fractionation, and characterization of proteoglycans from bovine tracheal cartilage. Biochim Biophys Acta 285:181.

73. Hewitt AT, Kleinman HK, Pennypacker JP, and Martin GR. (1980). Identification of an adhesive factor for chondrocytes. Proc Natl Acad Sci (USA) 77:385-388.

74. Hewitt AT, Varner HH, and Martin GR. (1981). Isolation of chondronectin. In Immunochemistry of the Extracellular Matrix. Vol. I. Ed. Furthmayr H. CRC Press Inc., Boca Raton. pp. 135-141.

75. Higgins PJ and Bunn HF. (1981). Kinetic analysis of the nonenzymatic glycosylation of hemoglobin. J Biol Chem 256:5204-5208.

76. Horn N. (1976). Copper incorporation studies on cultured cells for prenatal diagnosis of Menke's disease. Lancet 1:1156-1158.

77. Howard GA, Bottlemiller BL, Turner RT, Rader JI, and Baylink DJ. (1981). Parathyroid hormone stimulates bone formation and resorption in organ culture: Evidence for a coupling mechanism. Proc Natl Acad Sci (USA) 78:3204-3208.

78. Hunter RE, Bradford DS, and Oegema TR. (1978). Biochemistry of the intervertebral disc in scoliosis. Trans Orthop Res Soc 3:135.

79. Hynes RO. (1982). Fibronectin and its relation to cellular structure and behavior. In Cell Biology of Extracellular Matrix, Ed. Hay ED. Plenum Press, New York and London. pp. 295-334.

80. Jordan RE, Hewitt N, Lewis W, Kagan H, and Franzblau C. (1974). Regulation of elastase-catalyzed hydrolysis of insoluble elastin by synthetic and naturally occurring hydrophobic ligands. Biochemistry 13:3497-3503.

81. Kakkar VV, Hughes RD, Gimson AES, and Williams R. (1982). Fibronectin in fulminant hepatic failure. Brit Med J 285:1231-1232.

82. Kanwar YS, Linker A, and Farquhar MG. (1980). Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. J Cell Biol 86:688-693.

83. Kefalides NA. (1974). Biochemical properties of human glomerular basement membrane in normal and diabetic kidney. J Clin Invest 53:403-407.

84. Kenney MC, Lewis DA, Atton BJ, and Riggins RS. (1982). Structural properties of type I collagen isolated from chickens with scoliosis. Connective Tissue Res 10:129-135.

85. Kischer CW and Hendrix M-JC. (1983). Fibronectin in hypertrophic scars and keloids. Cell Tissue Res 231:29-37.

86. Klein L, Butcher DL, Sudilovsky O, Kikkawa R, and Miller M. (1975). Quantification of collagen in renal glomeruli isolated from human nondiabetic and diabetic kidneys. Diabetes 24:1057-1065.

87. Kleinman HK, Klebe RJ, and Martin GR. (1981). Role of collagenous matrices in the adhesion and growth of cells. J Cell Biol 88:473-485.

88. Kofoed JA and Tocci AA. (1973). The acidic glycosaminoglycans of gingiva of diabetic rats. J Periodont Res 8:323-326.

89. Kraemer PM. (1971). Heparin sulfates of cultured cells. I. Membrane-associated and cell-sap species in Chinese hamster cells. Biochemistry 10:1437-1444.

90. Kream BE, Rowe DW, Gworek SC, and Raisz LG. (1980). Parathyroid hormone alters collagen synthesis and procollagen in RNA levels in fetal rat calvaria. Proc Natl Acad Sci (USA) 77:5654-5658.

91. Kuivaniemi H, Peltonen L, Palotic A, Kaitila I, and Kivirikko KI. (1982). Abnormal copper metabolism and deficient lysyl oxidase activity in a heritable connective tissue disorder. J Clin Invest 69:730-733.

92. Laurell C-B and Eriksson S. (1963). The electrophoretic α1-globulin pattern of serum in α1-antitrypsin deficiency. Scand J Clin Lab Invest 15:132-140.

93. le Pape A, Guitton J-D, and Muh J-P. (1981a). Modification of glomerular basement membrane cross-links in experimental diabetic rats. Biochem Biophys Res Commun 100:1214-1221.

94. le Pape A, Muh J-P, and Bailey AJ. (1981b). Characterization of N-glycosylated type I collagen in streptozotocin-induced diabetes. Biochem J 197:405-412.

95. Lesot H, Kuhl U, and von der Mark K. (1983). Isolation of a laminin-binding protein from muscle cell membranes. EMBO J 2:861.

96. Lichtenstein JR, Martin GR, Kohn LD, Byers PH, McKusick VA. (1973). Defect in conversion of procollagen to collagen in a form of Ehlers-Danlos syndrome. Science 182:298.

97. Lilja S and Barrach H-J. (1981). An electron microscopical study of the influence of different glycosaminoglycans on the fibrillogenesis of collagen type I and II in vitro. Virchows Arch [Path] 390:325-338.

98. Lin H, Benson DR, Riggins RS, Rucker RB, and Abbott UK. (1980). Plasma free hydroxyproline, growth and sexual maturity in the scoliotic chicken. Proc Soc Exp Biol Med 165:345-348.

99. Lindahl U and Hook M. (1978). Glycosaminoglycans and their binding to biologic macromolecules. Ann Rev Biochem 47:385-417.

100. Linsenmayer TF. (1982). Collagen. In Cell Biology of Extracellular Matrix, Ed. Hay ED. Plenum Press, New York and London. pp. 5-38.

101. Linsenmayer TF, Fitch JM, Schmid TM, Zak NB, Gibney E, Sanderson RD, Mayne R. (1983). Monoclonal antibodies against chicken type V collagen: Production, specificity, and use for immunocytochemical localization in embryonic cornea and other organs. J Cell Biol 96:124-132.

102. Lipson SJ and Reilly DT. (1982). Lysosomal, collagen and biomechanical changes in an animal model of intervertebral disc degeneration. Trans Orthop Res Soc 7:219.

103. Lohmander LS, Fellini SA, Kimura JH, Stevens RL, abd Hascall VC. (1983). Formation of proteoglycan aggregates in rat chondrosarcoma chondrocyte cultures treated with tunicamycin. J Biol Chem 258:12280-12286.

104. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ. (1951). Protein measurement with the folin phenol reagent. J Biol Chem 193:265-275.

105. Madia AM, Rozovski SJ, and Kagan HM. (1979). Changes in lung lysyl oxidase activity in streptozotocin-diabetes and in starvation. Biochim Biophys Acta 585:481-487.

106. Malathy K and Kurup PA. (1972). Metabolism of glycosaminoglycans in alloxan diabetic rats. I. Changes in tissue glycosaminoglycans. Diabetes 21:1162-1167.

107. Malinoff H and Wicha MS. (1983). Isolation of a cell surface receptor protein for laminin from murine fibrosarcoma cells. J Cell Biol. 96:1475.

108. Malmstrom A, Carlstedt I, Aberg L, and Fransson LA. (1975). The copolymeric structure of iduronic acid- and glucuronic acid-containing units in soluble and cell associated glycan. Biochem J 151:477-489.

109. Martinez-Hernandez A, Huffer WE, Neldner K, Gordon S, and Reeve EB. (1976). Resolution and repair of elastic tissue calcification in pseudoxanthoma elasticum. Arch Pathol Lab Med 102:303-305.

110. Matalan R and Dorfman A. (1969). Acid mucopolysaccharides in cultured human fibroblasts. Lancet 2:838-841.

111. Mayne R. (1983). A summary of different types of collagen and collagenous peptides. In 42nd Symposium of the Society for Developmental Biology, Irvine, CA.

112. Mayne R, von der Mark K. (1983). Collagens of cartilage. In Cartilage Vol. I, Ed. Hall BK. Academic Press, New York. pp. 181-214.

113. McKusick VA. (1972). Heritable Disorders of Connective Tissue, 4th Ed. C.V. Mosby Co., St. Louis.

114. Melnick GF, Ladoulis CT, and Cavallo T. (1981). Decreased anionic groups and increased permeability precedes deposition of immune complexes in glomerular capillary wall. Am J Pathol 105:114-120.

115. Meyer K. (1971). Hyaluronidase. Enzymes 5:307-320.

116. Morrison JGL, Wilson Jones E, McDonald DM. (1977). Juvenile elastoma and osteopoikilosis (the Buschke-Ollendorff syndrome). Br J Dermatol 97:417-422.

117. Mosher DF and Williams EM. (1978). Fibronectin concentration is decreased in plasma in severely ill patients with disseminated intravascular coagulation. J Lab Clin Med 91:729-735.

118. Muir IHM. (1980). The chemistry of ground substance of joint cartilage. In The Joints and Synovial Fluid II, Ed. Sokoloff L. Academic Press, New York. pp. 27-94.

119. Muir H and Hardingham TE. (1975). Structure of proteoglycans. In Biochemistry of Carbohydrates, Ed. Whelan WH. Butterworths, London. pp. 153-222.

120. Myllyla R, Risteli L, and Kivirikko KI. (19750. Assay of collagen-galactosyltransferase and collagen-glucosyltransferase activities and preliminary characterization of enzyme reactions with transferases from chick-embryo cartilage. Eur J Biochem 52:401-410.

121. Narayanan AS and Page RC. (1977). Serum modulates collagen types in human gingiva fibroblasts. FEBS Lett 80:221-224.

122. Neville DM. (1971). Molecular weight determination of protein dodecyl sulfate complex by gel electrophoresis in a discontinuous buffer system. J Cell Biol 246:6328.

123. Ninomiya Y, Hata R, Nagai Y, Tajimo S, Nishikawa T, and Hatano H. (1982). Glycosaminoglycan metabolism by scleroderma fibroblasts in culture. Biochem Res 3:70-82.

124. Norling B, Glimelius B, Westermark B, and Wasteson A. (1978). A chondroitin sulfate proteoglycan from human cultured glial cell aggregates with hyaluronic acid. Biochem Biophys Res Commun 84:914-921.

125. Oegema TR and Thompson RC. (1981). Characterization of a hyaluronic acid dermatan sulfate proteoglycan complex from dedifferentiated human chondrocyte cultures. J Biol Chem 256:1015-1022.

126. Orkin RW and Toole BP. (1980). Isolation and characterization of hyaluronidase from cultures of chick embryo-skin and muscle-derived fibroblasts. J Biol Chem 255:1036-1042.

127. Orkin RW, Pratt RM, and Martin GR. (1976). Undersulfated chondroitin sulfate in cartilage matrix of brachymorphic mice. Develop Biol 50:82-94.

128. Orkin RW, Underhill CB, and Toole BP. (1982). Hyaluronate degradation in 3T3 and simian virus transformed 3T3 cells. J Biol Chem 257:5821-5826.

129. Parsons DB, Brennan MB, Glimcher MJ, and Hall J. (1982). Scoliosis: Collagen defect in the intervertebral disc. Trans Orthop Res Soc 7:52.

130. Paterson RA and Heath H. (1967). The effect of β,β'-iminodipropionitrile treatment and alloxan diabetes on the glycosaminoglycans of the rat retina and aorta. Biochim Biophys Acta 148:207-214.

131. Pedrini VA, Ponseti IV, and Dohrman SC. (1973). Glycosaminoglycans of intervertebral disc in idiopathic scoliosis. J Lab Clin Med 82:938-950.

132. Pedrini-Mille A, Pedrini VA, Tudisco C, Ponseti IV, Weinstein SL, and Maynard JA. (1983). Proteoglycans of human scoliotic intervertebral disc. J Bone Joint Surg 65-A:815-823.

133. Peltonen L, Kuivaniemi H, Palotie A, Horn N, Kaitila H, and Kivirikko KI. (1983). Alterations in copper and collagen metabolism in Menkes syndrome and a new subtype of the Ehlers-Danlos syndrome. Biochemistry 22:6156-6163.

134. Pinnell SR and Martin GR. (1968). The cross-linking of collagen and elastin: Enzymatic conversion of lysine in peptide linkage to -aminoadipic and semialdehyde (allysine) by an extract from bone. Proc Natl Acad Sci (USA) 61:708-713.

135. Pinnell SR, Krane SM, Kenzora JE, and Glimcher MJ. (1972). A heritable disorder of connective tissue: Hydroxylysine-deficient collagen disease. New Eng J Med 286:1013-1020.

136. Ponseti IV, Pedrini V, Wynne-Davies R, and Duval-Beaupere. (1976). Pathogenesis of scoliosis. Clin Orthop 120:268-280.

137. Pope FM, Martin GR, Lichtenstein JR, Penttinen RP, Gerson B, Rowe DW, and McKusick VA. (1975). Patients with Ehlers-Danlos syndrome type IV lack type III collagen. Proc Natl Acad Sci (USA) 72:1314.

138. Rao CN, Barsky SH, Terranova VP, and Liotta LA. (1983). Isolation of a tumor cell laminin receptor. Biochem Biophys Res Commun 111:804.

139. Reissig JL, Strominger JL, and Leloir LR. (1955). A modified colorimetric method for the estimation of N-acetylamino sugars. J Biol Chem 217:298-305.

140. Rennard SI, Stier L, and Crystal RG. (1982). Intracellular degradation of newly synthesized collagen. J Invest Dermatol 79:77s-82s.

141. Roden L, Baker JR, Cifonelli A, and Mathews HB. (1974). Isolation and characterization of connective tissue polysaccharides. Method Enz 28:73-141.

142. Rohrbach DH, Hassell JR, Kleiman HK, and Martin GR. (1981). Alteration in the basement membrane (heparan sulfate) proteoglycan in diabetic mice. Diabetes 31:185-188.

143. Ross R and Bornstein P. (1969). The elastin fiber. I. The separation and partial characterization of its macromolecular components. J Cell Biol 40:366-381.

144. Royce PM, Camakaris J, and Danks DM. (1980). Reduced lysyl oxidase activity in skin fibroblasts from patients with Menkes' syndrome. Biochem J 192:579-586.

145. Saba TM, Blumenstock FA, Scovill WA, and Bernard M. (1978). Cryoprecipitate reversal of opsonic-2-surface binding glycoprotein deficiency in septic surgical and trauma patients. Science 210:622-624.

146. Sage H, Pritzl P, and Bornstein P. (1980). A unique pepsin-sensitive collagen synthesized by aortic endothelial cells. Biochemistry 19:5747-5755.

147. Riggins RS, Abbott UK, Ashmore CR, Rucker RB, and McCarrey JR. (1977). Scoliosis in chickens. J Bone Joint Surg 59-A:1020-1026.

148. Risteli J, Kovisto VA, Akerblom HK, Kivirikko KI. (1976). Intracellular enzymes of collagen biosynthesis in rat kidney in streptozotocin diabetes. Diabetes 25:1066-1070.

149. Robert B, Robert L, Legrand Y, Pignaud G, and Caen J. (1971). Elastolytic protease in blood platelets. Ser Haemato 4:175-185.

150. Robert B, Szigeti M, Derouette J, Robert L, Bouissou H, and Fabre MT. (1971). Studies on the nature of the microfibrillar component of elastic fiber. Eur J Biochem 21:507-516.

151. Robinson J, Viti M, and Hook M. (1984). Structure and properties of an undersulfated heparan sulfate proteoglycan synthesized by a rat hepatoma cell line. J Cell Biol 98:946-953.

152. Roden L. (1980). Structure and metabolism of connective tissue proteoglycans. In Biochemistry of Glycoproteins and Proteoglycans, Ed. Lennarz WA. Plenum Press, New York. pp. 267-371.

153. Sage H, Trueb B, and Bornstein P. (1983). Biosynthetic and structural properties of endothelial cell type VIII collagen. J Biol Chem 258:13391.

154. Sage H, Balian G, Vogel AM, and Bornstein P. (1984). Type VIII collagen: Synthesis by normal and malignant cells in culture. Lab Invest 50:219-231.

155. Sakamoto M, Sakamoto S, Brickley-Parsons D, and Glimcher MJ. (1979). Collagen synthesis and degradation in embryonic chick-bone explants. J Bone Joint Surg 61-A:1042-1053.

156. Sanberg LB, Soskel NT, Leslie JG. (1981). Elastin structure-biosynthesis and relation to disease states. New Eng J Med 304:566-579.

157. Schafer IA, Sitabkha L, and Pandy M. (1984). Isolation and preliminary characterization of proteoglycan aggregates from cultured fibroblasts. J Biol Chem 259:2321-2330.

158. Schiller S and Dorfman A. (1963). The distribution of acid mucopolysaccharides in skin of diabetic rats. Biochim Biophys Acta 78:371-373.

159. Schiller S and Hille . (1969). Nucleotide diphosphate sugar pyrophosphatase activity in the skin of alloxandiabetic rats. Biochim Biophys Acta 177:377.

160. Schneir M, Bowersox J, Ramamurthy N, Yavelow J, Murray J, Edlin-Folz E., and Golub L. (1979). Response of rat connective tissues to streptozotocin diabetes: Tissue specific effects on collagen metabolism. Biochim Biophys Acta 583:95-102.

161. Schneir M, Ramamurthy N, and Golub L. (1982). Skin collagen metabolism in the streptozotocin induced diabetic rat: Enhanced catabolism of collagen formed both before and during the diabetic state. Diabetes 31:426-431.

162. Schnider SL and Kohn RR. (1980). Glucosylation of human collagen in aging and diabetes mellitus. J Clin Invest 66:1179-1181.

163. Schofield JD, Freeman IL, and Jackson DS. (1971). The isolation, and amino acid and carbohydrate composition, of polymeric collagens prepared from various human tissues. Biochem J 124:467-473.

164. Scott JE, Orford CR, and Hughes EW. (1981). Proteoglycan-collagen arrangements in developing rat tail tendon. BIochem J 195:573-581.

165. Shaw SN and Amos H. (1973). Insulin stimulation of glucose entry in chick fibroblasts and HeLa cells. Biochem Biophys Res Commun 53:357-365.

166. Siegel RC. (1974). Biosynthesis of collagen cross-links: Increased activity of purified lysyl oxidase with reconstituted collagen fibrils. Proc Natl Acad Sci (USA) 71:4826-4830.

167. Siegel RC. (1979). Lysyl oxidase. Inter Rev Conn Tiss Res 8:73-118.

168. Siegel RC and Fu JCC. (1977). Collagen cross-linking: Purification and substrate specificity of lysyl oxidase. J Biol Chem 251:5779-5785.

169. Siegel RC and Martin GR. (1970). Collagen cross-linking: Enzymatic synthesis of lysine-derived aldehydes and the production of cross-linked components. J Biol Chem 245:1653-1658.

170. Siegel RC, Page RC, and Martin GR. (1970). The relative rate activity of connective tissue lysyl oxidase and plasma amino oxidase on collagen and elastin substrates. Biochim Biophys Acta 222:552-555.

171. Silbert JE. (1973). Biosynthesis of mucopolysaccharides and protein-polysaccharides. In Molecular Pathology of Connective Tissue, Ed. Perez-Tamayo R and Rojkind M. Marcel Dekker, Inc., New York. pp. 323-353.

172. Sjoberg I and Fransson LA. (1977). Synthesis of glycosaminoglycans by human embryonic lung fibroblasts. Biochem J 167:383-392.

173. Smith BD, Byers P, and Martin GR. (1972). Production of procollagen by human fibroblasts in culture. Proc Natl Acad Sci (USA) 69:3260-3262.

174. Spiro RG and Spiro MJ. (1971). Effect of diabetes on the biosynthesis of glomerular basement membrane. Studies on glucosyl transferase. Diabetes 20:641-648.

175. Stern B, Glimcher MJ, and Goldhaber P. (1965). The effect of various oxygen tensions on the synthesis and degradation of bone collagen in tissue culture. Proc Soc Exp Biol Med 121:869-872.

176. Tanzer ML. (1973). Cross-linking of collagen. Science 180:561-566.

177. Tanzer ML and Mechanic G. (1970). Isolation of lysinonor-leucine from collagen. Biochem Biophys Res Commun 39:183-189.

178. Taylor LW. (1971). Kyphoscoliosis in a long-term selection experiment with chickens. Avian Dis 15:376-390.

179. Taylor TKF, Ghosh P, and Bushell GR. (1981). The contribution of the intervertebral disk to the scoliotic deformity. Clin Orthop 156:79-90.

180. Terranova VP, Rao CN, Kalebic T, Margulies IM, and Liotta LA. (1983). Laminin receptor on human breast carcinoma cells. Proc Natl Acad Sci (USA) 80:444-448.

181. Timpl R, Wiedemann H, van Delden V, Furthmayr H, Kuhn K. (1981). A network model for the organization of type IV collagen molecules in basement membranes. Eur J Biochem 120:203-211.

182. Toole BP. (1982). Glycosaminoglycans in morphogenesis. In Cell Biology of Extracellular Matrix, Ed. Hay ED. Plenum Press, New York and London. pp. 259-294.

183. Traub W and Piez KA. (1971). The chemistry and structure of collagen. Adv Protein Chem 25:243-252.

184. Truelstad RL and Hayashi K. (1979). Tendon collagen fibrillogenesis: Intracellular subassemblies and cell surface changes associated with fibril growth. Dev Biol 71:228-242.

185. Tyagi JS, Hirano H, Merlino GT, and Pastan I. (1983). Transcriptional control of the fibronectin gene in chick embryo fibroblasts transformed by Rous sarcoma virus. J Biol Chem 258:5758-5793.

186. Uitto J and Bauer EA. (1982). Diseases associated with collagen abnormalities. In Collagen in Health and Disease, Ed. Weiss JB and Jayson MIV. Churchill Livingstone, Edinburgh. pp. 289-312.

187. Uitto J, Santa Cruz DJ, and Eisen AZ. (1980). Connective tissue nevi of the skin: Clinical, genetic, and histopathologic classification of harmatomas of the collagen, elastin, and proteoglycan type. J Am Acad Dermatol 3:441-461.

188. Uitto J, Perejda AJ, Grant GA, Rowold EA, Kilo C, and Williamson JR. (1982a). Glycosylation of human glomerular basement membrane collagen: Increased content of hexose in ketoamine linkage and unaltered hydroxylysine-O-glucosides in patients with diabetes. Conn Tiss Res 10:287-296.

189. Uitto J, Ryhanen L, Abraham PA, and Perejda AJ. (1982b). Elastin in diseases. J Invest Dermatol 79:160s-168s.

190. Underhill CB and Toole BP. (1982). Transformation-dependent loss of the hyaluronate-containing coats of cultured cells. J Cell Physiol 110:123-128.

191. Vater CA, Harris ED, and Siegel RC. (1979). Native cross-links in collagen fibrils induce resistance to human synovial collagenase. Biochem J 181:639-645.

192. Venn G, Metha MH, and Mason RM. (1983). Solubility of spinal ligament collagen in idiopathic and secondary scoliosis. Clin Orthop 177:294-301.

193. Villee DB and Powers ML. (1977). Effect of glucose and insulin on collagen secretion by human skin fibroblasts in vitro. Nature 268:156-157.

194. Vracko R. (1973). Basal lamina layering in diabetes mellitus: Evidence for accelerated rate of cell death and cell regeneration. Diabetes 23:94-104.

195. Wagner CW, Ledbetter S, Hassell JR, Martin GR, and Rohrbach D. (1983). Reduced synthesis of basement membrane heparan sulfate proteoglycan core protein in diabetic tissue. J Cell Biol 97:454a.

196. Weil R, Nozawa M, Koss M, Weber C, Reemtsma K, and McIntosh R. (1976). The kidney in streptozotocin diabetic rats. Arch Pathol Lab Med 100:37-49.

197. Weinstein SL and Ponseti IV. (1983). Curve progression in idiopathic scoliosis. J Bone Joint Surg 65A:447-455.

198. Weinstein SL, Zavala DC, and Ponseti IV. (1981). Idiopathic scoliosis: Long-term follow-up and prognosis in untreated patient. J Bone Joint Surg 63A:702-712.

199. Weiss JB and Smith I. (1967). Sensitive location reagent for the simultaneous detection of sugars, amino sugars, and sialic acids. Nature 215:638.

200. Westberg NG. (1980). Diabetic nephropathy. Pathogenesis and prevention. Acta Endocrinologica 94 (Suppl 238): 85-101.

201. Westberg NG. (1976). Biochemical alterations of human glomerular basement membrane in diabetes. Diabetes 25 (Suppl 2):920-924.

202. Wicha M, Liotta LA, Garbisa S, and Kidwell WR. (1979). Basement membrane collagen requirements for attachment and growth of mammary epithelium. Exp Cell Res 124:181-190.

203. Wight TN and Hascall VC. (1983). Proteoglycans in primate arteries. III. Characterization of the proteoglycans synthesized by arterial smooth muscle cells in culture. J Cell Biol 96:167-176.

204. Williams CJ and Prockop DJ. (1983). Synthesis and processing of a type I procollagen containing shortened pro $\alpha 1(I)$ chains by fibroblasts from a patient with osteogenesis imperfecta. J Biol Chem 258:5915-5921.

205. Winterbourne DJ and Mora PT. (1981). Cell selected from high tumorigenecity or transformed by simian virus 40 synthesize heparan sulfate with reduced degree of sulfation. J Biol Chem 256:4310-4320.

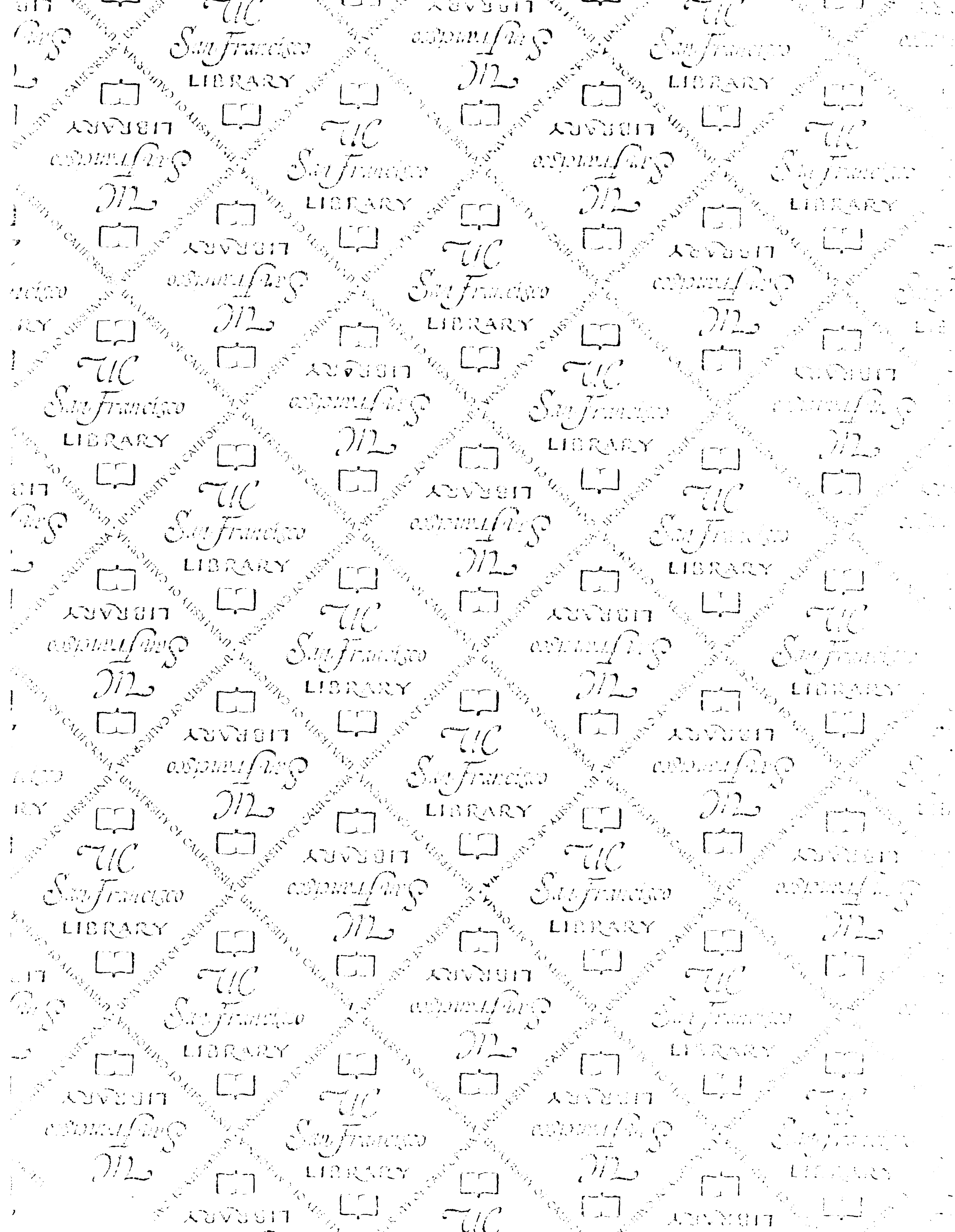
206. White AA and Panjabi MM. (1978). Clinical Biomechanics of the Spine. J.B. Lippincott, Philadelphia.

207. Wynne-Davies R. (1968). Familial (idiopathic) scoliosis. J Bone Joint Surg 50-B:24-30.

208. Yamada KM. (1982). Fibronectin and other structural proteins. In Cell Biology of Extracellular Matrix, Ed. Hay ED. Plenum Press, New York and London. pp. 95-114.

209. Zaleske DJ, Ehrich MG, and Hall JE. (1980). Association of glycosaminoglycan depletion and degradative enzyme activity in scoliosis. Clin Orthop 148:177-181.

210. Zorab PA, Clark S, Cotrel Y, and Harrison A. (1971). Bone collagen turnover in idiopathic scoliosis estimated from total hydroxyproline excretion. Arch Dis Child 46:828-832.


UC
San Francisco
LIBRARY
UNIVERSITY OF CALIFORNIA

FOR REFERENCE

NOT TO BE TAKEN FROM THE ROOM

BRODART CAT. NO. 23 012 PRINTED IN U.S.A.