Cell Biology of Extracellular Matrix

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Preface

At a recent meeting to discuss the domains of cell biology, I put forth a case for the extracellular matrix, even though my argument ran the risk of falling on deaf ears. After all, the matrix is EXTRAcellular, outside the cells. In this book, however, the authors make a compelling case for the relevance of the matrix to cellular concerns. Not only are numerous cell types, including many epithelia, quite caught up in the business of manufacturing matrix components, but also most of them contain matrix molecules in exoskeletons that are attached to the plasmalemma and that organize or otherwise influence the affairs of the cytoplasm. The idea of this book is to present the extracellular matrix to cell biologists of all levels. The authors are active and busy investigators, recognized experts in their fields, but all were enthusiastic about the prospect of writing for this audience. The chapters are not "review" articles in the usual sense, nor are they rehashes of symposium talks; they were written specifically for this book and they present the "state of the art" in engaging style, with ample references to more technical or historical reviews. The book is rich in electron micrographs and diagrams and for many of the latter, as well as for the design of the cover, we are indebted to Sylvia J. Keene, medical illustrator for the Department of Anatomy at Harvard Medical School. We also owe special thanks to Susan G. Hunt of this Department, who did a masterful preliminary job of editing the manuscripts, and to the editors and staff of Plenum Press, who were supporting and helpful throughout the enterprise. We think you will enjoy this treatise.

Elizabeth D. Hay

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Cell Biology of Extracellular Matrix

Introductory Remarks

ELIZABETH D. HAY

Cytoskeleton, cell shape, cell migration, control of cell growth and differentiation, these are all subjects that to be fully understood today require a consideration of the extracellular matrix (ECM): its composition, its role in development, and its relation to the cell surface. The ECM is the structurally stable material that lies under epithelia and surrounds connective tissue cells, but the old concept of the ECM as an inert supporting material, created by the cells as a mere scaffolding on or in which to reside, is now bygone. Surely, collagens are sources of strength to the tissues, elastin and proteoglycans are essential to matrix resiliency, and the structural glycoproteins help to create tissue cohesiveness. But the cell, having produced these extracellular macromolecules, and spoken out in one way or another on the question of their assembly, does not then divorce itself of them. The cell continues to interact with its own ECM products, and with the ECM produced by other cells. 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 to and dependent on the composition and organization of the matrix.

The ECM is composed of a far greater variety of molecules than we would have guessed even a decade ago. Discounting all the molecules that must pass through the matrix to reach the cells or are temporarily trapped in the ECM, we are left with a basic structural composition of at least four major classes of macromolecules. In Chapter 1, Dr. Linsenmayer brings us up to date on the chemistry and molecular biology of the five or more different (genetically distinct) types of collagen molecules. Then (Chapter 2), Drs. Vincent and Gretchen Hascall describe the remarkable progress that has been made in recent years in our knowledge of proteoglycan composition and assembly. In Chapter 3, Drs. Franzblau and Faris describe a unique protein of the ECM, elastin, and give us insights into its ultrastructure and the development of elastic fibers. The final class of ECM molecules, the structural glycoproteins, are relatively large molecules with sugar side chains that are longer

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than those found in collagen and are often rich in sialic acid. The structural glycoproteins are discussed in Chapter 4 by Dr. Yamada, who has pioneered many of the recent discoveries on the structure and function of fibronectin.

A book on the cell biology of extracellular matrix would, of course, consider the manner in which cells synthesize ECM, influence its organization, and bring about its degradation. These subjects are covered in the second section of the book. In Chapter 5, Dr. Dorfman describes the biosynthesis of glycosaminoglycans (GAG) and the protein cores that, together with GAG, form the proteoglycan (PG) monomers that become assembled into the PG aggregates of the ECM; in both Chapters 2 and 5, the authors take care to relate biochemical aspects of PG organization and synthesis to tissue and cell ultrastructure. The role of the endoplasmic reticulum and Golgi complex in the synthesis and secretion of collagens is developed in Chapter 6 by Dr. Olsen, who also informs us of the very recent and exciting advances that have been made in the area of collagen gene structure and function. Drs. Trelstad and Silver (Chapter 7) present a very original synthesis of our understanding of the physical chemistry, molecular biology, and morphology of collagen assembly. Finally (Chapter 8), Dr. Gross acquaints us with what is known and not known about collagenases and the process of ECM degradation (and remodeling) that somehow, in amazing fashion, is controlled by the cells.

The matrix, as we indicated earlier, is, in a sense, talking back to the cells that create (and reside on or in) its interstices. In the third section of the book, we consider the phenomenon of cell–extracellular matrix communication in more detail. Dr. Toole (Chapter 9) tells us about the role of hyaluronic acid and PG in morphogenesis, cell migration, and cell proliferation, and he presents a model of interaction of hyaluronic acid with the cell surface. Dr. Hynes (Chapter 10) then presents a model of interaction of fibronectin with other ECM molecules and with the cell surface (the models are not necessarily coexclusive). Chapter 10 also reviews *in vitro* studies of the role of fibronectin in cell growth, cell migration, platelet adhesion, etc., and speculates on its possible *in vivo* functions. In Chapter 11, Dr. Farquhar convinces us that the PG component of the glomerular basement membrane (GBM) plays an important role in the filtering function of the glomerulus, and in the last chapter, I give an overview of the effects of collagens on cell differentiation, cell shape, and cell metabolism, and speculate further on the relation of the ECM to the cell surface.

We are fortunate in being able to bring together so many investigators who are pioneers in their fields and were so willing to make the advances in, and future of, these important subjects clear to the cell biologist. In editing this endeavor, I have not attempted to impose my own views of terminology on these distinguished authors. Thus, the reader will find that some terms are used interchangeably, the best example being the terms *basal lamina* and *basement membrane*. The basal (basement) lamina or basement membrane is a zone about 100 nm wide under epithelia and around muscle cells; it consists of a central compact sheet of collagen (and probably other glycoproteins), called the lamina densa, that is separated from the cell by a less electron-dense zone, the lamina rara externa, and from the underlying connective tissue by a second electron-

lucid zone, the lamina rara interna. Both the laminae rarae externa and interna contain a layer of PG granules connected by small filaments to the cell, on the one hand, and to underlying collagen fibrils, on the other; these structures are not visible unless special fixatives are used, hence the term *rara* (or *lucida*) is used to refer to the "empty" zone (Fig. i-1).

The term *basement membrane* was originally used by light microscopists to refer to the whole condensation of connective tissue (basal lamina and the

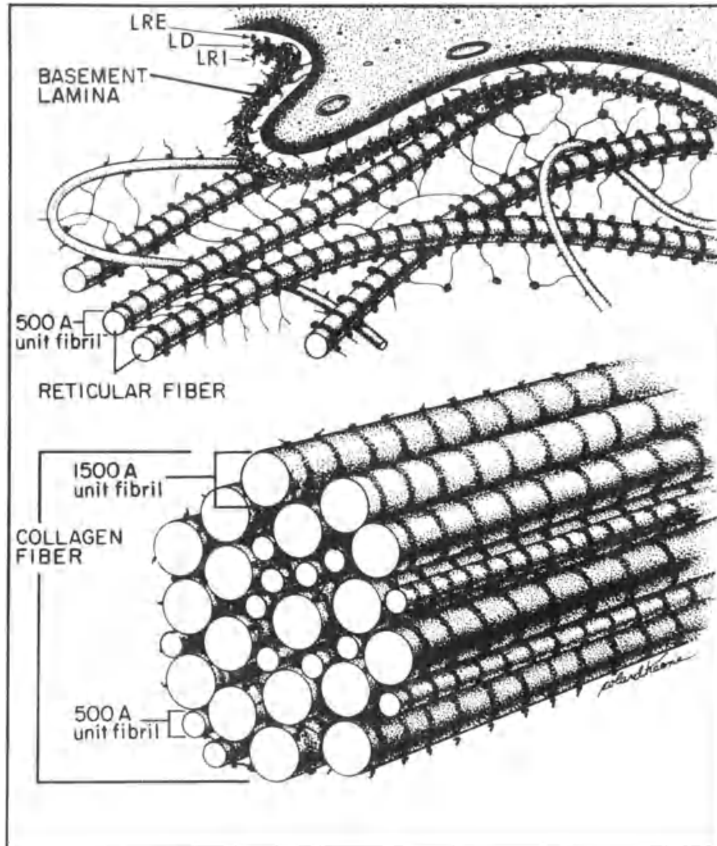

Figure i-1. Diagrams depicting relations of proteoglycans, collagen fibrils, and basement lamina in the tissues. The drawing at the top includes part of the basal cytoplasm of an epithelial cell. The outermost layer of the basal lamina, the lamina rara externa (LRE), is attached to the epithelial cell by small filaments presumably composed of glycoprotein. The LRE and LRI (lamina rara interna) each contain a layer of proteoglycan granules. The lamina densa (LD) contains collagen and possibly other glycoproteins (see Chapter 11). Collagen fibrils associated with the basement lamina are small (50 nm in diameter) and may form small bundles called reticular fibers that stain with silver salts. The fibrils are covered with proteoglycan granules and are connected by ruthenium red-staining filaments that may consist of hyaluronic acid (see Chapter 2). Collagen fibers are larger than reticular fibers; they contain less proteoglycan and a greater variety of unit collagen fibrils (50–1500 nm in diameter). (From Hay *et al.*, 1978, referenced in Chapter 7.)

associated collagen fibrils, or reticular lamina) under an epithelium. Only in the glomerulus and a few other locations is the basal lamina free of collagen fibrils and thus truly equivalent to the basement membrane visualized previously by light microscopists. Another terminology problem is that in the glomerulus, the basal laminae of the epithelium and endothelium are fused, obliterating the layer we called the lamina rara interna above; the latter term is given to the PG-rich, juxtaendothelial zone of the glomerular basement membrane (GBM). In spite of the historical precedence of that use (in the GBM), most morphologists find it helpful to refer to the inner layer (facing connective tissue) of simple basement laminae as the internal lamina rara (Fig. i-1).

The term *connective tissue* is often used to refer to the collagen fibrils and other ECM molecules that surround cells of the fibroblast family. Some authors, however, include the basal lamina, as this structure is composed of molecules similar to those of the ECM proper and "connects" epithelium and muscle to the ECM proper. By connective tissue "cells," most authors mean cells of the fibroblast family (osteoblasts, chondroblasts, fibroblasts), but muscle and epithelial cells also secrete ECM and, in this sense, are part of the cellular component of connective tissue. Unlike the connective tissue cells proper (the fibroblast family), however, muscle and epithelial cells are separated from collagen fibrils by basal laminae; why this should be is not immediately obvious, but it is likely that these groups of cells have distinct cell surfaces that differ in their interactions with ECM (Chapter 12). Collagen fibrils are 10 nm in diameter or wider (above 25 nm in diameter, they appear striated); the term *filament* is usually used in this book to refer to a fibrous structure less than 10 nm in diameter. Collagen fibrils are often organized into small fibers called reticular fibers that are argyrophilic as seen in the light microscope, and into larger collagen fibers; the diameters of the fibrils composing these fibers vary in a predictable way (Fig. i-1).

Cell Biology of Extracellular Matrix is, as we noted above, a book written by experts, all of whom have contributed measurably to our understanding of the ECM. They have agreed to present their material succinctly and in a manner understandable and relevant to the cell biologist who has not been working in the field. The chapters are not "review" articles; for full coverage of historical and other details, I have asked the authors to refer the reader to the numerous, more technical reviews that appear in the journals and books of the trade and are readily accessible to the interested reader desiring more depth. This book is written for the cell biologist, then, but it does present the "state of the art" in ample detail to serve as a ready reference for all who wish to think and talk intelligibly about the ECM. Many of the techniques used to explore the ECM are presented in considerable detail, in footnotes, in figure legends, or in the text. The book is also fairly lavishly illustrated; I am a visual person myself and I have often added electron micrographs to the more biochemically oriented chapters. There is much in this book about disease, as well as health. This is, in short, a book that we believe will convince even the most doubting that to understand the cell is to understand the extracellular matrix.

Chapter 1

Collagen

T. F. LINSENMAYER

1. Introduction

Collagenous proteins occur in all eukaryotic phyla except for the protozoans, sometimes constituting more than half of the total protein in adult organisms and usually found as the principal component of the extracellular matrix. Traditionally, the role attributed to collagen is a structural one. The molecule is thought to give support and tensile strength to the mesoglea of coelenterates, the cuticle of annelid worms, and the skin, bone, tendon, and cornea of vertebrates. During the past several years it has been demonstrated that collagen is actually a heterogeneous class of molecules with common chemical and physical properties. Thus, individual organisms contain different genetic types of collagen that occur in tissue-specific patterns and arise during development in a defined temporal sequence. Also, collagen(s) has been shown to be involved either directly or indirectly as an agent in promoting cell attachment and differentiation, as a chemotactic agent for both macrophages and fibroblasts, as an antigen in immunological processes, and as a causal agent in certain pathological conditions. Thus, in addition to its structural role, collagen potentially has numerous developmental and physiological functions, many of which remain to be elucidated (Chapter 12).

In the present chapter, we will examine the biochemical characteristics of the molecule and how molecules associate with one another, giving rise to higher-order forms of molecular arrangements. Then, we will consider the characteristics of the different genetic types of collagen and how these collagens function as antigens in eliciting immunological responses. Finally, some of the uses of anti-collagen antibodies for exploring collagen organization in cells and tissues will be discussed.

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2. The Collagen Molecule

2.1. Triple-Helical Region

The strength, rigidity, and highly asymmetric length-to-width ratios that are usually associated with collagen fibers or bundles of fibers are, to a great extent, reflected in the structure of collagen molecules (Fig. 1-1). All of the known collagen molecules are rodlike structures, and with few possible exceptions are 3000 Å long and 15 Å in diameter (Gross *et al.*, 1954). Due to this asymmetry, native collagen molecules in solution exhibit a high radius of gyration, and thus impart a high degree of viscosity to the solution compared to more symmetrical, globular molecules. Whereas a solution of the globular

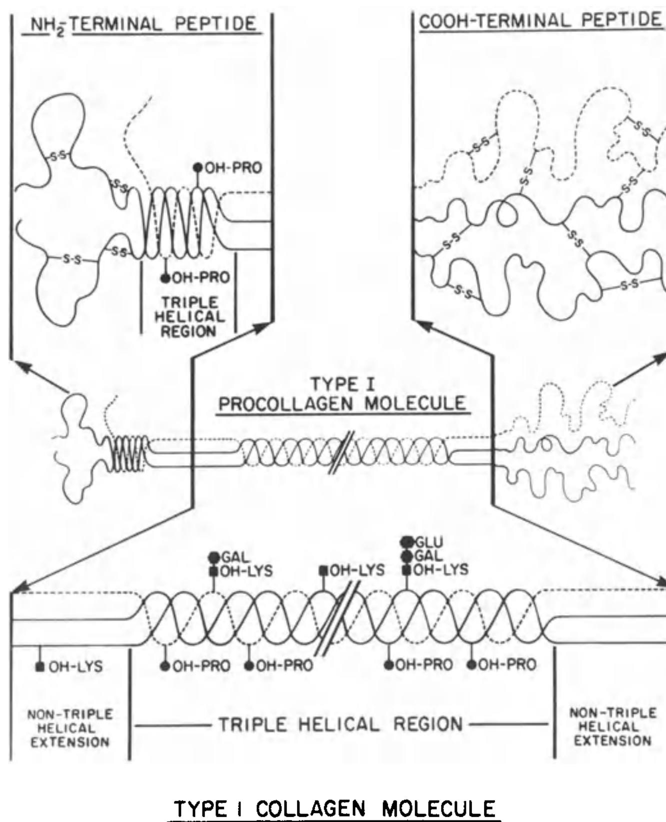

Figure 1-1. Diagram of the major characteristics of the type I collagen molecule and its procollagen form. OH-PRO, hydroxyproline; OH-LYS, hydroxylysine; –S–S–, disulfide bonds; GAL, galactose; GLU, glucose. The extension peptide sugars are discussed in Chapter 6. NH_2-terminal propeptide and COOH-terminal propeptide show the two pieces that are cleaved from procollagen during its processing into a collagen molecule. (Modeled after Prockop *et al.*, 1979.)

protein bovine serum albumin at 1 mg/ml shows virtually no increase in viscosity, a solution of collagen at 1 mg/ml has an easily detectable increase in viscosity, as determined in either a viscometer or simply by observing the rate at which an air bubble rises through the solution. A collagen solution of 3–4 mg/ml, which approaches saturation, has such a high viscosity that an air bubble rises only slowly through the solution. That this viscosity is due to the asymmetric shape that exists only when the molecule is in the native conformation can be demonstrated by raising the solution above the denaturation temperature of the collagen molecules (~ 40°C). As denaturation occurs and the conformation of the molecules breaks down, the viscosity of the solution largely disappears, and will only return under conditions that allow renaturation and reformation of the native molecules.

The shape and most of the properties of native collagen molecules are determined by the triple-helical region, which composes more than 95% of the molecule (Fig. 1-1). This region consists of three separate chains (α chains), each of which contains approximately 1000 amino acids twisted in the form of a left-handed helix termed a polyproline type II helix (for review see Ramachandran and Ramakrishnan, 1976). These three helical chains are then wrapped around one another in a higher-order ropelike fashion to produce the tight, triple-helical structure of the molecule (see Traub and Piez, 1971; Fietzek and Kuhn, 1975). This conformation is stabilized by interchain hydrogen bonds. These are the bonds that are disrupted during thermal denaturation and unfolding of the molecule. The triple-helical conformation is wound such that the peptide bonds linking adjacent amino acids of the chains are buried within the interior of the molecule. Thus, the triple-helical region is highly resistant to attack by strong, general proteases such as pepsin. If the molecule is denatured, however, the liberated chains are susceptible to proteolysis and are reduced to small peptides. The only enzymes that are able to efficiently attack the helical portion of native collagen molecules are the collagenases (see Chapter 8).

Folding of the component chains into the proper helical conformation requires that glycine be present as every third amino acid residue (approximately 333 residues per chain). The chains are therefore composed of a series of triplet Gly-X-Y sequences in which X or Y can be any amino acid. Frequently, X is proline and Y is hydroxyproline, each occurring at about 100 sites per chain (see, for example, the sequence shown in Fig. 1-2). The presence of other

Gly - X - Y - Gly - X - Y - Gly - X - Y

-Gly - Pro - Ser - Gly - Pro - Arg - Gly - Leu - Hyp -
-Gly - Pro - Hyp - Gly - Ala - Hyp - Gly - Pro - Gln -
-Gly - Phe - Gln - Gly - Pro - Hyp - Gly - Glu - Hyp -
-Gly - Glu - Hyp - Gly - Ala - Ser - Gly - Pro - Met -

Figure 1-2. Amino acid sequence of the cyanogen bromide peptide α1-CB2, derived from the α1 chain of rat skin type I collagen. Note the Gly-X-Y repeating triplets and the presence of hydroxyprolines (Hyp) in the Y position.

amino acids at the X and Y positions does not seem to follow any recognizable or repeating pattern (see, for example, the sequence shown in Hulmes et *al.*, 1973; Piez, 1976). Having a large number of hydroxyproline residues in the Y position seems to add stability to the helical structure. Hydroxyproline is formed by the posttranslational enzymatic conversion of proline in the Y position to hydroxyproline (described below). If collagen is synthesized under conditions in which this conversion is blocked, the resulting molecules have a lowered melting temperature (Berg and Prockop, 1973a,b) that is thought to be proportional to the degree of inhibition of proline hydroxylation. At present, the only other amino acid with a known functional significance is lysine. Lysine and its enzymatically modified forms participate in covalent crosslinking between chains and molecules, and as sites for sugar attachment (discussed below).

Information about the linear sequence of amino acids in the triple-helical region can be obtained by electron microscopy if molecules are precipitated in the form of segment-long-spacing (SLS) crystallites (for review see Gross, 1974) and then are positive stained with heavy metals such as tungstate.* When an acidic solution of collagen is dialyzed against ATP (Schmitt et *al.*, 1953), the collagen precipitates in the form of SLS crystallites 2800 Å long of variable width. In each crystallite, the molecules are arranged side by side with their NH_2- and COOH-terminal ends in register (Fig. 1-3). Amino acids of like charge are thus in apposition. Positive staining of crystallites reveals the presence of about 58 dark-staining bands (Bruns and Gross, 1974). By comparing band positions with amino acid sequence data, it has been deduced that each dark-staining band represents a region with a high density of polar amino acid residues, whereas the light-staining bands represent regions containing apolar amino acids (Chapman, 1974). SLS crystallites of the different collagen types have unique charge profiles that can be demonstrated in this manner (Trelstad et *al.*, 1970); from this we would expect that similarly stained native fibrils of different collagens would be distinguishable, but they are not. SLS preparations and the characteristic banding patterns have been used to order cyanogen bromide peptides in sequencing studies (Rauterberg and Kuhn, 1969, 1971), to identify the large propeptide extension pieces in the precursor forms of collagens (Chapter 6), and to localize the cleavage site of the animal collagenases (Chapter 8).

2.2. Non-Triple-Helical Region

In addition to the triple-helical region, collagen molecules found in extracellular matrices also have short (~ 20 amino acids), nonhelical extension

* The terms positive *staining* and *negative staining* are used to refer to treatments of molecules or larger structures that have been spread out on the surface of a film-covered grid. In positive staining, the molecules themselves are actually stained, usually by interaction of heavy metals with charged regions of the molecule. In negative staining, electron-dense material is deposited in unoccupied areas in and around the molecules.

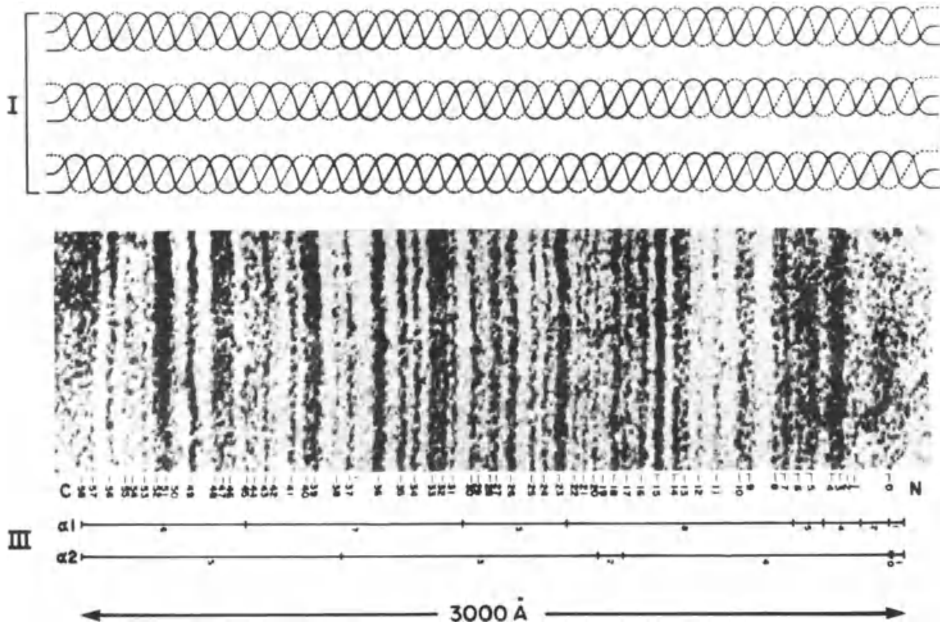

Figure 1-3. A positive-stained segment-long-spacing crystallite, with a diagram of its molecular packing arrangement Each of the 58 stained bands is assigned a number. C, carboxy-terminal end; N, amino-terminal end. Also shown along the length of the crystallite are the relative sizes and positions of the cyanogen bromide peptides of the $\alpha1$ and $\alpha2$ chains, designated by numbers on chains. (Micrograph courtesy of Dr. R. Bruns.)

peptides (originally called telopeptides) at the NH_2- and COOH-terminal ends of each component α chain (Fig. 1-1) (for review see Traub and Piez, 1971; Piez, 1976). These terminal extension peptides play important functional roles, for they represent the sites where hydroxylysine-derived (Fig. 1-1) intramolecular and intermolecular cross-links are formed when molecules are arranged in native fibrils. The cross-links stabilize the molecular arrangement within fibrils and, depending on the type of cross-link compound formed, they greatly decrease the solubility of the molecules. As the cross-link-containing extension peptides are not in the triple-helical conformation, they are susceptible to proteolytic degradation under conditions in which the triple-helical body of the molecule is left intact. This represents the basis for limited pepsin digestion, one of the most effective procedures for extracting otherwise insoluble collagens from tissues (Miller, 1972). For example, when tissues are incubated with the enzyme pepsin at 4°C and pH 2, the cross-link regions are degraded while the major, helical portions of the molecules remain intact. After the solution is neutralized to inactivate the pepsin, most of the collagen molecules are readily soluble in neutral salt solutions. The pepsin treatment also partially purifies the collagen preparation by degrading many of the noncollagenous proteins. Thus, this procedure has become one of the most frequently used techniques for collagen extraction.

3. Posttranslational Modifications

3.1. Proline

About half of the 200 or so prolines that are incorporated into newly forming α chains are inserted in the Y position of the Gly-X-Y triplets, and these can be enzymatically converted to the 4-isomer of hydroxyproline, 4-hydroxyproline (for reviews see Grant and Prockop, 1972; Cardinale and Udenfriend, 1974). The enzyme responsible for this conversion is prolyl hydroxylase (see Chapter 6). Its substrate specificity is limited to peptide-linked proline residues that precede a glycine residue (i.e., the Y position). In the presence of molecular O_2, α-ketoglutarate, and the cofactors ascorbic acid and ferrous iron, it converts these proline residues into hydroxyprolines. Although prolyl hydroxylase will work on small polypeptides, in general its activity increases with increasing peptide size. Moreover, from sequence data, it is known that there are sites of incomplete hydroxylation on the chains (Balian *et al.*, 1972).

In addition to 4-hydroxyproline, different collagens have many small and more variable amounts of a second isomer, 3-hydroxyproline, which in different collagens is found at about one to seven residues per chain. All known 3-hydroxyprolines are found in the X position of Gly-X-Y triplets, and thus are probably formed by the action of a different prolyl hydroxylase enzyme. At present not much is known about the hydroxylation enzyme responsible for producing 3-hydroxyproline, and no function has been attributed to the 3-hydroxyproline residues found in collagen chains.

It is known that the formation of hydroxyproline is necessary for helix stability and also for efficient secretion of procollagen, the precursor form of collagen, into the extracellular space (Berg and Prockop, 1973a,b). Thus, it is possible to interfere with the synthesis of collagen, or to change the properties of the collagen that is produced, by modifying the insertion of prolines using amino acid analogues or by deleting one of the substrates or cofactors required for the enzymatic formation of hydroxyproline. Incubating cells in an N_2 environment *in vitro* limits the availability of O_2. The compound $\alpha\alpha$-dipyridyl, when added to culture medium, chelates available iron. Both of these procedures inhibit the formation of hydroxyproline, which causes the cells to accumulate an unhydroxylated form of collagen called protocollagen (Hurych and Chvapil, 1965; Prockop and Juva, 1965; Kivirikko and Prockop, 1967). The administration of proline analogues, such as L-azetidine-2-carboxylic acid or *cis*-hydroxyproline, to animals or *in vitro* cultures causes the synthesis of aberrant collagen chains that are inefficiently secreted (Rosenbloom and Prockop, 1971; Uitto *et al.*, 1972; Harsch *et al.*, 1972). These analogues cannot be considered to be specific for collagen, however, because proline is found in other proteins whose synthesis might also be affected. A cofactor that can easily become limiting is ascorbic acid, as it is rapidly destroyed under tissue culture conditions; in long-term cultures fresh ascorbate is added daily (see Chapter 6).

3.2. Lysine

The amino acid lysine, once incorporated into nascent collagen chains, can undergo one or more sequential enzymatic conversions. Lysine residues in the Y position of Gly-X-Y triplets can be converted to hydroxylysines by the enzyme lysyl hydroxylase (see Fig. 1-1, OH-LYS) (Miller, 1971; Kivirikko et *al.*, 1973; Popenoe and Aronson, 1972). The substrate specificity of the enzyme (i.e., lysine preceding a glycine in peptide linkage) and the cofactor requirements, which include molecular O_2, ferrous iron, α-ketoglutarate, and ascorbic acid, are the same as for prolyl hydroxylase (Chapter 6). A special case in which the hydroxylation of lysine before glycine rule does not hold is found in the non-triple-helical extensions at both the NH_2 and the COOH terminals. The hydroxylysines in these regions are very important in that once they have undergone a further enzymatic modification, they can participate in cross-link formation. In both of these regions, a single lysine residue, not followed by a glycine, is frequently hydroxylated. Possibly these hydroxylysines are formed by the action of a hydroxylase enzyme other than the one that acts on lysines in the helical region.

The hydroxylation of lysine is quite variable (Spiro, 1969; Kivirikko et *al.*, 1973), and the content of hydroxylysine differs greatly among the different genetic types of collagens. The hydroxylysine content can even be different for the same genetic type of collagen extracted from different tissue sources. For example, the major collagen types extracted from tissues rich in basement membrane probably represent several genetically different molecules. Each of these has a relatively high content of hydroxylysine when compared to the chief collagen molecule extracted from skin and bone, termed type I collagen (Spiro, 1973; Kefalides, 1973). Although the type I molecule extracted from skin and bone represents the same gene product, or a very similar gene product, bone collagen in general is more highly hydroxylated than skin collagen. The functional significance of the hydroxylysine in collagen is still unknown.

Once the lysine has been hydroxylated it can undergo further post-translational enzymatic modifications such as glycosylations. The glycosylations are catalyzed by two glycosyltransferase enzymes, which catalyze the transfer of uridine diphosphate sugars onto hydroxylysines in peptide linkage (R. G. Spiro and Spiro, 1971; M. J. Spiro and Spiro, 1971). The first enzyme, a galactosyltransferase, attaches galactose onto hydroxylysine, and the second, a glucosyltransferase, attaches a glucose residue onto the already bound galactose. Thus, the two glycosylated derivatives are galactosylhydroxylysine (Fig. 1-1, GAL, OH-LYS) and glucosylgalactosylhydroxylysine (Fig. 1-1, GLU, GAL, OH-LYS). These are the only two forms of glycosylation found in a collagen molecule. As is true for hydroxylysine itself, the amounts of the two glycosylated derivatives vary greatly among the different genetic types of collagen. They also can vary within the same collagen type from different tissues. It has been suggested that the concentration of the glycosylated derivatives may have some influence in controlling collagen fibril diameter, as the more highly glycosylated collagen molecules tend to be found in tissues with narrow

fibrils (Schofield *et al.*, 1971). This hypothesis, however, remains to be critically tested.

From a functional standpoint, the best understood modification of lysine and hydroxylysine is the conversion to their respective aldehyde forms, allysine and hydroxyallysine. These chemically active compounds are necessary for the process of intramolecular and intermolecular cross-link formation (for reviews see Tanzer, 1973, 1976). The enzyme responsible for this conversion, lysyl oxidase, promotes the conversion of the ϵ-amino group of lysine and hydroxylysine to highly reactive aldehyde forms. It acts preferentially on these two amino acids when they are found in the NH_2- and COOH-terminal extensions, that is, where cross-linking originates (Fig. 1-1). Unlike the other enzymes that perform posttranslational modifications on collagen, this one works not intracellularly but extracellularly, and it appears to work preferentially on collagen molecules after they have assembled into fibrillar forms (see Seigel, 1979). It seems, then, that one way intermolecular cross-links can be made is for these highly reactive aldehyde compounds, once they themselves have been formed in the terminal extension of one molecule, to react spontaneously with other lysines or hydroxylysines within the triple-helical region of an adjacent molecule.

An example of such a cross-link would be for the aldehyde functional group of a hydroxylysine in the terminal extension of one molecule to form a Schiff's base condensation product with the amino group of a hydroxylysine in the body of an adjacent molecule. It is thought that such a Schiff's base compound, once formed, can undergo an internal oxidation–reduction reaction, called an Amadori rearrangement, giving rise to a stable keto–amine cross-link. While numerous other potential intermolecular and intramolecular cross-link compounds have been isolated and characterized, they are all thought to originate with the formation of lysyl oxidase-catalyzed aldehydes.

The enzyme lysyl oxidase itself can be irreversibly blocked by a class of inhibitors called lathyrogens, one of which is a substance termed β-aminopropionitrile. When injected into animals or added to cell or organ cultures, β-aminopropionitrile renders the newly synthesized collagen molecules devoid of cross-links and thus extractable in cold neutral salt solutions (Levene and Gross, 1959). The use of this compound and the discovery of its mechanism of action have greatly aided biochemical and metabolic studies on collagen.

4. Molecular Arrangement in Native Fibrils

Collagen molecules will spontaneously form native fibrils when allowed to aggregate under the proper *in vivo* or *in vitro* environmental conditions (Chapter 7). Depending on whether native fibrils are positive stained or negative stained, they display several morphological forms, two of which are shown in Figure 1-4. Such preparations have been used to construct models for the packing of collagen molecules within fibrils (Schmitt *et al.*, 1955; Gross, 1956; Hodge and Petruska, 1963; Bruns and Gross, 1974). In positive-stained prepara-

tions, the stain binds to regions rich in polar amino acids just as it does in the SLS crystallites discussed earlier (Fig. 1-3). The positive-staining pattern of the native fibril reflects the summation of charged residues along each molecule. In negative-stained preparations, the stain permeates the fibrils and becomes trapped in "hole zones," which are formed by incomplete overlapping of adjacent collagen molecules within the repeating structure. When fibrils are both positive and negative stained, it is possible to see both the repeating units of positive-stained bands and the hole zones created by the molecular arrangement. The most widely accepted model for the packing of molecules in native fibrils (as originally devised by Gross, 1956, and co-workers, and modified by Hodge and Petruska, 1963) is diagrammatically shown in the lower part of Fig. 1-4. The individual molecules are approximately 4.4 times the length (67 nm) of the repeat period, called D. They are staggered about one-quarter their length, and a short empty space is present between the NH_2 terminal of one molecule and the COOH terminal of the next. The empty spaces between adjacent molecules are the hole zones in which stain accumulates (i.e., the dark bands in negative-stained preparations). The regions in which no stain accumulates (i.e., the light bands) are the regions in which molecular overlapping is complete. These are termed "overlap zones." In the negative-stained fibrils, then, the basic repeating unit (D) consists of one hole zone, which is about 0.6 D unit long, plus one overlap zone, which is 0.4 D unit long. The 67-nm repeat period can also be identified in the banding pattern of positive-stained fibrils, and the relationship of the two patterns becomes clearly visible in fibrils that are both positive and negative stained, as shown in Fig. 1-4B.

The presence of a hole zone may be necessary for the enzymatic formation of the allysine and hydroxyallysine required for intermolecular cross-link formation. It is known that the lysyl oxidase enzyme acts preferentially to form these compounds in the NH_2- and COOH-terminal, non-triple-helical extension peptides of molecules after they have aggregated into the native fibrillar form. Why the enzyme prefers to act on molecules in native fibrils is still unknown. However, as the terminal extension peptides reside at the borders of each hole zone, the physical presence of the hole zone may be necessary for the enzyme to diffuse into the interstices of fibrils, where it can act on its substrate. It is also thought that only when molecules are in the native fibrillar form are the aldehydes, once formed, brought into juxtaposition with the proper residues in adjacent molecules so that cross-links can then form spontaneously.

5. Procollagen

Cells producing collagen in culture and animals with certain genetic defects in collagen processing secrete collagen molecules with much higher molecular weights than the corresponding molecules extracted from extracellular matrices (for recent reviews see Fessler and Fessler, 1978; Prockop *et al.*, 1979; Chapter 6, this volume). These higher molecular weight forms are now known to represent the procollagen (precursor) form of the processed

molecules found in the extracellular matrix (Layman *et al.*, 1971; Bellamy and Bornstein, 1971; Lenaers *et al.*, 1971; Stark *et al.*, 1971). In procollagen molecules, each chain has large additional extension-peptides, called propeptides, at both NH_2 and COOH terminals (Fig. 1-1) (Tanzer *et al.*, 1974, 1975; Byers *et al.*, 1975; Fessler *et al.*, 1975). As is the case for most proteins destined for extracellular secretion, an even higher molecular weight form of collagen precursor chain has recently been identified in cell-free protein-synthesizing systems containing collagen mRNA (Chapter 6). Procollagen itself is much longer lived, so that once it is secreted by cells in culture it can be isolated from the medium. To isolate procollagen from culture medium, it is necessary to prevent enzymatic removal of the propeptide extensions by inhibiting both specific and nonspecific proteolysis.

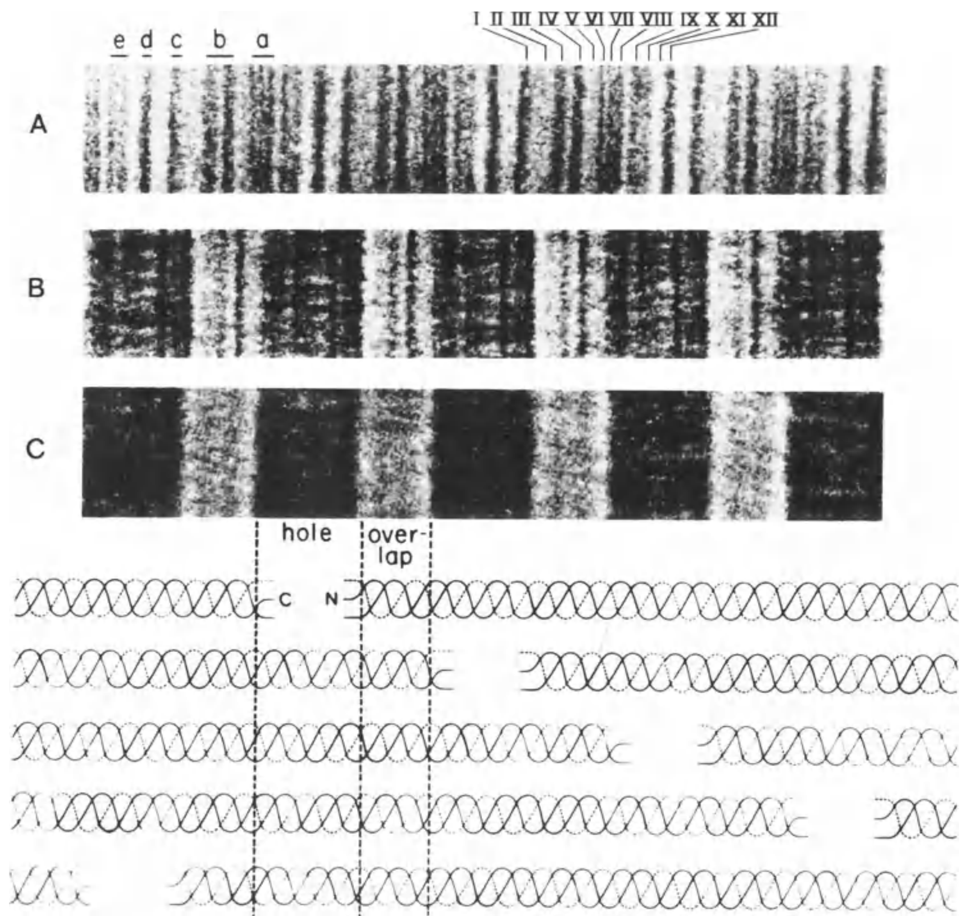

Figure 1-4. Electron micrographs of native collagen fibril stained either (A) positive, (B) positive and negative, or (C) negative. The quarter-stagger arrangement of collagen molecules within a fibril is shown diagrammatically. Vertical lines between molecules designate sites of cross-linking. (Micrographs courtesy of Dr. R. Bruns.)

At least two different enzymes seem to be involved in the normal processing of procollagen to collagen, one to remove the NH_2-terminal propeptides piece and a second to remove the COOH-terminal one. They are called procollagen proteases (Lapiere *et al.*, 1971; Chapter 6, this volume). The involvement of separate enzymes has been inferred from analysis of the collagenous molecules extractable from the skin of cattle with dermatosparaxis. The extracellular sequence for removal of the propeptides involves first the removal of all three chains in the NH_2-terminal extension as a single piece (Morris *et al.*, 1975; Davidson *et al.*, 1975). This procollagen intermediate(s) with only the NH_2-terminal propeptide removed is termed P_C-collagen, indicating that it is a procollagen form with an intact COOH-terminal extension but no NH_2-terminal one. Subsequently, the COOH-terminal extensions are also removed en bloc, resulting in a completely processed collagen molecule. In the normal sequence of conversion, molecules with only the NH_2 propeptide intact are usually not found, as this is the first to be removed. Such molecules, called P_N-collagen, are the product of a genetic abnormality such as dermatosparaxis, in which the skin is extremely fragile and easily torn (Chapter 6).

6. Genetic Types of Collagen

For a number of years, investigators were impressed with the apparent uniformity of the collagen extracted from different species of animals, and from different tissues within the same species of animal. It is true that during evolution, the basic size, helical structure, and amino acid composition of many collagens have remained highly conserved. Also, the tissues that were usually examined, adult skin, bone, tendon, and cornea, do indeed contain predominantly the same genetic type of collagen.

These results, however, are somewhat deceiving in that with improved extraction and analytical procedures there are now known to be at least seven to nine different types of collagen within an individual organism, and others are being discovered with disturbing rapidity. Analyzing these different collagens with respect to their biochemical characteristics and syntheses, their distribution in adult and embryonic tissues, and their possible functional roles in both normal and pathological conditions, constitutes a great portion of the research done on collagen today.

6.1. The Interstitial Collagens: Types I, II, and III

6.1.1. Type I Collagen

The most ubiquitous type of collagen isolated from many adult connective tissues such as skin, bone, tendon, and cornea, is type I collagen. Type I collagen forms striated fibrils 20–100 nm in diameter, which may aggregate to form larger collagen fibers. Much of the information that has already been discussed in this chapter has been obtained from preparations of type I collagen or procollagen.

From a biochemical standpoint, the most striking characteristic of this collagen is that each molecule contains two genetically different types of α chains: one called the $\alpha 1$ type I or $\alpha 1(I)$ chain, and the second called the $\alpha 2(I)$ chain.* Each type I molecule is composed of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain, the complete molecule being abbreviated $[\alpha 1(I)]_2\alpha 2(I)$. When type I collagen is thermally denatured, the $\alpha 1(I)$ and $\alpha 2(I)$ chains can be separated from one another either by ion-exchange chromatography on O-(carboxymethyl)-cellulose (CM-cellulose) columns (Piez et *al.*, 1963) or by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 5–7% gels (Laemmli, 1970) (Fig. 1-5A). Both $\alpha 1(I)$ and $\alpha 2(I)$ have a net basic charge, but $\alpha 2(I)$ is more basic and thus elutes later from the column.

If the type I molecules are obtained from animals or cultures that have been treated with β-aminopropionitrile to prevent cross-linking, by far the major amounts of material correspond to single $\alpha 1(I)$ and $\alpha 2(I)$ chains. Stoichiometrically, these chains appear in a 2 : 1 ratio reflecting their distribution within each molecule. If, however, the molecules are extracted from sources in which cross-linking has been less well blocked or not blocked at all, there are increasing amounts of cross-linked dimers composed of two α chains (termed β chains), trimers composed of three α chains (termed γ chains), and even higher molecular weight aggregates. Dimers (β chains) can either be composed of two $\alpha 1(I)$ chains, in which case they are designated $\beta_{1,1}$ chains, or they can be composed of one $\alpha 1(I)$ chain and one $\alpha 2(I)$ chain, in which case they are designated $\beta_{1,2}$ chains. On SDS-PAGE the two types of β chains migrate much more slowly than α chains, and on CM-cellulose columns they elute in the positions designated in Fig. 1-5. On SDS-PAGE the various combinations of γ chains and higher-order aggregates migrate only slightly from the origin, or in some cases do not enter the gel at all. It can also be seen in Fig. 1-5 that the patterns obtained from the separation of α chains can be one of the diagnostic methods for identifying the different genetic types of collagen (discussed below).

That the $\alpha 1(I)$ and $\alpha 2(I)$ chains represent separate gene products can also be demonstrated by a number of methods that give information on the primary structure, amino acid compositions, and sequences of the chains. The methods include (1) amino acid analysis; (2) amino acid sequencing; (3) banding patterns of SLS crystallites; and (4) mapping of the mixture of peptides derived from cyanogen bromide (CNBr) cleavage. These same methods have also been applied to identifying and analyzing the different genetic types of collagen (types I–V, discussed below).

The amino acid compositions of the $\alpha 1(I)$ and $\alpha 2(I)$ chains of the type I molecule can function as standards to which the compositions of other collagen types can be compared. Partial amino acid analyses of each of these chains are given in Table I. They are listed as the number of residues of each amino acid present per 1000 total amino acids. For collagen, this is an especially con-

* The chains of type I collagen were originally termed $\alpha 1(I)$ and $\alpha 2$, as type I collagen was the only one known to contain two different α chains. Now, however, it seems that two different α chains may occur in other collagens and so $\alpha 2$ must be designated $\alpha 2(I)$, etc. The whole type I molecule then becomes $[\alpha 1(I)]_2\alpha 2(I)$. For recent nomenclature, see Bornstein and Sage (1980).

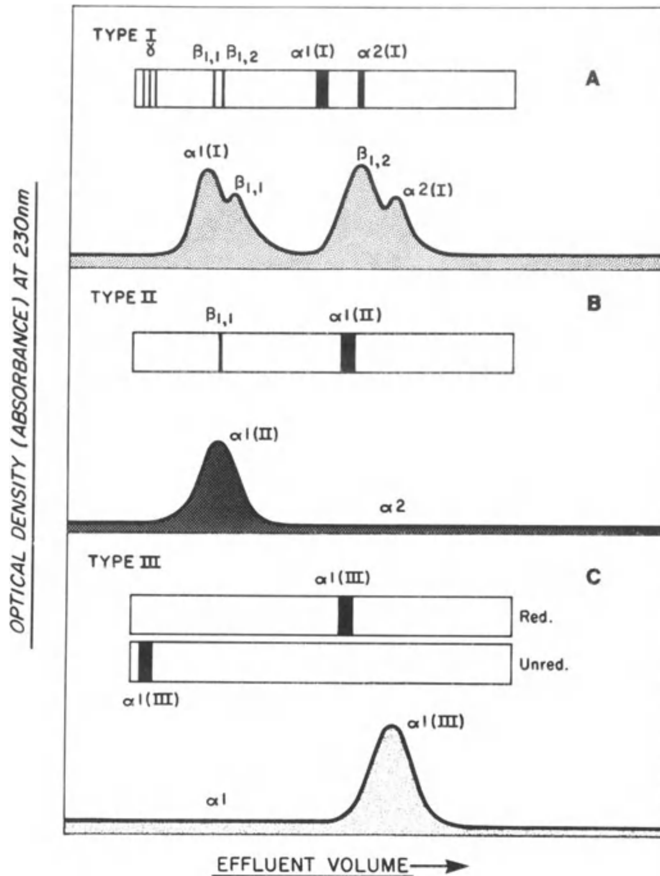

Figure 1-5. Diagram showing the positions of the composite chains of collagen types I (Fig. A), II (Fig. B), and III (Fig. C) when separated by either CM-cellulose column chromatography or SDS-polyacrylamide gel electrophoresis. The individual chains are labeled "α," the dimers "β," and the trimers "γ." Red, reduced; unred, unreduced.

venient way of expressing compositions, as each collagen chain contains just slightly more than 1000 amino acids. Thus, the number of amino acid residues per 1000 amino acids also closely approximates the number of residues of each amino acid per α chain. As can readily be seen in Table I, glycine accounts for one-third of the total amino acids of both the α1(I) and the α2(I) chains. This reflects the presence of the repeating Gly-X-Y triplets. In addition, both chains have about half of their total proline content as hydroxyproline, and some of their lysine content as hydroxylysine. Characteristically, as for most collagens, the two chains have no cysteine, and very small amounts of the aromatic amino acids tyrosine and tryptophan. They differ from one another, however, in their concentrations of a number of the other amino acids.

The differences between α1(I) and α2(I) chains are also reflected in the primary sequence of amino acids within the chains (see Piez, 1976). From

Table I. Partial Amino Acid Compositions of Collagen Chains

	$\alpha 1(I)$	$\alpha 2(I)$	$\alpha 1(II)$	$\alpha 1(III)$
4-Hydroxyproline	99	90	100	128
Proline	117	115	115	109
Threonine	19	19	26	15
Serine	29	29	26	41
Glutamic acid	78	67	87	73
Glycine	333	333	333	350
Alanine	125	102	105	90
Hydroxylysine	5	8	20	7
Lysine	30	24	13	22

amino acid sequencing studies it is known that within the two chains there are numerous positions at which differences occur. When the amino acid sequences of the $\alpha 1(I)$ and $\alpha 2(I)$ chains are compared with those of the α chains from other genetically different types of collagen, there are sites where the same amino acids are found. In some of these different types of α chains there are enough homologies that they probably arose by evolutionary gene duplication followed by subsequent divergence.

For the cell and developmental biologist, who usually deals with radiolabeled collagens or at most very small chemical amounts of material, CNBr peptide mapping is the major option available for obtaining the identities of α chains and molecules from primary-sequence information (see, for example, Mayne *et al.*, 1975; Linsenmayer *et al.*, 1977). In some cases, analysis of the banding pattern of SLS crystallites can also be used (see, for example, Linsenmayer, 1974). CNBr cleaves protein chains at sites where methionine residues occur (see Fig. 1-3), thus degrading the chains into a mixture of peptides. The CNBr peptides within the mixture then can be separated from one another by ion-exchange column chromatography or by SDS-PAGE (see Miller, 1976). The result is a diagnostic "map" or "fingerprint" of the peptides. Because collagen α chains contain approximately 10 methionine residues, CNBr cleavage results in a convenient number of peptides for separation. Neither the single-step CM-cellulose separation generally used for such analyses nor the SDS-PAGE separation completely separates and displays all the peptides.* However,

* For experiments involving radiolabeled collagens produced in cell or organ cultures, it is frequently desirable to include several milligrams of unlabeled "carrier" collagen of a known type to facilitate purification of the labeled material (see, for example, Mayne *et al.*, 1975; Linsenmayer *et al.*, 1973a,b). The carrier collagen also serves as an internal standard to which the labeled material can be compared during subsequent analysis. When CM-cellulose chromatography is used for peptide separation, an optical density profile of the unlabeled carrier peptides can be obtained by continuously monitoring the column effluent spectrophotometrically at wavelengths of 222–230 nm. The radiolabeled material is subsequently assayed by collecting suitably sized fractions which are then assayed by scintillation counting. Likewise, when SDS-PAGE is used, the carrier peptide bands can be visualized by staining the gel with Coomassie blue. Subsequently, the radiolabeled bands can be visualized by fluorography (Bonner and Laskey, 1974; Laskey and Mills, 1975). In this procedure the gel is impregnated with a fluor that is sensitive to β radiation, and then it is placed into contact with X-ray film for a determined exposure time. Once the film is developed, each radioactive band becomes visible as an exposed region on the film.

the patterns obtained are different enough to be diagnostic for all the known interstitial collagens and their component chains.

6.1.2. Type II Collagen

With the discovery that cartilage contains a collagen molecule quite different from type I, it became apparent that collagen constitutes a class of closely related, yet genetically distinct molecules. The new molecule, termed type II collagen (Miller and Matukas, 1969; Trelstad *et al.*, 1970), was thought to be found only in embryonic and adult cartilage. Indeed, the synthesis of type II collagen was considered to be the sine qua non for chondrocyte differentiation, and it still is one of the best molecular criteria we have for cartilage.

More recently, however, type II collagen has been found as a major collagen type in embryonic notochord (Linsenmayer *et al.*, 1973b; Miller and Matukas, 1969) and its adult derivative, the nucleus pulposis (Eyre and Muir, 1974); the vitreous body of the eyes of both embryos and adults (Smith *et al.*, 1976; Swann *et al.*, 1972); and the corneal stroma of early chick embryos (Linsenmayer *et al.*, 1977), including Descemet's membrane (von der Mark *et al.*, 1977; Hendrix *et al.*, 1981). Type II fibrils in cartilage are generally thin (10–20 nm), but the 25-nm-thick type II collagen fibrils in embryonic cornea cannot be distinguished from type I fibrils. Because type II collagen is synthesized by widely diverse cell types, it may be that this collagen does in fact constitute a class of genetically distinct molecules. From sequence data there is some evidence for genetic heterogeneity of the type II collagen extracted from cartilage itself (Butler *et al.*, 1977).

Type II collagen can be extracted from lathyritic animals [i.e., animals receiving the cross-linking inhibitor β-aminopropionitrile (Trelstad *et al.*, 1970)], but studies were greatly aided when it was discovered that native type II molecules could be extracted by limited pepsin digestion of cartilage after removal of proteoglycan (Miller, 1972). The type II molecule can then be cleanly separated from type I by a fractional salt precipitation technique (Trelstad *et al.*, 1970). When compared to type I collagen, the most striking biochemical feature of the type II molecule is that it is composed of a single type of α chain called $\alpha 1$(II). The molecule is composed of three of these $\alpha 1$(II) chains and is abbreviated $[\alpha 1(\mathrm{II})]_3$.

Thus, when a purified sample of type II collagen is thermally denatured and the chains are analyzed on CM-cellulose columns or by SDS-PAGE (Fig. 1-5B), only a single peak or band is seen, which is found in the same region as the $\alpha 1$(I) chain. Although the $\alpha 1$ chains from type I and II collagens have similar sizes and net charge characteristics, by other criteria it can be concluded that these two chains undoubtedly represent separate gene products. The amino acid compositions of the $\alpha 1$(I) and $\alpha 1$(II) chains are significantly different (Table I), especially in relative content of threonine, alanine, hydroxylysine, and lysine. Obviously, then, the amino acid sequence of $\alpha 1$(II) chains also differs from that of $\alpha 1$(I) chains, as do their CNBr peptide patterns when separated either on columns or on gels. Also, the banding patterns of SLS crystallites show differences between type II and type I molecules, even though

the striated fibrils formed by the two molecules have the same staining pattern. Most of the SLS bands are similar, but there are reproducible diagnostic differences in band density (Trelstad *et al.*, 1970). The advantage of SLS-crystallite analysis is that it can be performed with small amounts of material and circumvents the need for using biochemical approaches, but it is not quantitative.

The posttranslational modifications of lysines in type II collagen are more complete than they are in type I. For example, in the type II chains synthesized by cartilage, most of the lysines that are potentially hydroxylatable (i.e., that occur in the Y position of Gly-X-Y triplets) are converted to hydroxylysine. The large amount of hydroxylysine in type II collagen also reflects an increased amount of glycosylation. In type II collagen, the content of galactosylhydroxylysine and glucosylgalactosylhydroxylysine is about 10 times higher than in type I collagen. The suggestion has been made that glycosylation affects fibrillogenesis and that there is an inverse relationship between the amount of glycosylation of collagen molecules and the diameter of the fibrils formed by these molecules. If this is true, collagen molecules with more glycosylation should form narrower fibrils. Consistent with this hypothesis is the observation that the type II collagen within cartilage and vitreous is present in the form of very fine fibrils that usually lack a clear-cut banding pattern. However, as mentioned above, type II collagen fibrils in embryonic cornea are striated. Moreover, when type II collagen molecules are precipitated from solutions of collagen, they can make fibrils with various diameters that exhibit a native banding pattern (Bruns *et al.*, 1973). Thus, this hypothesis for the role of collagen glycosylation still remains to be critically tested.

6.1.3. Type III Collagen

Type III collagen has a tissue distribution similar to type I, except for bone, tendon, and cornea, in which it is either absent or present only in very small amounts. Loose connective tissue, blood vessel walls, dermis, and placenta are rich sources of type III collagen (Miller *et al.*, 1971; Chung and Miller, 1974; Epstein, 1974). Immunocytochemical staining experiments with specific antibodies suggest that type III is the principal component of the small (50 nm wide), argyrophilic collagen fibers that characterize reticular connective tissues.

Type III collagen is most easily obtained by limited pepsin digestion of reticular connective tissue, followed by salt fractionation to separate it from other collagens, chiefly type I. Pure type III collagen when analyzed by SDS-PAGE is seen to migrate chiefly as trimeric, γ-chain-sized molecules in the unreduced state, and as a single band of α-chain-sized molecules when analyzed under reducing conditions. This same material elutes from CM-cellulose columns as a single peak near the region where the $\alpha 2$ chain of type I collagen elutes (Fig. 1-5C). Thus, type III collagen is composed of a single type of chain termed $\alpha 1(\mathrm{III})$. The native molecule contains three of these chains and is abbreviated $[\alpha 1(\mathrm{III})]_3$. The reason the denatured but unreduced type III molecule migrates as a trimeric component is that the component α chains are connected by intramolecular disulfide cross-links that occur between the chains in the triple-helical portion of the molecule.

The amino acid composition of type III collagen (Table I) shows several unique features including high levels of 4-hydroxyproline, more than 333 glycines, and the presence of half-cystines, which participate in the intramolecular disulfide cross-links. The CNBr-peptide and SLS-banding patterns also show differences between this collagen and the other genetic types. The presence of more than 333 glycine residues in the α chain means that some of the Gly-X-Y triplets have an additional glycine at the X or Y position. It has been suggested that these extra glycine residues may cause localized helix instability, resulting in increased susceptibility to proteolytic cleavage and more rapid turnover of matrices containing this collagen type. No definitive data, however, exist on the relative turnover rates of different types of collagen *in vivo*.

6.1.4. Type I Trimer

The last collagen type related to those just described is termed the type I trimer (Mayne *et al.*, 1975, 1976). This molecule, which appears to be composed of three α 1(I) or α 1(I)-like chains, was initially found in cultures of chondrocytes that had been allowed to grow to senescence or that had been chemically forced to dedifferentiate by BrdUrd treatment. Subsequently, type I trimer molecules have been described in long-term cultures of a teratocarcinoma cell line and a mouse blastocyst cell line. Because all of these sources are potentially abnormal cells that have been in culture for a large number of passages, the possibility was raised that the abnormal cells somehow misassembled molecules by putting three α 1(I) chains in the same molecule rather than combining them with an α 2 chain. Recently, however, this molecule has been described *in vivo* in pathological conditions such as wound healing and inflamed gingiva, and even in normal tissues in small amounts. The criteria that have been applied to analyzing the composition of this molecule, α-chain separations and CNBr peptide analysis, have clearly demonstrated that the molecule is composed of three molecules that resemble α 1(I) chains. Whether this molecule represents a misassembly of authentic α 1(I) chains or, alternatively, is the normal product of a completely different structural gene, will require the application of methods with higher resolution, such as amino acid sequencing or possibly gene mapping by somatic cell genetics.

6.2. Collagen Types IV and V

Results of the studies that have been performed on the interstitial collagens during the past few years have usually been in agreement about these molecules. On the other hand, it has been difficult to study collagen molecules of basement membrane origin. Much of the difficulty is due to the nature of basement membranes themselves. For one thing, the basement membrane, or basal lamina, defines a morphological entity, visible only at the level of the electron microscope. Thus, ultrastructural monitoring of extraction procedures is necessary to ascertain the purity of the isolated membranes. Except for the

lens capsule and kidney glomerulus, it is difficult to find a pure source of basement membrane without contaminating reticular fibrils. Second, it is not only possible but probable that basement membranes in different tissues may be quite different from one another. Lastly, the collagen molecules of proposed basement membrane origin are generally quite difficult to solubilize, requiring limited proteolytic digestion with pepsin plus reduction and alkylation. Thus, the molecules that are extracted from tissues undoubtedly have been modified to a great degree by the extraction procedures, so it is difficult to draw conclusions as to how these molecules are arranged within the basement membrane itself.

The lens capsule and kidney glomerulus were the two sources initially employed for studies on basement membrane collagens (see Kefalides, 1973, 1975). Limited pepsin digestion resulted in a single type of collagen molecule, composed of three identical α chains with molecular weights similar to the α chains of other collagens. This is the molecule that has been classically termed type IV collagen. The amino acid composition of this molecule revealed clear-cut differences from the interstitial collagens. Characteristically, the molecule has elevated amounts of 3-hydroxyproline and cysteine, is less than one-third glycine (about 320 residues per 1000), and has a large amount of glycosylation. More recently, studies using these and other tissues have revealed several additional collagenous components, some with molecular weights ranging from 50,000 to 140,000. It also seems that some type IV molecules, at least, are composed of two different α chains (Daniels and Chu, 1975; Dixit, 1978; Glanville et *al.*, 1979; Sage et *al.*, 1979; Crouch et *al.*, 1980).

When these same tissues are not exposed to proteolytic enzymes, but instead are extracted by detergent solubilization plus reduction and alkylation, an even greater heterogeneity is noted. This type of extraction produces numerous peptides ranging in molecular weight from 30,000 to 220,000, and some even larger (Hudson and Spiro, 1972; Sato and Spiro, 1976). Many of the peptides apparently contain both collagenous and noncollagenous regions. Thus, it has been suggested that at least some of the basement membrane collagenous components represent procollagen or "procollagenlike" molecules with alternating triple-helical and non-triple-helical regions, some possibly containing additional glycopeptides attached along their lengths. Biosynthetic studies have, in some cases, supported the similarities between nonproteolysis-extracted basement membrane collagens and procollagenlike molecules (Minor et *al.*, 1976; Timpl et *al.*, 1978).

Recently, much work has been done on characterization of noninterstitial collagen molecules using the highly vascular human placenta as starting material (see, for example, Burgeson et *al.*, 1976; Chung et *al.*, 1976; Glanville et *al.*, 1979; Sage et *al.*, 1979). Again, pepsin extraction yields numerous collagenous components including large amounts of type I and III collagens. These two interstitial collagens can be separated from the remaining basement-membrane-like collagen molecules by fractional salt separation. Then, the latter molecules can be separated from one another, again by fractional salt separation followed by various chromatographic procedures. This results in a number of

different collagenous components—the best characterized being the αA and αB chains, referred to together as type V collagen (Burgeson *et al.*, 1976; Chung *et al.*, 1976). These chains migrate on SDS gels with apparent molecular weights approximately that of an α1(I) chain. The αA chain migrates slightly slower than an α1(I) chain and the αB chain migrates somewhat slower yet. Also, amino acid analysis shows them to be distinctly different from other known α chains. A third chain, the αC chain, has also been reported to occur in some tissues (Sage and Bornstein, 1979; Brown *et al.*, 1978). The αA, αB, and αC chains of type V are also termed α1(V), α2(V), and α3(V), respectively (see Bornstein and Sage, 1980, pp. 963–964). Originally, it was observed that αA and αB chains occurred in a ratio of one αA to two αB chains, suggesting that they occur in a triple-helical molecule with the conformation $\alpha A(\alpha B)_2$; however, they may also occur in separate molecules with other conformations such as $(\alpha B)_3$. Preliminary immunohistochemical studies suggest that type V collagen may not be confined to basement membranes, but further work is needed in this area.

Other heterogeneous components, too numerous to describe in the present review, have also been isolated from sources of basement membranes (Chapter 11). Clearly, basement membranes are complex structures and the analysis of their collagenous components is one of the most difficult and intriguing problems in extracellular matrix biology.

7. Immunology of Collagen

At one time collagen was thought to be nonantigenic, and although it is still considered to be a rather poor immunogen, we now know that animals can produce antibodies to a number of different sites within both collagen and procollagen molecules (for recent reviews see Timpl, 1976; Linsenmayer, 1981). Indeed, antibodies can now be produced that are specific for the different genetic types of collagens and some procollagens. When these type-specific anti-collagen antibodies are properly purified, they can be used as tools for the immunocytochemical localization of the different genetic types of collagen in tissue sections and in cell cultures. It is of considerable interest that autoimmune responses to collagens have been implicated in some pathological conditions such as certain forms of rheumatoid arthritis; the reader is referred to Gay and Miller (1979) for discussion of this aspect of collagen immunology.

Collagen molecules have several different antigenic regions or domains, and procollagen molecules have two additional ones (Fig. 1-6) (Beil *et al.*, 1973; Timpl *et al.*, 1973; Sherr *et al.*, 1973). In general, the procollagen form is most antigenic, due mainly to the large propeptide extensions at the NH_2- and COOH-terminal ends (Timpl *et al.*, 1973; Park *et al.*, 1975; Murphy *et al.*, 1975; Olsen *et al.*, 1977). Thus, when whole procollagen is used as an immunogen, most of the antibodies are directed against these large, globular extensions and not against the collagenous body of the molecule. Antibodies specific for procollagens can also be elicited by immunization with the isolated propeptide

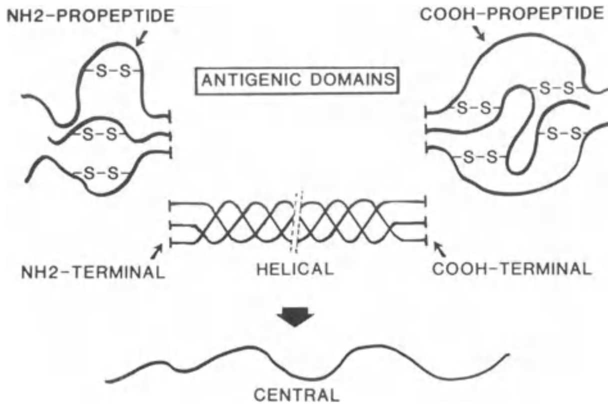

Figure 1-6. Antigenic domains of procollagens and collagens. The individual domains specific for procollagen are the amino (NH_2-propeptide) and carboxy (COOH--propeptide) extensions found only in the procollagen form. Native collagen molecules have determinants in the helical region, as well as in both the amino (NH_2-terminal) and the carboxy (COOH-terminal) nonhelical extensions. Central determinants are found in the chains after denaturation of the molecules. (After Timpl, 1976.)

extensions themselves. Both the NH_2- and the COOH-terminal extensions are independently antigenic. Even when whole procollagen molecules are used for immunization, antisera produced against procollagens are only weakly reactive, if at all, against processed collagen molecules.

When native, completely processed collagen molecules are used as immunogens, there are still two classes of antibodies that can be elicited: those directed against sites along the triple-helical body of the molecule and those directed against the small NH_2- and COOH-terminal, non-triple-helical extension peptides (Beil *et al.*, 1973). In the extension peptides, the antigenic sites are determined largely by the primary amino acid sequences, which are different for each chain. Thus, in type I collagen, antibodies can be directed against sites in either the $\alpha 1(I)$ or the $\alpha 2(I)$ chain at either the NH_2- or the COOH-terminal extensions. These antibodies react equally well with native or denatured molecules so they fall into the general category known as sequential determinants. Sequential determinants depend only on the primary sequence of amino acids within a chain, and do not depend on the conformational state of the chains.

On the other hand, antibodies produced against sites along the triple-helical body of native molecules do not react with thermally denatured molecules or isolated α chains. These antibodies are directed against the class known as helical determinants, and tend to be conformationally dependent (i.e., they require the native conformation for generation of the antigenic site). When such conformation-dependent antibodies are produced against type I collagen, they react equally well with native molecules, and with renatured molecules if the renatured ones contain the correct chain composition, $[\alpha 1(I)]_2\alpha 2(I)$. They react only poorly, if at all, with artificially renatured molecules composed solely of $\alpha 1(I)$ chains (i.e., $[\alpha 1(I)]_3$ molecules) or $\alpha 2(I)$ chains (i.e., $[\alpha 2(I)]_3$ molecules) (Hahn and Timpl, 1973). Clearly then, for generation of the antigenic site, the anti-collagen antibodies directed against helical determinants require both the proper amino acid sequences of the different

chains, plus the triple-helical conformation to bring the required amino acids from each chain into proper juxtaposition. In general, these antibodies have a high degree of specificity for the genetic type of collagen used as an immunogen; most of the antibodies produced to the triple-helical portion of the molecule do not cross-react with the other genetic types of collagens (Hahn *et al.*, 1974, 1975). They may, however, cross-react with type I molecules from certain other species. It should be realized that *a priori*, no absolute statement can be made about the degree of molecular and species cross-reactivity that will be present for any individual antiserum.

One final class of antigenic determinant, termed the central antigenic determinant (Fig. 1-6), is exposed when the triple-helical portion of the molecule is uncoiled. Antibodies to central determinants then are produced when animals are immunized with thermally denatured collagens, isolated α chains, or segments of α chains such as individual CNBr peptides. The antibodies to these are produced against sequential determinants and usually do not bind to native molecules. Presumably, then, they recognize some portion of the primary amino acid sequence within a single α chain that is exposed upon denaturation and may be either altered or masked when folded into the triple-helical conformation. These antibodies tend to have a good deal of cross-reactivity both with chains of the same collagen type from different species of animals and with chains of different collagen types from the same species. This is probably due to that fact that many different collagens have short regions within their primary sequences that are common to one another.

For the investigator wanting to utilize immunological techniques, the most formidable task is the production of a useful titer of specific antibody to the desired antigen, with no significant cross-reactivity to other antigens. In the case of collagens, this becomes a formidable task for two reasons. First is the problem of noncollagenous contaminants—as collagen is such a poor immunogen, eliciting a useful antibody response requires injecting relatively large amounts of the antigen. So, even when the highest purity collagen preparation is used for immunization, there is the possibility of producing unwanted antibodies to quantitatively minor, yet highly antigenic contaminants that might be present. Second is the problem of producing antibodies that cross-react with several of the different genetic types of collagen. Because the antibodies to a given type of collagen are directed against a heterogeneous group of determinants along the molecule, many of those produced will be against sites that are found only in the type of collagen used for immunization and thus will have the desired specificity. Others, however, may be against sites common to several different types of collagens, and these will have undesirable cross-reactivity.

Two general methods are now available for producing antibodies with the desired specificity: (1) the conventional method, which involves raising antisera in animals and then using affinity chromatography on collagen immunoabsorbants to remove the unwanted antibodies, and (2) the newly devised hybridoma technology for the *in vitro* production of monoclonal antibodies. Both methods have desirable features plus some potential drawbacks.

7.1. Conventional Antibodies

Standard procedures have been devised for animal immunization with collagen and for purifying the resulting antibodies so that they will have enough specificity to be used with a reasonable degree of confidence in immunological procedures (Wick et *al*., 1975; Timpl, 1976). The animal species in which the antibodies are produced will, to some degree, determine the type of the antibodies obtained. For example, it is known that when native type I collagen molecules are injected into rabbits, the resulting antibodies are predominantly directed against the extension peptides (i.e., terminal sites). When the same collagen, however, is injected into guinea pigs, rats, or mice, the antibodies produced are predominantly against sites in the triple-helical region (i.e., they are helical determinants). The usual injection regimen involves several administrations of 5- to 10-mg quantities of collagen given at 2-week intervals, the first with complete Freund's adjuvant and later ones with incomplete adjuvant. Subsequent affinity chromatographic purification* of the antibodies on collagen immunoabsorbants is performed using the IgG fraction isolated from antisera by ammonium sulfate precipitation and DEAE-cellulose chromatography (Fig. 1-7). Each purification step should be monitored by a procedure to determine the purity of the resulting antibodies. Useful assays include passive hemagglutination, hemagglutination inhibition, and various radioimmune assays. In this way, it can be determined when the cross-reactive antibodies have been removed, at least within the limits of the assay procedure employed. Once the desired degree of purity is obtained, the antibodies can be used in procedures such as immunocytochemistry.

7.2. Monoclonal Antibodies

Recently, an alternative to the conventional method of antibody production has become available in the form of monoclonal antibodies as produced by

* Affinity chromatography is usually performed using a solid matrix such as CNBr-activated Sepharose 4B to which specific genetic types of collagen have been covalently bound. A schematic representation of the purification of antibodies specific for type I collagen is shown in Fig. 1-7. The first absorptions are performed on affinity columns of the heterologous types of collagen. This step is to remove those antibodies that cross-react with the other known collagens. For example, to obtain a preparation of non-cross-reactive type I collagen antibodies, the IgG should be passed sequentially over affinity columns of the other known, heterologous collagen types, such as types II and III. In this purification step, those antibodies that cross-react with the other types of collagen will bind to the column and the desired specific antibodies will pass directly through in the effluent. The effluent antibodies then are passed over a column of the homologous-type collagen, in this case type I. Here, the desired, specific antibodies will bind to the column and the noncollagen antibodies will pass through unretarded. Subsequently, the bound, type I-specific antibodies can be released from the column and obtained in active form by elution with a low-pH buffer or a chaotropic agent like 3 M KSCN. This direct purification step reduces the possibility of contaminant antibodies that may have been produced if a highly antigenic, minor contaminant in the immunogen collagen had elicited a major antibody response.

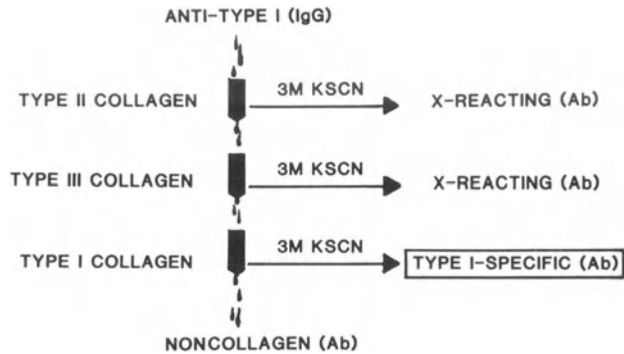

Figure 1-7. Method for purifying type I-specific collagen antibodies. The IgG is isolated from an animal immunized with type I collagen, and is absorbed on columns of type II and type III collagens, which remove the cross-reacting, anti-collagen antibodies. Lastly, the antiserum is directly absorbed on a column of type I collagen. The noncollagen antibodies wash through the column, while the type I-specific antibodies bind and can be subsequently eluted with 3 M KSCN. Ab, antibody; 3M KSCN, 3 molar potassium thiocyanate. (After Timpl *et al.*, 1977.)

mouse lymphocyte–myeloma hybrids, or hybridomas. A hybridoma represents the fusion product between a mouse lymphocyte and a cell of an established mouse myeloma cell line (Kohler and Milstein, 1976; for review see Melchers *et al.*, 1978; Linsenmayer and Hendrix, 1981). The lymphocyte parent supplies the information to produce antibody with a desired specificity, and the myeloma parent* conveys immortality on the antibody-synthesizing cell. The hybridoma producing the specific desired antibody can be individually selected* and propagated by cloning (Fig. 1-8).

Monoclonal antibodies to chick collagens type I and II have been produced (Linsenmayer *et al.*, 1979; Linsenmayer and Hendrix, 1980, 1981). It is still too early to make definitive statements about all the characteristics of anti-collagen

* The mouse myeloma parent that has been generally used produces and secretes its own IgG antibody molecule of unknown specificity, so the hybridomas make a mixture of functional and nonfunctional antibodies. Recently, more preferable parental mouse myeloma cell lines have been selected that do not secrete, and in some cases do not synthesize their own antibodies until a productive fusion occurs with a lymphocyte. To allow for subsequent selection of the hybrid cells, once formed, all these myeloma lines are deficient in the enzyme hypoxanthine phosphoribosyl transferase (HPRT), which means that they are unable to utilize the salvage pathway of purine synthesis if the *de novo* synthetic pathway is blocked by the folic acid antagonist aminopterine. Thus, the parent myeloma cells are killed in a selective medium containing hypoxanthine, aminopterine, and thymidine (HAT), whereas normal cells and hybrids containing a lymphocyte genome will survive due to their ability to synthesize a functional HPRT enzyme and thus effectively utilize the salvage pathway.

In a hybridoma, the information for producing the specific antibody is provided by the mouse lymphocytes. The lymphocytes are obtained from the spleen of mice that have undergone a regimen of collagen injections in order to build up their anti-collagen lymphocyte precursor population. Lymphocytes themselves do not divide in culture so the only ones that will propagate are those that have undergone a successful fusion with a myeloma cell. Details of cloning are given in Fig. 1-8. Because the hybridoma cells secrete their proteins directly into their culture environment, useful quantities of antibody can be harvested directly from the spent culture medium in which the hybridomas have been growing. However, antibody titers more than three orders of magnitude greater can be obtained from the ascites fluid of mice that have received intraperitoneal injections of hybridoma cells 2 weeks earlier.

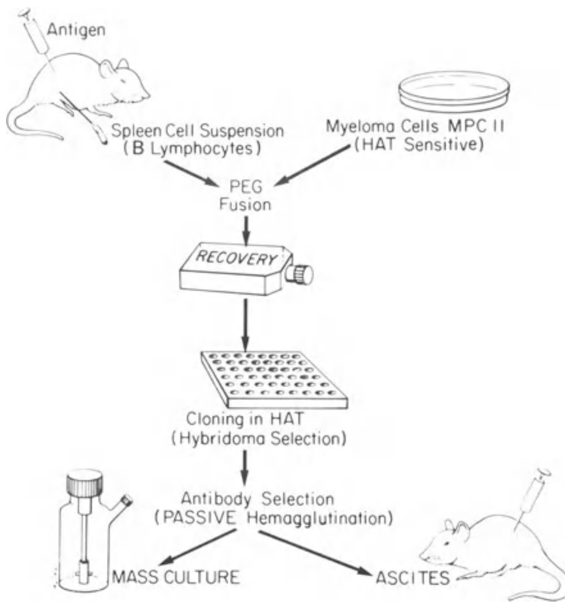

Figure 1-8. Diagram of a method that has been used by the author for producing monoclonal antibodies to chick collagen types I and II. HAT, hypoxanthine, aminopterine, thymidine; PEG, polyethylene glycol. Myeloma cells are fused in medium containing PEG and then, to select for hybridomas, the cells are divided into culture wells containing HAT medium. Subsequently, to identify the hybrid cells producing anti-collagen antibodies, the medium from wells is screened by passive hemagglutination and the specific antibody-producing cells are isolated by cloning. Cloning the desired hybridomas eliminates the possibility of the production of cross-reactive antibodies, plus it greatly decreases the amount of specific antibody obtained, as all of the progeny are producing a single specificity of antibody.

monoclonal antibodies, as compared to antibodies produced by conventional methods. However, each method, and the antibodies produced by each method, may have certain advantages and disadvantages. An obvious advantage of the monoclonal antibodies is the degree of specificity obtainable when the entire antibody population is homogeneous. In addition, the specificity of the antibody can be precisely characterized. This degree of binding specificity and antibody characterization can never be reached with a conventional, heterogeneous antibody population, no matter how exhaustive a purification procedure is used. In addition, once a monoclonal antibody is fully characterized and found to be one with desired characteristics, it is available indefinitely with no variation. On the other hand, the conventional antibodies are initially quicker and easier to produce, and due to their heterogeneous avidities to a variety of sites along collagen molecules, they may be more efficient, for example, in reactions requiring precipitations of collagen molecules.

Whether conventionally produced or monoclonal, the most frequent use made of the anti-collagen antibodies is for the localization of the different genetic types of collagens within tissues and organs, or cells in culture. Studies have included adult tissues, developing embryos, and pathological tissues. Fluorescence microscopy (Fig. 1-9) is the usual method employed to visualize antibody binding, but the peroxidase–anti-peroxidase method has been used for light and electron microscopy, and ferritin-conjugated antibody techniques have been used for visualization with the electron microscope (Sternberger, 1979). The sections shown in Fig. 1-9 were reacted first with a specific monoclonal antibody (to type I or type II collagen) and then with a rhodamine-tagged

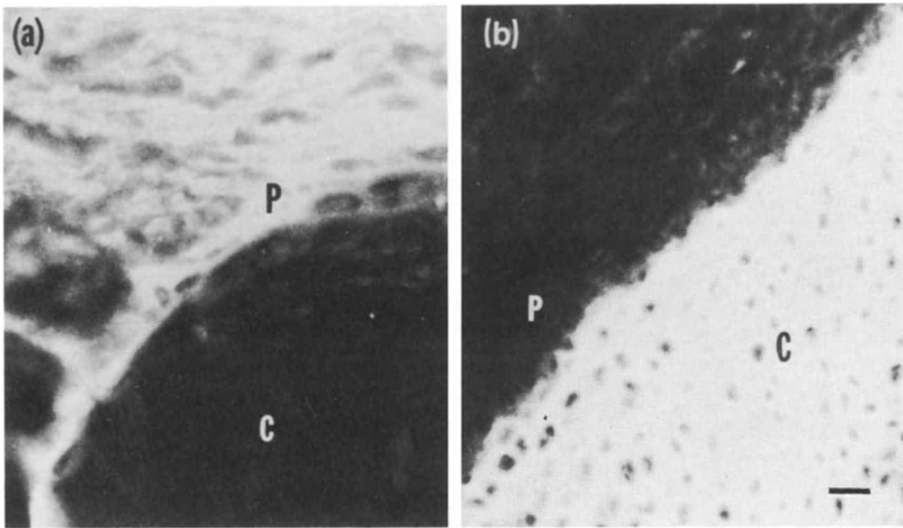

Figure 1-9. Fluorescence micrographs of embryonic chick tibias stained with monoclonal antibody ascites fluid to (a) type I collagen and (b) type II collagen, using a double-antibody sandwich technique. The second antibody is rhodamine-conjugated rabbit IgG directed against mouse IgG. It attaches to the mouse anti-collagen and renders its location visible by fluorescence. Anti-type I stains the perichondrium (P), and anti-type II, the cartilage (C). In addition, some anti-type I staining extends down into the cartilage for one or two cell layers. Because the embryonic cartilage is undergoing active appositional growth, in which perichondrial cells are continuously becoming chondrocytes, this type I staining within the cartilage matrix probably represents a zone where both type I and type II collagen occur, type I representing residual collagen of perichondrial origin and type II being synthesized by the cells as they become chondrocytes. Bar = 10 μm. (From Linsenmayer and Hendrix, 1980.)

antibody directed against the first antibody. The sections contain embryonic cartilage (C), which is known from biochemical studies to contain type II collagen but no type I, whereas the perichondrium and surrounding connective tissue contain type I (plus type III and possibly other types not yet identified) but no type II. When the primary monoclonal antibody is directed against type II collagen, only the cartilage is stained. Conversely, when the primary monoclonal antibody is directed against type I collagen, the fluorescence is localized in the perichondrium and the surrounding connective tissue (Fig. 1-9).

It is also possible to detect the distribution of two different collagen types within the same tissue section by using a double immunofluorescence staining procedure. In such a procedure, the primary anti-collagen antibodies to the individual collagen types are produced in two different species of animal, for example, rabbit and goat. Then, two different anti-IgG, secondary antibodies are used, each specific for one of the species of primary antibody used and each coupled to a different fluorescent dye (usually rhodamine and fluorescein). Sections of embryonic, cartilaginous tissues treated in this way show staining for both collagen types I and II in the perichondrium–cartilage interface zone, as was just described using the individual antibodies on different sections

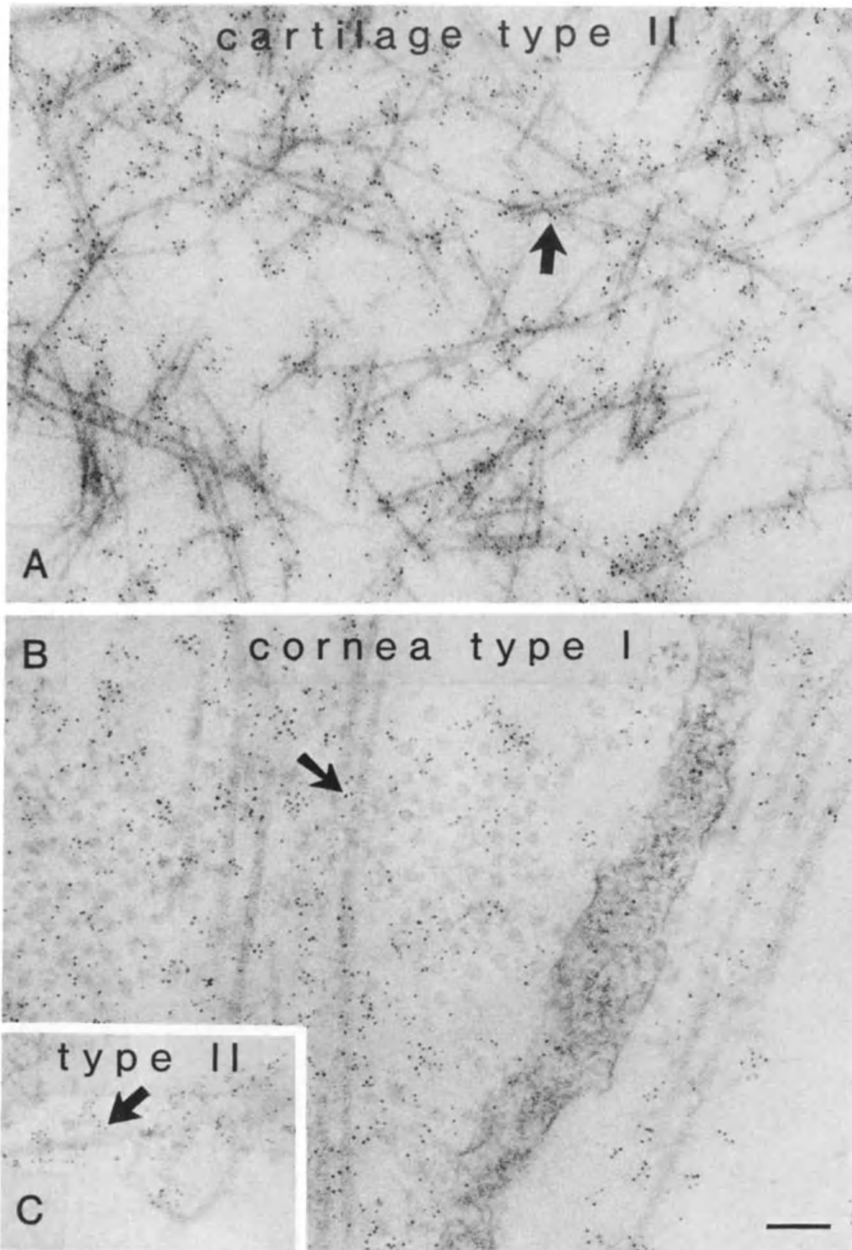

Figure 1-10. Electron micrographs of (A) embryonic chick tibia stained with rabbit antibody to type II collagen; (B) embryonic chick cornea stained with rabbit antibody to type I collagen; and (C) embryonic chick cornea stained with rabbit antibody to type II collagen. Ferritin-conjugated goat IgG directed against rabbit IgG was used as the second antibody. The arrows point to ferritin particles, showing the location of type I collagen in striated fibrils (B), and type II in striated fibrils of the cornea (B) and faintly striated fibrils of cartilage (A). Bar = 100 nm. (From Hendrix et *al.*, 1981.)

(H. von der Mark *et al.*, 1976; K. von der Mark *et al.*, 1976). Double immunofluorescence with anti-collagen antibodies has been used to demonstrate that an individual fibroblast in tissue culture can simultaneously produce collagen types I and III (Gay *et al.*, 1976). Thus, a single cell seems capable of synthesizing at least two different collagen types. Other studies have used similar procedures to determine the tissue localizations of the three interstitial collagens, plus type IV, and molecules containing A and B chains (for review, see Linsenmayer, 1981).

Applications to the electron microscopic level usually employ ferritin-labeled antibodies (Fig. 1-10), but other labels are possible (hemocyanin, silicon spheres, colloidal iron, etc.). The antibodies may be applied to thin sections of frozen blocks (Roll *et al.*, 1980) or to vibratome sections of tissue that are subsequently embedded in plastic for thin sectioning (Hendrix *et al.*, 1981). Applied to cartilage, such methods permit the nonstriated or faintly striated 10- to 20-nm-diameter fibrils of the matrix to be clearly identified as collagenous (Fig. 1-10A). Applied to embryonic cornea (Fig. 1-10B,C), the ferritin technique reveals that in regions where types I and II collagen coexist (von der Mark *et al.*, 1977), striated collagen fibrils contain both types (see Hay *et al.*, 1979, for review). The possibility that a single fibril contains both collagens could be investigated in the future by double labeling. If so, this would mean that minor differences in charge pattern (Trelstad *et al.*, 1970) do not prevent quarter-staggering of the molecules into fibrils. It has been possible to permeabilize fibroblasts by partial homogenization and to prove that Golgi vacuoles contain procollagen using appropriate antibodies (Chapter 6). Clearly, the immunohistochemical applications of anti-collagens are potentially some of the most interesting to cell biologists.

8. Concluding Remarks

Work over the last decade has provided much information about the general biochemical characteristics and structure of collagen molecules, the degree of diversity among the various genetic types of collagens, both in primary structure and posttranslational modifications, and in the tissue distributions and localizations of these different types of collagens. Many questions, however, remain unanswered. Foremost is the question of why different genetic types of collagen exist. If collagen is simply a structural molecule giving support to tissues and organs, we should need only one type. Structures such as bone, tendon, and adult cornea seem to do well with little else than type I collagen. One might hypothesize that the different genetic types of collagens have informational roles that influence developmental processes, but if this is so, why is there no clear-cut, tissue-specific distribution of different genetic types? Type II collagen was originally thought to be specific for cartilage, but now has been discovered in two other widely divergent tissues, the vitreous body of the eye and the avian primary corneal stroma. Type IV collagens appear to have wide distributions throughout many different types of basement mem-

branes. On the other hand, it is interesting to speculate that there might indeed be tissue-specific collagens, and that the heterogeneity of collagen types is greater than has already been observed. It is possible that the type I of bone, tendon, and cornea and the type II of cartilage and vitreous in reality all represent separate gene products with differences that we have not yet detected.

Moreover, we do not know the function of many of the posttranslational modifications, such as the hydroxylations and glycosylations of lysine. These vary widely between the different genetic types of collagen, but can also vary within the same genetic type of collagen whenever it is isolated from different sources. Critical experiments to answer these questions have not even been posed, let alone performed.

Thus, there are many aspects of the function, synthesis, and assembly of the collagen molecule that we do not yet understand. How does one cell synthesize and assemble more than one type of collagen so that each molecule contains the proper chains? Indeed, how is a single type I collagen molecule assembled so that it always contains two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain? Is the molecule synthesized and assembled by a cell-mediated mechanism or is the process one of self-assembly, with the information for proper association being contained within the collagen chains themselves? What is the function(s) of the procollagen extension peptides? Do they contain the information of chain assembly? Do they act as a transport form of collagen? Do they function in fibrillogenesis by preventing molecular association until after their removal? Clearly, the list of unanswered questions is already large and hopefully will become even larger and more interesting as more cell biologists enter the field.

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Chapter 2

Proteoglycans

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1. Introduction

Proteoglycans are complex macromolecules that contain a core protein to which at least one glycosaminoglycan (GAG) chain is covalently bound. This simple definition encompasses a wide range of structures involving different core proteins, different classes of GAG, and different numbers and lengths of GAG chains. This enormous versatility at the molecular level allows proteoglycans to serve many diverse structural and organizational functions in tissues. These macromolecules are found in almost all mammalian tissues and are especially prominent in connective tissues. The purpose of this chapter is to define the chemical structures of some proteoglycans that have been investigated and the possible roles that they serve in tissue organization and function. For reviews and background information, see Muir and Hardingham (1975), Comper and Laurent (1978), Rodén (1980), Hascall (1977, 1981), Lindahl and Höök (1978), and Muir (1980).

2. Cartilage Proteoglycans

2.1. Structure

At present, proteoglycans isolated from cartilage have been the most widely studied, primarily because cartilage contains large amounts of proteoglycan and because alterations in the proteoglycans appear to occur in arthritic diseases. The structure of these proteoglycans is perhaps the most complicated found in this family of macromolecules. Figure 2-1 depicts part of an aggregate structure that contains four monomer proteoglycan molecules associated with hyaluronic acid (HA). At the right is a schematic model illustrating what is known about the general chemical structure of these macromolecules. The backbone core protein has a molecular weight of around 250,000 and extends approximately 300 nm in length. This is a large molecular weight even

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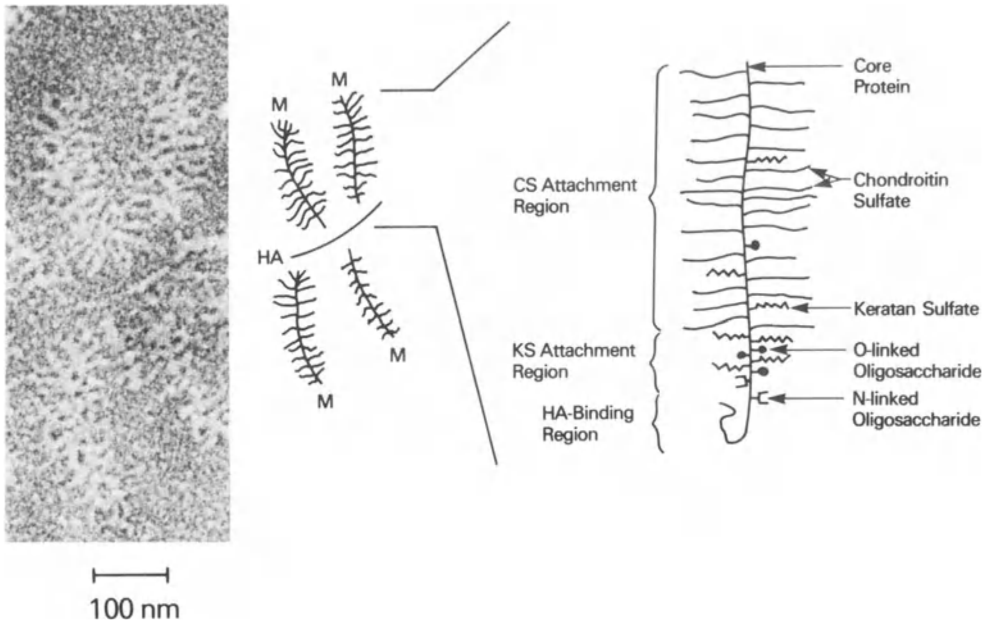


Figure 2-1. Cartilage proteoglycan, monomer structure. The electron micrograph shows a portion of an aggregate purified from a transplantable rat chondrosarcoma (Faltz et *al.*, 1979a). Four distinct monomers (M) with extended GAG chains are associated with a central strand of HA as is shown schematically in the line drawing in the middle. The chemical structure of a typical cartilage proteoglycan monomer is shown schematically at the right.

when compared to other large matrix proteins such as a collagen α chain (MW $\cong$ 100,000) or fibronectin (MW $\cong$ 220,000). Nevertheless, the core protein represents only 5–10% of the mass of an average proteoglycan molecule. The remainder is composed of a variety of complex carbohydrate structures that must be assembled on the core protein during posttranslational processing steps. Most of the mass of the proteoglycan consists of GAG chains, usually of two types, chondroitin sulfate and keratan sulfate (Fig. 2-2), and it is the extension of these chains away from the core protein that is responsible for the bottle-brush appearance of the macromolecules in the micrograph of Fig. 2-1.

The GAG consist of linear polymers of repeating disaccharides that contain a hexosamine and either a carboxylate or a sulfate ester, or both, which provide the chains with linear arrays of anionic groups (Fig. 2-2). It should be emphasized that with the possible exception of HA, it appears that GAG chains per se do not exist in tissues; rather, they are bound to core proteins as components of proteoglycans. An average of about 80 chondroitin sulfate chains, each with an average molecular weight of about 20,000, are present in a typical cartilage proteoglycan. Each chain is attached to the core protein through a glycosidic bond between the hydroxyl group of a serine and a xylose residue at the reducing end of the chain (Rodén and Smith, 1966; Fig. 2-2, this chapter). Two galactose residues and one glucuronic acid residue complete the specialized

linkage region upon which the repeating disaccharide units are subsequently assembled. This linkage structure is the same as that which provides the attachment of heparin and heparan sulfate chains in proteoglycans that contain these GAG. The biosynthesis of these structures is discussed in Chapter 5. The GAG chains extend out from the core protein because their polysaccharide backbones are relatively stiff and contain arrays of negatively charged groups. Generally,

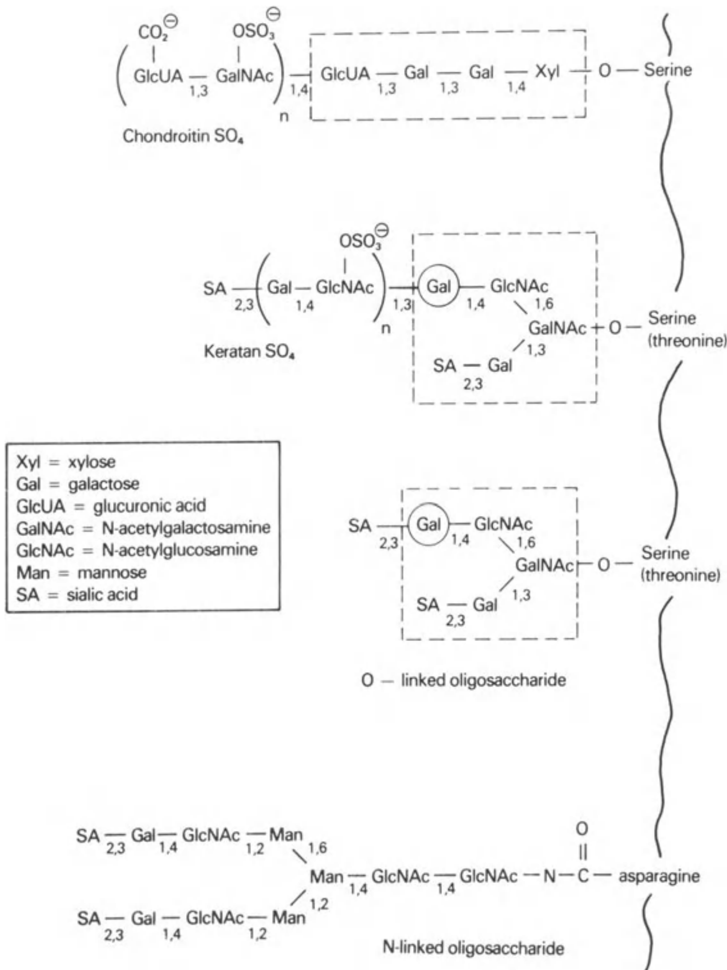


Figure 2-2. Structures of the complex carbohydrates attached to cartilage proteoglycans. The repeating disaccharide structures of chondroitin sulfate and keratan sulfate are indicated along with the specialized attachment regions by which the chains are covalently bound to the core protein (shown in the boxes). The O-linked oligosaccharides are related to the linkage region structure for keratan sulfate as indicated by the circled, similarly located galactose residues. The exact structures of the N-linked oligosaccharides for proteoglycans remain to be determined, but they are related to the structure shown, which is common for glycoproteins. The structure of HA is shown in Fig. 2-4.

chondroitin sulfate chains are the predominant component of the cartilage proteoglycans, and, as discussed below, they are critical for the structural function of the macromolecules in this tissue.

Keratan sulfate chains are bound to the core protein through an oligosaccharide structure that closely resembles "mucin-type" oligosaccharides, which are linked to protein by glycosidic bonds between *N*-acetylgalactosamine and hydroxyls of serine and threonine. An example of the structure of one of these oligosaccharides that is present on the core protein as well (De Luca *et al.*, 1980; Lohmander *et al.*, 1980) and its relationship to the linkage region for keratan sulfate are shown in Fig. 2-2. The repeating disaccharide structure of keratan sulfate is initiated on the circled galactose residue. For cartilage proteoglycans, the total number of keratan sulfate chains plus these mucin-type oligosaccharides remains relatively constant, about 100 per core protein, and is somewhat greater than the number of chondroitin sulfate chains. However, proteoglycans from embryonic or immature cartilages and from chondrosarcomas contain high proportions of the O-linked oligosaccharides relative to keratan sulfate chains. Conversely, keratan sulfate chains are predominant in proteoglycans from mature cartilages (Lohmander *et al.*, 1980; Sweet *et al.*, 1979). Therefore, with maturation of the cartilage, more of the O-linked oligosaccharides are used to initiate keratan sulfate chains. A large proportion of the keratan sulfate chains plus O-linked oligosaccharides are concentrated on the core protein in a middle region that contains only a few of the chondroitin sulfate chains, referred to as the keratan sulfate-attachment region (Heinegård and Axelsson, 1977; Fig. 2-1, this chapter). One end of the core protein, referred to as the HA-binding region, consists of a portion of the polypeptide of approximately 55,000 molecular weight with few or no attached GAG chains. This region has within it an active site that binds to HA through a noncovalent, highly specific interaction. As discussed below, this interaction is essential for forming proteoglycan aggregates in the organization of the cartilaginous extracellular matrix.

The polypeptide in the HA-binding region of cartilage proteoglycans contains covalently bound oligosaccharides of a different class, namely the complex "glycoprotein type" (De Luca *et al.*, 1980; Lohmander *et al.*, 1980, 1981). These oligosaccharides, which have structures similar to those shown in Fig. 2-2, are covalently bound to the core protein by *N*-glycosylamine linkages between *N*-acetylglucosamine and asparagine. They contain primarily sialic acid, galactose, mannose, and *N*-acetylglucosamine with a small amount of fucose (Kornfeld and Kornfeld, 1980). The biosynthesis of the *N*-linked oligosaccharides is presented in more detail in Chapter 5. There are about 15 of these oligosaccharides in the proteoglycan structure. Thus, the HA-binding region of the core protein resembles a glycoprotein in many respects.

2.2. Assembly, Secretion, and Aggregation

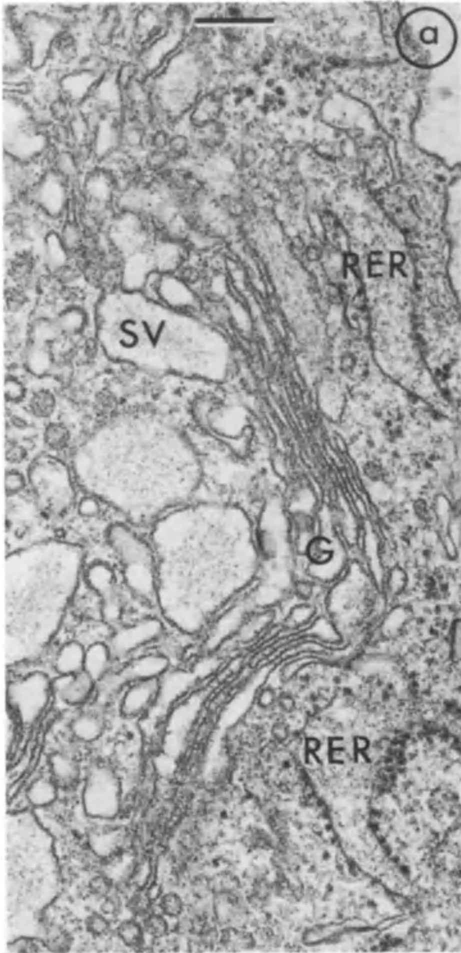
It is apparent from the chemical structure that assembling a proteoglycan molecule requires the orchestration of a large number of specialized biosyn-

thetic pathways involved in posttranslational processing. Many of these pathways are localized in different regions of the cell. There are probably many factors required to ensure that the different substituents are added onto the core in the proper sequence and that the partially completed macromolecule proceeds through the intracellular compartments in an orderly fashion.

The synthesis of the core protein begins on the ribosomes of the endoplasmic reticulum. It is quite likely that while the protein is still being elongated, initial stages in the assembly of the N-linked oligosaccharides begin through a highly coordinated series of enzymatic steps that are described in more detail in Chapter 5 (see Figs. 5-2, 5-5). Once released from the ribosome into the endoplasmic reticulum cisternae, the core protein begins a journey to the Golgi region of the cell where chondroitin sulfate chains and O-linked oligosaccharides are assembled onto the hydroxyls of appropriate serine, and serine and threonine residues respectively (Thonar et *al.*, 1981). In recent experiments, the core protein synthesized by chondrocytes isolated from a transplantable rat chondrosarcoma has been identified prior to the addition of the chondroitin sulfate chains (Kimura et *al.*, 1981a,b). The core protein, which had an apparent molecular weight on SDS-polyacrylamide gels of approximately 370,000, was specifically precipitated using antibodies against the hyaluronic acid-binding region of the core. It was able to interact with hyaluronic acid and link protein when combined with carrier proteoglycan aggregate. Further, after blocking new protein synthesis with cycloheximide, the kinetics of loss of the core protein, labeled during a 30-minute pulse prior to the addition of the cycloheximide, was the same as appearance of completed proteoglycan. Whether the size of the polypeptide in the completed proteoglycan is as large as that of the core protein precursor, or whether some processing steps occur involving trimming of the polypeptide remains to be determined. In all probability, the keratan sulfate chains are also elongated on some of the partially completed O-linked oligosaccharides in the Golgi region as well.

Once the GAG chains have been initiated on a core protein, the proteoglycan is completed very rapidly. For example, radiosulfate-labeled proteoglycans recovered directly from chondrocytes exposed in culture to [^{35}S]sulfate for only 1–2 min have molecular size distributions indistinguishable from completed proteoglycans isolated after much longer labeling times (Kimura et *al.*, 1981a). The completed molecules are collected into secretory vacuoles and over a time period of 10–30 min are secreted from the cell by exocytosis (Kimura et *al.*, 1979).

The organelles involved in the synthesis and secretion of proteoglycans are shown in Fig. 2-3. Cisternae of the rough endoplasmic reticulum lie close to the *cis* side of the Golgi apparatus, while forming secretory vacuoles are present on the *trans* side (Fig. 2-3a). Dense granules are apparent in *trans* Golgi saccules and in many secretory vacuoles (Figs. 2-3b,c). These granules are most pronounced when cartilage is fixed in ruthenium red, a cationic stain that precipitates polyanions (Luft, 1971a,b). The predominant polyanion of cartilage is proteoglycan, and the observation of stained granules in secretory vacuoles (Thyberg et *al.*, 1973) and in the extracellular matrix reflects the distribution of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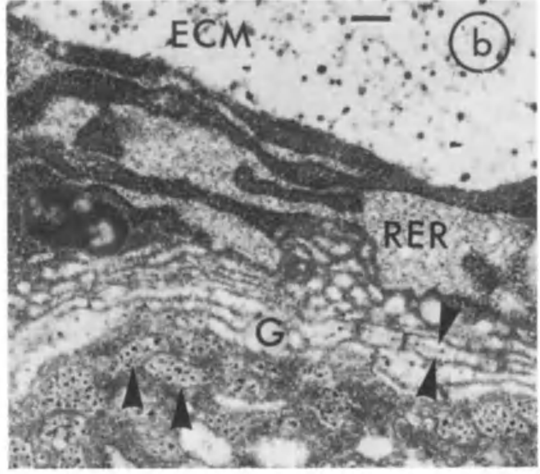
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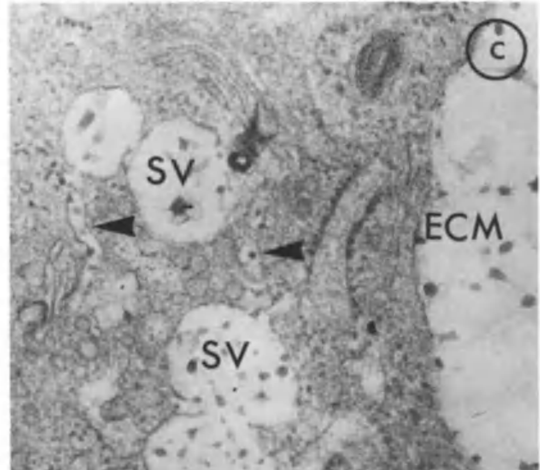
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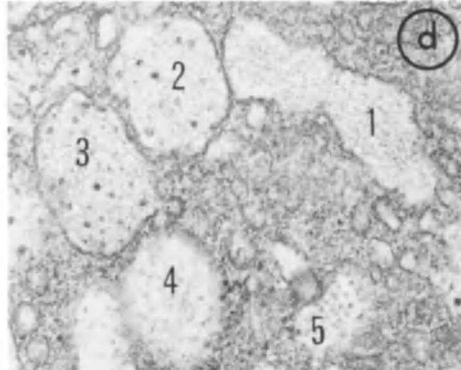
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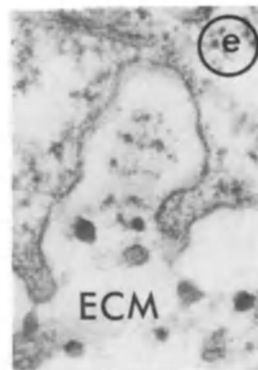
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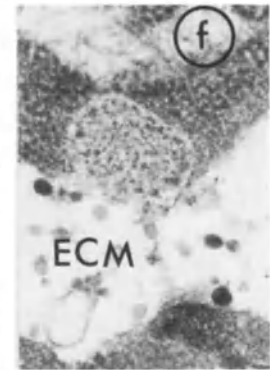
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proteoglycans in this tissue. The presence of these granules in Golgi saccules and secretory vacuoles, but not in rough endoplasmic reticulum, suggests that the proteoglycan acquires its polyanionic GAG while in the Golgi apparatus. This is consistent with biochemical evidence from cell fractionation studies showing that GAG synthesis occurs in smooth microsomal fractions, which include Golgi saccules (Kimata et *al.*, 1971; Silbert and Freilich, 1980; Freilich et *al.*, 1977), and from autoradiographic studies showing the site of uptake of radiolabeled sulfate to be the Golgi apparatus (Godman and Lane, 1964; Fewer et *al.*, 1964; Martinez et *al.*, 1977).

Some of the fine filaments in the Golgi saccules of Figs. 2-3b,d may represent collagen molecules (Weinstock and Leblond, 1974; Nist et *al.*, 1975; Ross and Benditt, 1965). The presence of both filamentous and granular material in many secretory vacuoles suggests that these components are packaged together for secretion (Revel and Hay, 1963; Hascall, 1980a). The varying proportions of filaments to granules in different secretory vacuoles may indicate that the ratio of the two components is not fixed. Some vacuoles (f, Fig. 5-6) contain only collagenlike rods (Fig. 7-8), which is consistent with the idea that some of them contain only collagen (Chapter 5).

Secretion occurs by the process of exocytosis, during which the secretory vacuoles travel to the cell surface where their membranes fuse with the plasma membrane, and their contents are released to the extracellular space (Figs. 2-3e,f). Occasionally, secretory vacuoles show alignment of granules spaced at approximately 70-nm intervals on filaments about 300 nm long (Fig. 2-3e). This organization is similar to the distribution of some matrix granules on collagen fibrils in the extracellular matrix (see Fig. 2-9).

As discussed in Chapters 1 and 6, the collagen molecules undergo several changes, eventually being assembled into the highly ordered fibrils that form a network structure typical for cartilage. Proteoglycans must also be organized into the extracellular matrix through a highly specific aggregation process involving two additional macromolecules, HA (Hardingham and Muir, 1972) and link protein (Heinegård and Hascall, 1974). The structure of these ag-

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← **Figure 2-3.** Morphological components involved in synthesis and secretion of proteoglycans. (a) The Golgi area of a chondrocyte, showing a stack of Golgi saccules (G), some of which are flat and others dilated. On the cis side of the stack lie cisternae of rough endoplasmic reticulum (RER), while the *trans* side has forming secretory vacuoles (SV). In this cell, the contents of the vacuoles are fine filamentous material. (b) In some cells the secretory vacuoles contain large dense granules (arrowheads) similar in size and appearance to proteoglycan matrix granules of the extracellular matrix (ECM). An elongated filamentous material is present in one Golgi saccule (apposed arrowheads). (c) Dense granules similar to matrix granules also occur in flattened Golgi saccules (arrowheads). (d) The contents of secretory vacuoles range from fine filaments in longitudinal array (vacuole 1) through tangles of filaments and granules (2, 3, 4) to occasional images of granules aligned on fine filaments (5). (e) Exocytosis of a secretory vacuole, whose contents are dense granules spaced about 70 nm apart on a bundle of filaments about 300 nm long, suggests proteoglycans on collagen fibrils. (f) A more common image of exocytosis is a vacuole containing a tangle of filaments and granules. Fixed in ruthenium red to enhance proteoglycans. Compare Fig. 5-6. All × 50,000 except (b), which is × 25,000. Bar = 200 nm.

gregates is shown schematically below an electron micrograph of an isolated aggregate in Fig. 2-4 (Rosenberg et *al.*, 1975; Kimura et *al.*, 1978; Faltz et *al.*, 1979a). At intervals averaging 30 nm, monomer proteoglycans interact with a long central strand of HA. The active site in the HA-binding region of the core protein has subsites for five repeating disaccharide units as indicated in Fig. 2-4 (Hardingham and Muir, 1973; Hascall and Heinegård, 1974; Christner et *al.*, 1979). The active-binding site exhibits a high affinity for this decasaccharide or larger oligomers of HA but much lower affinity for shorter oligomers. The interaction is highly specific, for no other anionic biopolymer has been found that will compete with HA for binding, and it has a dissociation constant of about 10^{-6}–10^{-7} M (Christner et *al.*, 1979).

When the third constituent, the link protein, is present, the proteoglycan molecules are essentially locked into place on the HA, and the dissociation constant becomes too low to measure conveniently (Kimura et *al.*, 1979; Hascall and Heinegård, 1974; Hardingham, 1979). The link protein has two forms: a smaller one of molecular weight about 42,000 and a larger one of molecular weight about 50,000 (Keiser et *al.*, 1972; Baker and Caterson, 1979). The larger form appears to contain an extra portion of polypeptide as well as some oligosaccharides (Baker and Caterson, 1979). The link protein binds to a length of HA adjacent to the HA-binding region of the proteoglycan (Kimura et *al.*, 1979; Faltz et *al.*, 1979b), and it also interacts with portions of the polypeptide in the HA-binding region (Heinegård and Hascall, 1979). The association between the three molecules, then, yields a stable structure, and while stretches of HA between adjacent proteoglycans are accessible to enzymes that digest HA, 40–50 monosaccharides in the contact regions with the polypeptides are not (Faltz et *al.*, 1979b). The minimum distance between proteoglycans on the HA is apparently limited by the hydrodynamic volume of the individual monomers, which is determined primarily by the extended lengths of the chondroitin sulfate chains radiating out from the core protein.

In the electron micrograph of the spread aggregate shown in Fig. 2-4, the chondroitin sulfate side chains of each monomer are collapsed along the core protein, unlike the monomers shown in Fig. 2-1. This allows the structure of the aggregate to be visualized more easily. The central HA filament is clearly visible, as are some of the regions where monomers are in contact with the central filament. This aggregate contains almost 40 monomers, and the entire structure would have a molecular weight approaching 10^8.

The micrograph in Fig. 2-5 shows a thin section through the extracellular matrix of a cartilage. The thin fibrils of the collagen network (CF) can be seen. The dense matrix granules represent proteoglycans, as determined by numerous experiments involving staining, extraction, and enzymatic digestion of the matrix (Luft, 1971b; Thyberg et *al.*, 1973; Anderson and Sajdera, 1971; Matukas et *al.*, 1967). They are spherical, polygonal, or cylindrical in shape, with dimensions similar to the proteoglycan monomers in the spread aggregate in Fig. 2-4, if one realizes that the granules are thin *sections* through proteoglycans. When the tissues are processed for electron microscopy, the GAG chains usually collapse onto the protein core because of the complete dehydration of the sample and the lack of a good fixative for the polysaccharide moiety. Thus,

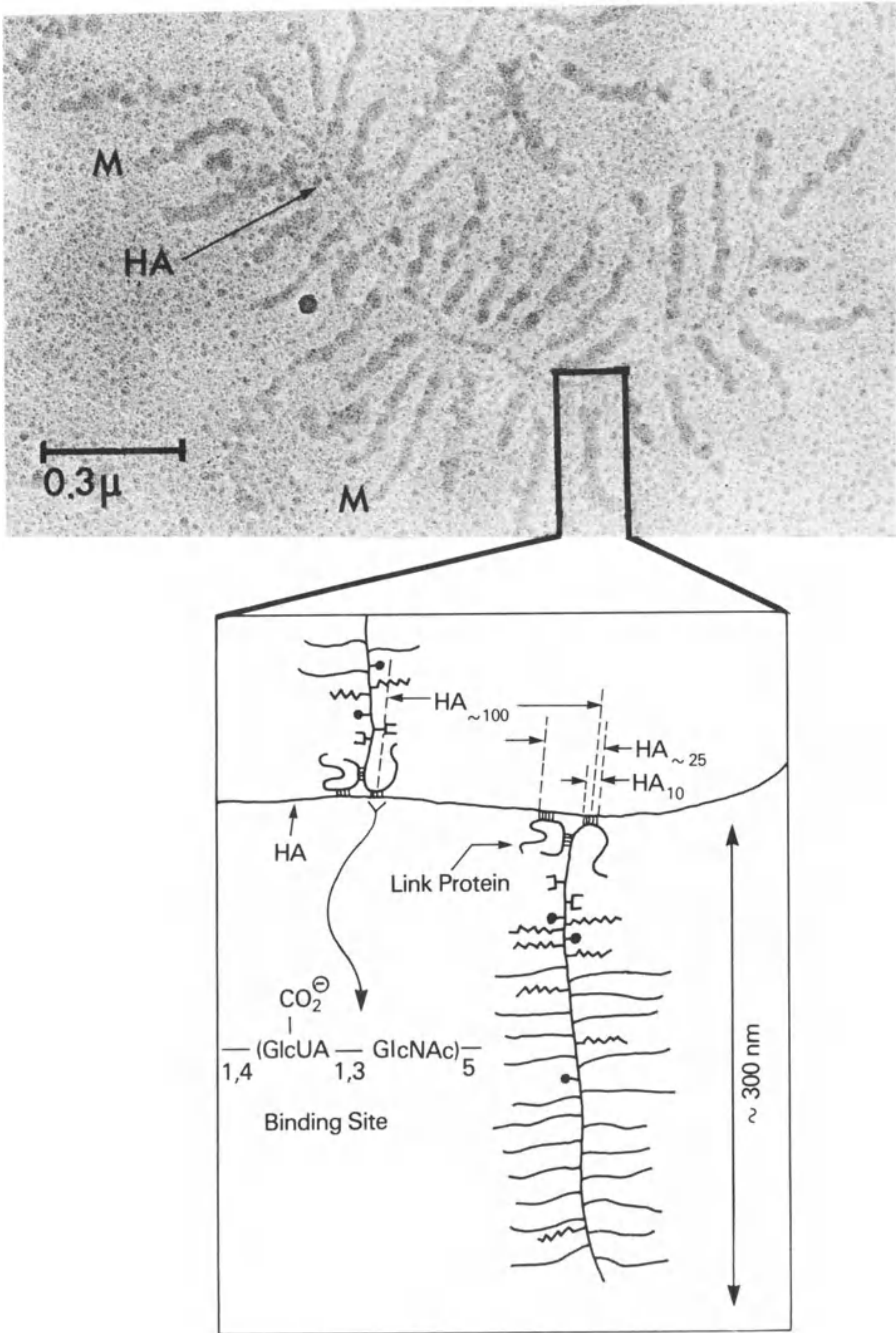


Figure 2-4. Structure of cartilage proteoglycan aggregates. The electron micrograph shows an aggregate isolated directly from a transplantable rat chondrosarcoma without prior dissociation (Faltz et *al.*, 1979a) and spread in a cytochrome *c* monolayer (Rosenberg et *al.*, 1975). Monomers (M) are aligned on a central filament of HA. The schematic model for the structure of the aggregate is discussed in the text. Bar = 0.3 μm.

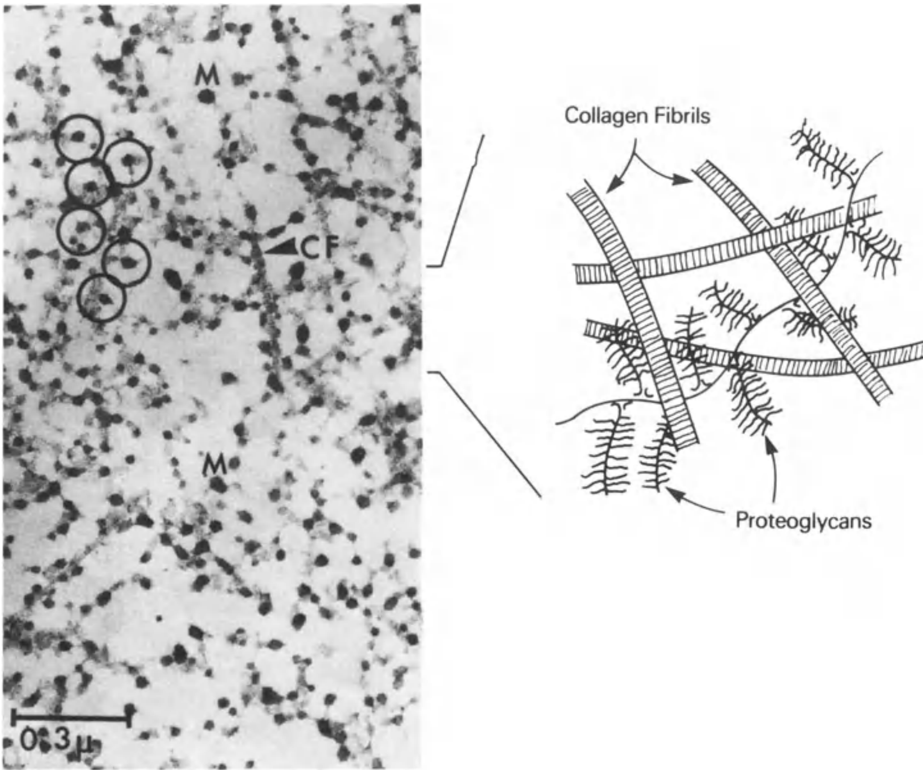


Figure 2-5. Organization of structural elements of cartilage extracellular matrix. The electron micrograph of a portion of the extracellular matrix shows thin collagen fibrils (CF), which occasionally show characteristic periodic banding, and matrix granules, which represent condensed proteoglycan monomers (M). The circles indicate the space that the expanded proteoglycans would occupy. The tissue is a composite structure of collagen fibrils with intertwining proteoglycan aggregates as shown schematically. Bar = 0.3 μm.

the matrix granules represent proteoglycan monomers with chondroitin sulfate chains condensed (Hascall, 1980b). In their expanded native state, each molecule would occupy the much larger volume indicated by the circles in Fig. 2-5, which represent the diameters of the monomers if the chondroitin sulfate chains were extended as in Fig. 2-1. Thus, the extended structures of the proteoglycans would occupy all the interstitial space in the matrix, and the aggregate structures would be intertwined throughout the collagen network. For comparison, Fig. 2-6 shows portions of a chondrocyte and its extracellular matrix at the same magnification as an aggregate (insert). It is apparent that the volume defined by an aggregate structure in the matrix is very large, comparable to such cell organelles as mitochondria and the Golgi apparatus.

It is clear from experiments with chondrocyte cultures that the proteoglycan monomers are not aggregated when they leave the cell (Kimura *et al.*, 1979, 1980). Thus, assembly of these large aggregates occurs some time after secretion

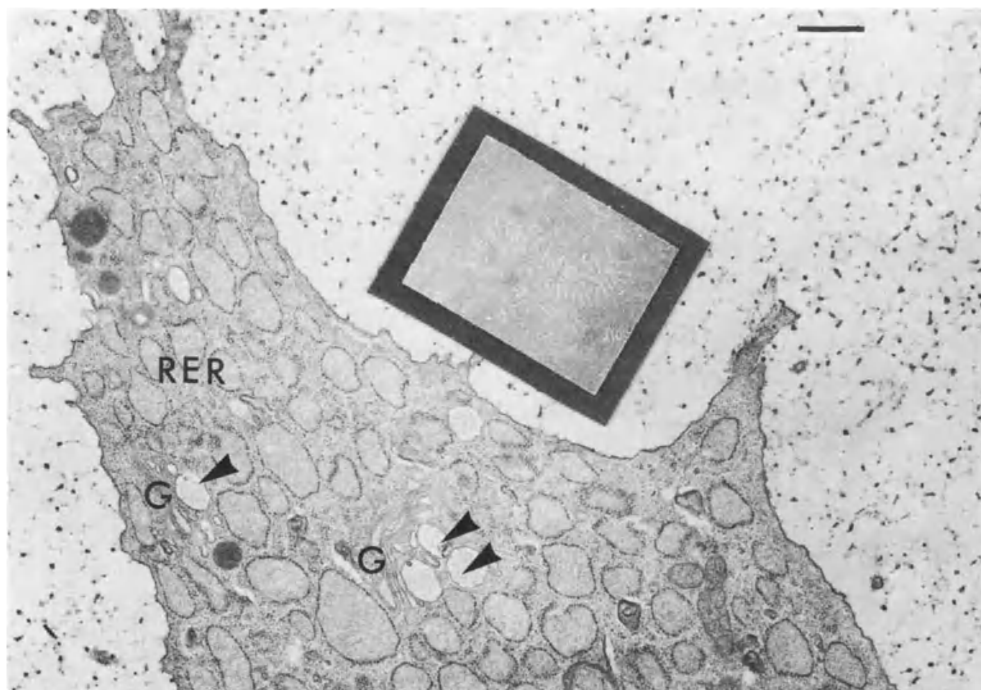


Figure 2-6. Comparison of size of aggregates with chondrocyte and extracellular matrix. The insert shows a spread aggregate at the same magnification as the surrounding thin section of a portion of a chondrocyte and its extracellular matrix. Golgi regions (G), rough endoplasmic reticulum (RER), and secretory vacuoles (arrowheads) are indicated. Bar = 0.6 μm.

for regulating assembly of the aggregates and for organizing them within the collagen network are not yet understood. It is likely, however, that the aggregation process is critical for holding the proteoglycans in the tissue, as the unbound monomers can diffuse out of the matrix. Based on many experiments, interaction of proteoglycans and collagen *in vitro*, and on the close association of proteoglycans with collagen often observed morphologically (see Figs. 2-3 and 2-11 for example), it has been postulated that proteoglycans interact with collagen in the formation of the matrix. While there are no clear biochemical data that indicate that such interactions occur *in vivo*, and the functional aspects of the tissue as discussed below do not require such interactions, it is probable that interactions between these two classes of macromolecules occur and have prominent roles, especially during the initial development and deposition of the matrix (see Chapter 9).*

*Most recently, Scott *et al.* (1981) and Scott and Orford (1981) demonstrated the association of proteoglycans with the d band, or gap region, of collagen fibrils in developing rat tendon. They also correlated a decrease in proteoglycan content and a change in proteoglycan type with increases in fibril diameter during tissue maturation. From these data, they have postulated a role for some of the tendon proteoglycans, particularly those with dermatan sulfate, in controlling collagen fibril diameter.

2.3. Functional Aspects

Proteoglycans have extended structures in solution and occupy very large hydrodynamic volumes relative to their molecular weights (Hascall and Sajdera, 1970; Pasternack *et al.*, 1974; Reihanian *et al.*, 1979). A monomer of 2.5×10^6 molecular weight has a radius of gyration of about 60 nm. This dimension is defined primarily by the extended lengths of the chondroitin sulfate chains, about 50 nm for a chain of 20,000 molecular weight. The molecules are reversibly compressible when subjected to a compressive load, as schematically indicated in Fig. 2-7. As solvent is displaced from the molecular domain, however, the intramolecular interactions between the relatively stiff and polyanionic GAG chains must increase. When the load is removed, the molecular domains can once again expand. This property is critical for normal function of cartilages such as the articular cartilages on the surfaces of bones, which must act as cushions for variable, compressive loads. The cartilage matrix, then, is a composite structure. The collagen fibrils provide a network that defines tissue shape and resists tensile forces, and the interspersed proteoglycan aggregates provide a hydrated, viscous gel that absorbs compressive loads. The concentration of proteoglycans in most hyaline cartilages, particularly weight-bearing ones, is three to five times more than would be possible if the molecules were fully extended. Therefore, individual proteoglycans are restricted to domains as small as 20% of their maximum. This increases the charge density within their domains and, in analogy with a partially compressed spring, provides a molecular configuration that can resist an equivalent load with less total deformation. The intermolecular competition between proteoglycan molecules for available solvent in the matrix will suffice to reduce the domains of the molecules. The concentration of proteoglycans in a local region within the matrix, then, will affect the "springiness" of that region and will be dictated by the ability of the resident chondrocytes to synthesize and secrete the molecules into the high local concentration of proteoglycans in the adjacent matrix.

Alterations in the amount of proteoglycan in the cartilage matrix or in the

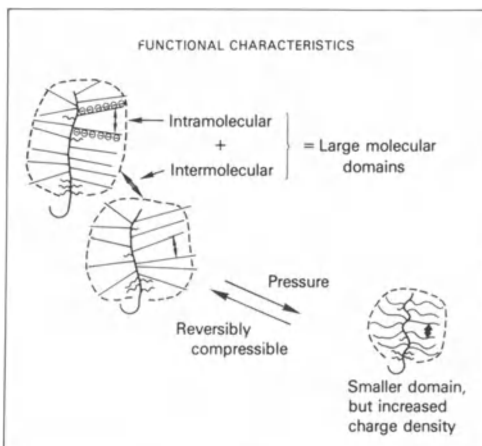


Figure 2-7. Functional characteristics of proteoglycans. (From Hascall, 1977, with permission.)

architecture of the individual molecules can directly affect tissue function. For example, long bones are formed on cartilage growth plates that determine to a large extent the final shape of the skeleton. Two lethal genetic anomalies have been found, nanomelia in chickens (Pennypacker and Goetinck, 1976) and cartilage matrix deficiency in mice (Kimata et *al.*, 1981; Kochar and Penner, 1980), in which hyaline cartilages contain very low amounts of proteoglycan (less than 5% of normal). In other respects, such as content of type II collagen and chondrocyte morphology, the cartilages in these genetic anomalies appear normal. In the absence of proteoglycans, however, the volumes of the cartilage matrices are drastically reduced and the cells are much closer together. The consequences of this genetic deficiency for the mouse mutant are shown in Fig. 2-8, where the morphology of a homozygous mutant embryo is compared with that of a heterozygous littermate. The cartilages in the limb from the homozygous mutant stain only weakly with Alcian blue, a dye that interacts with polyanions, compared with the phenotypically normal counterpart (Fig. 2-8c). This reflects the greatly reduced content of proteoglycan in the affected tissue. The bones constructed on the cartilage growth plates, as shown by the alizarin red stain (Figs. 2-8b,d), are foreshortened and malformed in the homozygous mutant embryos.

In another genetic anomaly in brachymorphic mice, there is a deficiency in one of the enzymes located in the pathway for biosynthesis of phosphoadenosine phosphosulfate (PAPS), which is the donor for introducing sulfate esters onto GAG chains (see Chapter 5). In tissues in the mutant where proteoglycan synthesis is high, such as growing cartilages, the concentration of PAPS becomes the limiting factor, and the GAG on the proteoglycans are undersulfated (Orkin et *al.*, 1976; Schwartz et *al.*, 1978; Sugahara and Schwartz, 1979). In the cartilage growth plates of this mutant, the proteoglycans contain about 15% fewer sulfate esters within their structure. It would be expected that these proteoglycans would occupy smaller volumes under the same loading conditions as their normal counterparts. This would account in large part for the 15–20% reduction in the width of the cartilage growth plates (Orkin et *al.*, 1977) and, therefore, the shortened limbs characteristic of these mice.

In adult hyaline cartilages, proteoglycans slowly turn over and are replaced by newly synthesized molecules, whereas the collagen network does not normally appear to turn over appreciably. The chondrocytes play a key role in maintaining the local structure of the matrix through regulation of both the synthetic and the catabolic pathways involved in this process. When the normal steady-state balance is altered, tissue function can be affected. For example, in an animal model for osteoarthrosis, the cruciate ligament of the knee is severed in dogs; this increases the laxity of the joint and alters the normal apposition and pressure relationships along the articular cartilages without altering the ability of the animal to walk or run. Invariably, over a year's time osteoarthritic lesions occur in central portions of the articular surfaces (McDevitt et *al.*, 1981). In these regions and adjacent areas, the rates of both synthesis and turnover of proteoglycans significantly increase long before a lesion occurs. In older cartilage or cartilage that has undergone considerable wear and tear, it appears that the chondrocytes are unable to replace proteoglycans sufficiently,

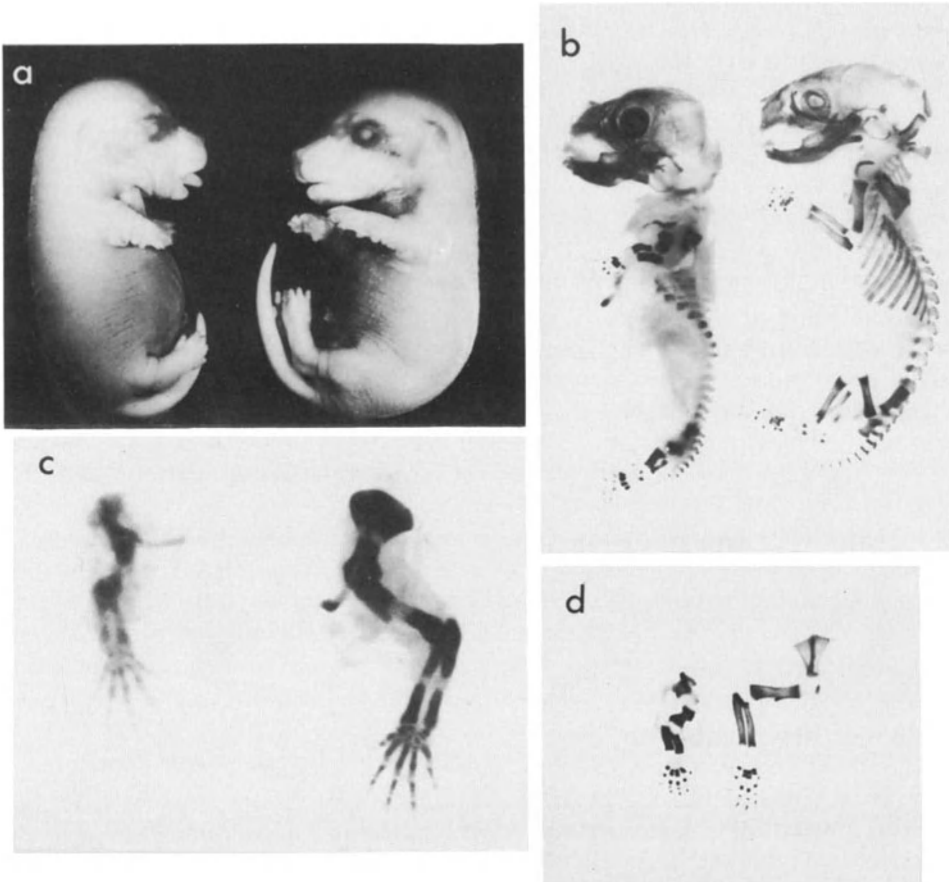


Figure 2-8. Comparison of skeletal structures for cartilage-matrix deficiency and normal mice. (a) Two littermates on the 18th day of gestation obtained from a mating between proven heterozygous mice (*+/cmd* × *+/cmd*). The fetus on the left is affected (*cmd/cmd*), showing stunted trunk and limbs, protruding tongue and abdomen. Fixed in Bouin's fixative before photography. × 2.5. (b) Newborn littermates from a heterozygous mating. Fixed in ethanol, cleared, and bones stained with alizarin red S dye. The fetus on the left is affected (*cmd/cmd*). Note that the ribs are shorter but more flared and thicker than those of the normal fetus (right). Skull bones are well developed but the mutant head as well as the jaws are reduced along the anteroposterior axis. Mutant fetus shows severe stunting of the girdle and the long bones of both the upper and the lower limbs. × 2.5. (c) Upper limbs of a normal (right) and a mutant (left) embryo on the 16th day of gestation. Cartilaginous skeleton stained with Alcian blue. Mutant cartilages show faint staining compared with intense staining in the normal cartilage. Note that an extreme reduction in the linear growth of the mutant limb cartilage is already apparent; yet, the primary centers of ossification in the diaphysis have appeared in the mutant just as in the normal limb at this stage. × 7. (d) Upper limbs of a normal (right) and a mutant (left) embryo stained with alizarin red to show the developing bones. (Photographs kindly provided by Dr. David Kochar.)

and they synthesize proteoglycans with less effective structures, such as molecules with fewer and shorter chondroitin sulfate chains. Tissue function is impaired and osteoarthritic lesions are a likely consequence. The mechanisms by which the cells regulate proteoglycan synthesis and turnover and modulate proteoglycan structure remain to be determined, although local environmental factors probably play important roles.

Proteases will break the backbone structure of proteoglycans and reduce the functionality of the macromolecules. The released GAG chains or peptide fragments with several chains do not have the large hydrodynamic domains characteristic of the intact proteoglycans. A trauma or inflammation can release degradative enzymes into the matrix, which can rapidly destroy the proteoglycan component and initiate repair processes. It is also probable that in some patients with various rheumatoid arthritic disorders, autoimmune mechanisms directed against proteoglycan antigens which would normally be sequestered from immune surveillance play a role in the vicious cycle of inflammation in the joints leading to invasion, degradation, and eventual removal of the cartilages (Glant et *al.*, 1980).

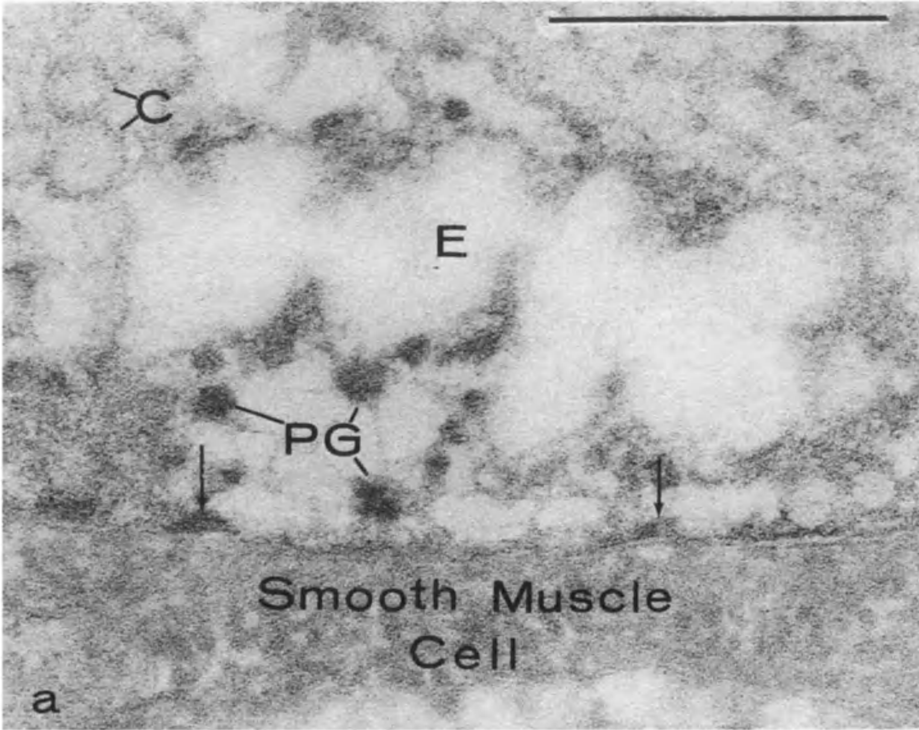
3. Proteoglycans in Other Tissues

3.1. Aorta

Some tissues have proteoglycans with features very similar to those of the cartilage proteoglycans. For example, a large proportion of the proteoglycans in aorta form aggregates with HA and link proteins similar or identical to those found in cartilage (Oegema et *al.*, 1979; Gardell et *al.*, 1980), and smooth muscle cells from aorta synthesize in culture a population of proteoglycans that have similar properties. In the extracellular matrix of the connective tissue in the aorta (Wight and Ross, 1975a,b; Fig. 2-9), the collagen fibrils are much thicker than in cartilage (Fig. 2-5). The proteoglycan matrix granules are very prominent and closely associated with both the collagen and the elastic fibers in the extracellular matrix. Often, the proteoglycans can be seen condensed on the surface of the collagen fibrils at the major periodic intervals of approximately 70 nm, similar to the patterns observed in Fig. 2-3e. This suggests that there are ordered interactions between the proteoglycan molecules and the collagen fibrils in the connective tissue of the aorta. This tissue, like cartilage, undergoes repetitive, transient pressure changes, and it is likely that the proteoglycans, in concert with the elastin component of the tissue (see Chapter 3), buffer these cyclical changes. Nevertheless, the proteoglycans in many other tissues have entirely different structures that clearly serve other functions.

3.2. Corneal Stroma

The corneal stroma contains the same kinds of GAG that are present in cartilage, namely chondroitin sulfate (as dermatan sulfate in which some of the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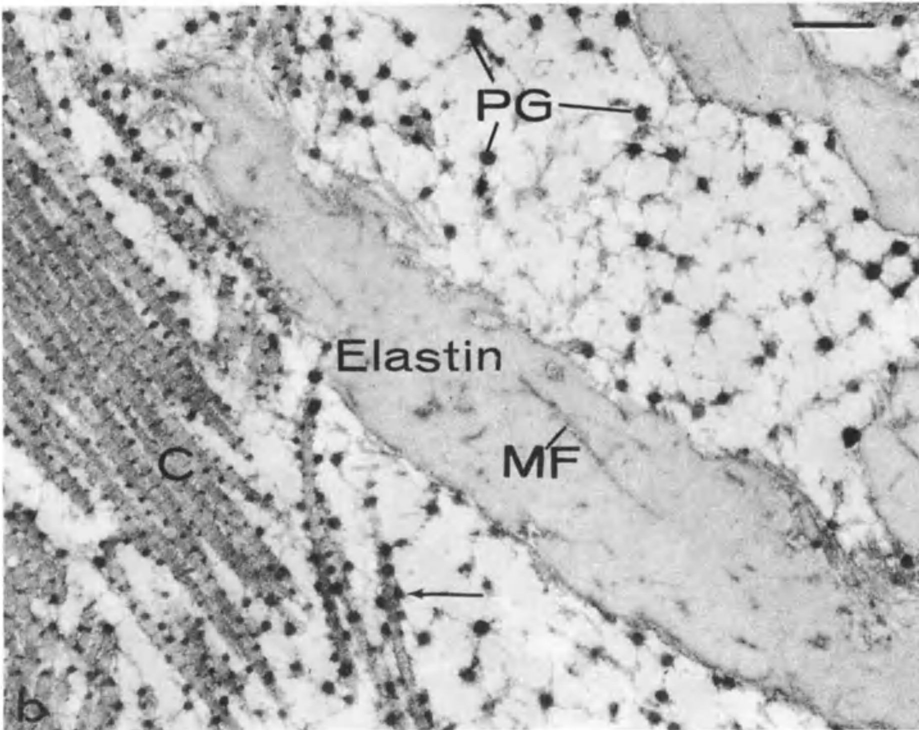
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MF
b

glucuronic acid residues are epimerized to iduronic acid; see Chapter 5) and keratan sulfate, but the proteoglycans are very different (Axelsson and Heinegård, 1978; Hassell et *al.*, 1979). There are two different classes of proteoglycans in the stroma, one that contains chondroitin sulfate and one that contains keratan sulfate (Fig. 2-10). Both types are small, and they are present in nearly equal amounts. The chondroitin sulfate proteoglycan contains 1–2 GAG chains of about 55,000 molecular weight. In addition, the core protein, which is about 80,000 molecular weight, contains oligosaccharides, probably of the N-asparagine-linked type. The overall average molecular weight of the intact macromolecule is about 150,000. The keratan sulfate proteoglycans are even smaller, with an average molecular weight of about 80,000. There are about one to three keratan sulfate chains per core, each with an average molecular weight of 5000–7000. The keratan sulfate chains are attached to the core protein through N-glycosylamine bonds between N-acetylglucosamine and asparagine. Details of the structure of the linkage region remain to be determined, but mannose residues are present, and it is likely that the "primer" oligosaccharide upon which keratan sulfate is synthesized in corneal stromacytes is related to glycoprotein-type oligosaccharides similar to those shown in Fig. 2-2. The fact that tunicamycin, a compound that inhibits the synthesis of this class of oligosaccharides (see Chapter 5), preferentially inhibits keratan sulfate synthesis in corneal explants in culture (Hart and Lennarz, 1978) is consistent with this suggestion.

The corneal stroma contains a very regular array of collagen fibrils of uniform diameter that are organized in alternating, nearly orthogonal layers of parallel fibrils, and this organization is critical for corneal transparency. The average space between adjacent collagen fibrils is approximately 30 nm, and thus on the basis of geometry alone, it would be most difficult to organize proteoglycan aggregates of the cartilage type in such a matrix without severely distorting the collagen framework. However, the hydrodynamic volumes of the corneal stroma proteoglycans are small enough to fit within the interfibrillar spaces in the matrix, where they probably contribute both to the high degree of order in the arrangement of the collagen network and to the refractive properties of the tissue. Interestingly, the highly hydrated, opaque embryonic cornea contains larger proteoglycan matrix granules (Fig. 2-11b) than does the compacted, more mature cornea (Fig. 2-11a).

In the human genetic deficiency corneal macular dystrophy, keratan

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← **Figure 2-9.** Electron micrographs of aorta. (a) This electron micrograph is from a portion of a thoracic aorta (*Macaca nemestrina*) fixed in the presence of ruthenium red. Ruthenium red deposits occur intermittently along the outer leaflet of the plasma membrane (arrows) of the intimal smooth muscle cell and can be found associated with other components of the intercellular matrix such as elastic fibers (E) and collagen fibrils (C). PG, proteoglycan. × 234,000, bar = 50 nm. (From Wight and Ross, 1975a.) (b) This electron micrograph is from a portion of the intercellular matrix of a thoracic aorta fixed in the presence of ruthenium red, demonstrating the intimate association of the proteoglycan (PG) granules with elastin and collagen (C). Note that the proteoglycan granules deposit at regular intervals along the collagen fibrils (arrow). MF, microfibril of elastic fiber. × 53,000, bar = 50 nm. (From Wight, 1980.)

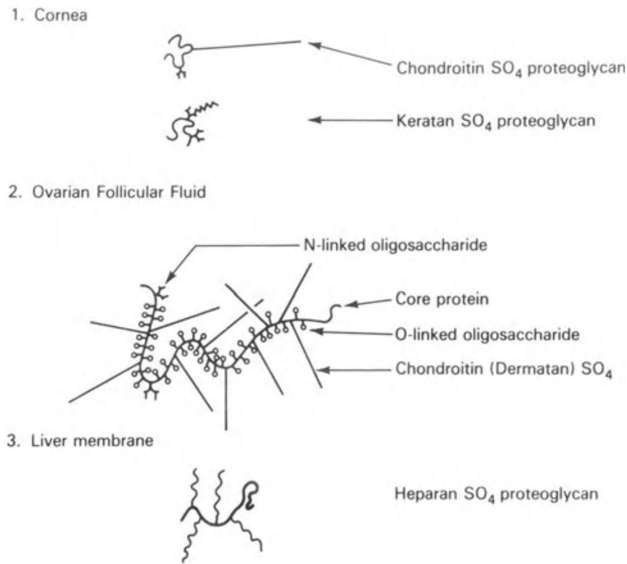

Figure 2-10. Schematic structures of proteoglycans from different tissues.

sulfate synthesis is deficient, although the core protein and associated oligosaccharides appear to be synthesized normally (Hassell *et al.*, 1980). As a consequence of the altered proteoglycan structure, precipitates form in the stroma and accumulate with time. This leads to increased corneal opacity, eventually requiring a corneal transplant to restore vision. The exact arrangement of the corneal proteoglycans vis-à-vis the collagen network in the extracellular matrix remains to be determined, but it is likely that the organization is very specific and involves precise interactions between portions of the proteoglycan structures, perhaps in the core proteins, and regions along the surfaces of the collagen fibrils (Fig. 2-11b).

3.3. Ovarian Follicular Fluid

Ovarian follicular fluid contains a predominant proteoglycan with yet a different structure (Fig. 2-10). The core protein, of about 300,000 molecular weight, is as large or larger than that of the cartilage proteoglycans, and the macromolecules have hydrodynamic volumes similar to those of cartilage proteoglycans (Yanagishita et *al.*, 1979; Yanagishita and Hascall, 1979). However, this follicular fluid proteoglycan has only 10–20 chondroitin sulfate chains (as dermatan sulfate), and the chains have a much larger average size, about 55,000 molecular weight. The macromolecules do not aggregate and do not appear to interact strongly with other macromolecules in the follicular fluid. The core protein is substituted with a very large number, 300–400, of mucin-type oligosaccharides linked to the protein through O-glycosidic bonds between *N*-acetylgalactosamine and the hydroxyls of serine and threonine. Quite likely,

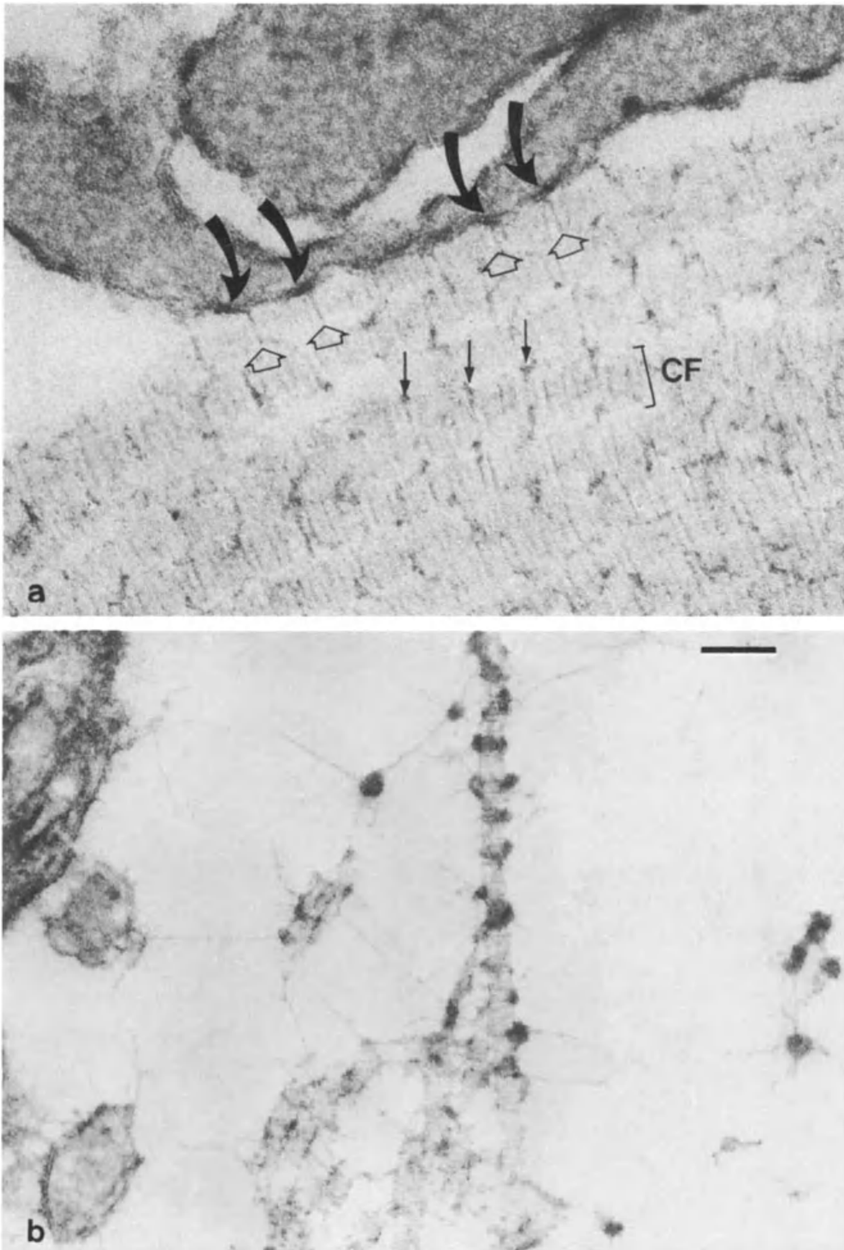

Figure 2-11. Morphology of corneal stroma. (a) In the compacted area of the 12-day-old embryonic avian cornea, the proteoglycan granules (small arrows) are small (~ 20 nm in diameter) and the collagen fibers (CF) to which they are attached are closely packed together. At this stage, the cornea is becoming transparent. The tissue was fixed in the presence of ruthenium red. The plasmalemma is stained at intervals (curved arrows) that relate to a repeating band (open arrows) in the adjacent collagen fibril. (b) In the 8-day-old embryonic avian cornea, proteoglycan granules are large (30–50 nm in diameter), and in the matrix between collagen fibrils (CF), the granules preserved by ruthenium red (open arrows) are connected by small filaments (curved arrows) that are hyaluronidase sensitive. The proteoglycan granules decorating the collagen fibrils occur at repeat intervals of ~ 60 nm, in the vicinity of the a–e band of the collagen striation period. Both × 140,000, bar = 72 nm. (From Hay, 1978.)

glycoprotein-type oligosaccharides are also present. If the chondroitin sulfate chains were not present, the macromolecule would in fact be classified as a typical mucin.

The structure of the follicular fluid proteoglycan is such that it would occupy large solvent domains, probably with a more open structure than the cartilage proteoglycan. This structure most likely is important for maintaining the expanded volume of the follicle and contributes directly to fluid viscosity. When follicles swell in response to reproductive hormones, the granulosa cells are stimulated to synthesize proteoglycans, and the concentration of proteoglycans, about 2 mg/ml, remains relatively constant as the net volume of the fluid increases. At the time of ovulation, in response to relative changes in the hormone levels, the granulosa cells synthesize plasminogen activator (Strickland and Beers, 1976). This converts plasminogen to the protease plasmin, which in turn may be involved in the sequence of events leading to ovulation. The follicular fluid proteoglycan is rapidly degraded by plasmin, and it is possible that its degradation and the concomitant reduction of fluid viscosity that would occur are important in the normal physiological process of ovulation.

3.4. Basal Lamina

The basal lamina (basement membrane) of many, if not all, tissues contains a row of proteoglycan granules in the internal lamina rara and another in the external lamina rara (Fig. 2-12). These granules form a regular array, with a spacing of about 60 nm between the granules (Trelstad *et al.*, 1974). Enzyme studies indicate that in the embryo these granules contain chondroitin sulfate (Trelstad *et al.*, 1974; Hay and Meier, 1974). As shown in Chapter 11, however, heparan sulfate proteoglycans are often present, such as those organized in the highly ordered lattice in the laminae rarae of the basement membrane in the kidney glomerulus (Kanwar and Farquhar, 1979). In their expanded states, then, these proteoglycans would provide an extended network of negative charges between the cell surface and the basement membrane in a variety of tissues. The potential functions of these proteoglycans for maintaining structural organization of the basal lamina and for selective filtration in the kidney are discussed in Chapter 11.

The basement membranes in developing mouse mammary glands (Gordon and Bernfield, 1980) contain heparan sulfate proteoglycans with a morphological appearance similar to the lattice arrangement described above. In the developing mouse salivary gland, however, the basement membranes contain chondroitin sulfate proteoglycans and HA (Cohn *et al.*, 1977). In each case, the intactness of the basement membrane, and probably of the proteoglycan component, appears to be critical in the morphogenesis of the lobulated structures of the glands (see Chapter 9). The macromolecular structures of these basement membrane proteoglycans have not yet been determined. Their strategic location and highly ordered arrangement suggest they play prominent roles in tissue function.

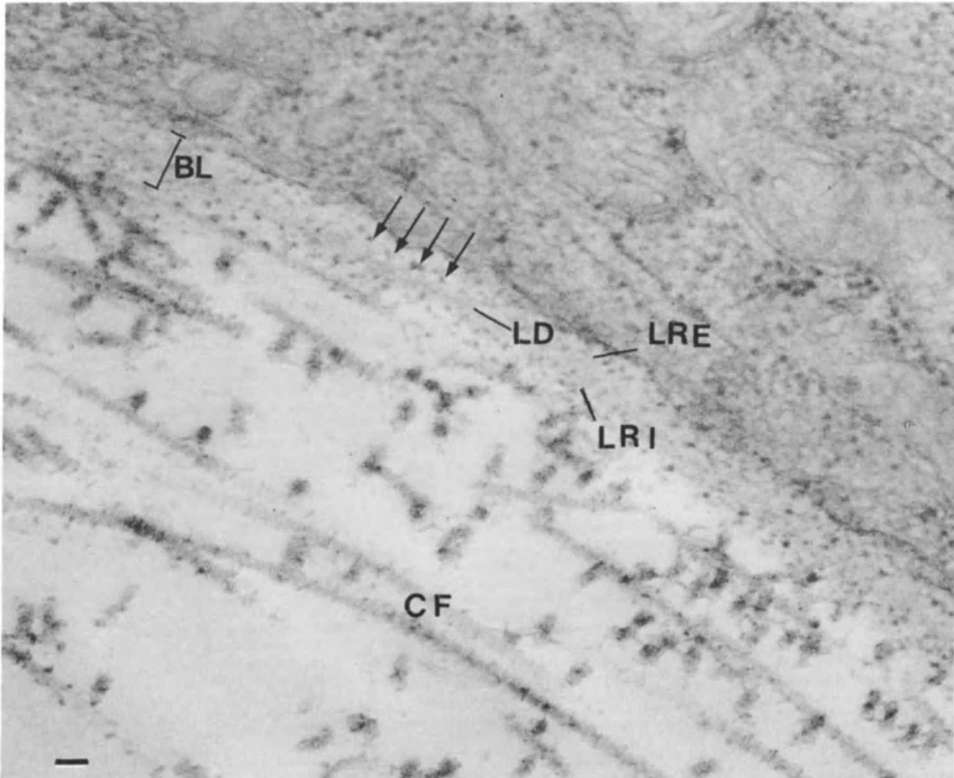

Figure 2-12. Morphology of the corneal basal lamina. The basal lamina (BL) consists of a central lamina densa (LD), which is ~ 60 nm wide, a lamina rara externa (LRE), and a lamina rara interna (LRI), each of which is ~ 25 nm wide. The proteoglycan granules (arrows) in the LRE and LRI are ~ 2 nm in diameter and are arranged in a lattice; each granule is ~ 60 nm away from its neighbor. The LD is a sheet of collagen (and other glycoproteins) with a proteoglycan lattice one granule thick on its outer (LRE) and inner (LRI) surfaces. Collagen fibrils (CF) are also decorated with proteoglycan granules. This 11-day-old embryonic avian cornea was fixed in ruthenium red. × 50,000, bar = 140 nm. (Courtesy of E. D. Hay.)

3.5. Plasma Membranes

Heparan sulfate proteoglycans are prominent components of many other tissues. One example that has been studied in some depth is the heparan sulfate proteoglycan derived from liver plasma membranes (Oldberg et *al*., 1979). It is a relatively small proteoglycan (Fig. 2-10) in which about four heparan sulfate chains, of about 14,000 molecular weight, are bound to each core protein, giving a total molecular weight of about 80,000. Detergents in combination with high-salt solutions were required to solubilize the proteoglycan, and it is possible that the core protein has hydrophobic regions, which can anchor the macromolecule in membranes. These proteoglycans, or closely related ones, may be important for facilitating cell adhesion, as heparan sulfate proteoglycans appear to interact with fibronectin–collagen complexes (see Chapter 4).

The liver membranes also contain a receptor that interacts with heparan sulfate (Kjellen *et al.*, 1980). Because each heparan sulfate proteoglycan contains more than one chain, it can bind to more than one receptor and possibly play a role in cell–cell adhesion as well. In a number of embryonic cell types, enzyme-resistant plaques have been identified by ruthenium red staining in the plasma membrane; it has been speculated that these plaques might contain the cell surface heparan sulfate (Hay and Meier, 1974; Wight and Ross, 1975a).

4. Concluding Remarks

This chapter has described a variety of proteoglycans found in different tissues. It has attempted to correlate their biochemical structures with their physiological function in different tissues and to demonstrate a widespread role for these molecules in filling and organizing extracellular matrices. Research on these molecules is still in its infancy and requires the development of many techniques for identifying, isolating, and characterizing them. Still, it is apparent already that these macromolecules play a wide variety of roles in tissue development and tissue function.

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Chapter 3

Elastin

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1. Introduction

A functional requirement of several mammalian tissues is an ability to stretch. The protein elastin, a naturally occurring rubberlike elastomer found in many extracellular matrices, such as those of blood vessels and lung, provides this property. It is the major protein component of the so-called elastic fibers of connective tissue. This chapter describes the biochemical and ultrastructural characteristics of these naturally occurring elastic fibers with particular emphasis on the insoluble amorphous elastin component. The other component of elastic fibers is a glycoprotein or a family of glycoproteins, which has been designated as the microfibrillar component. The microfibrils, which comprise approximately 10% of the total volume of the elastic fibers, are thought to provide both a matrix into which the insoluble protein elastin is incorporated, and a mechanism by which shape and size of elastic fibers may be controlled.

In the introductory paragraph to their treatise on elastic tissue, Richards and Gies (1902) wrote, "Comprehension of function is dependent on knowledge of structure and composition. Elastic tissues have received little analytic attention. They have been overlooked by reason, apparently, of their seeming metabolic passivity and because they serve mainly mechanical functions." It is still true today that one must have knowledge of the structure of a biological polymer to understand its function. And while it is also still true that relatively few investigators have actually worked on the structure and function of elastin, much has been accomplished since the turn of the century. The level of sophistication of our knowledge of amorphous elastin and its role in elastic fibers has advanced significantly from 1902.

It is important to mention at this point several pertinent reviews that have been published on the subject of elastin, and it would be foolhardy for this text to include many of the areas of elastin research already covered so well in previous articles. Those that must be mentioned include the classic review by Partridge (1962) and others by Franzblau (1971), Sandberg (1976), Rucker and

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Tinker (1977), and Burke and Ross (1979). The proceedings of a conference on elastin and elastic fibers have also been published (Sandberg et *al.*, 1977).

The scope of this chapter is to introduce the biochemical and ultrastructural properties of elastin and elastic fibers, describe the state of the art with respect to the biosynthesis of elastin, and relate the function of elastin to its molecular properties.

2. Isolation of Elastin

Elastin is an insoluble protein found in most connective tissues in conjunction with other structural elements such as collagen and proteoglycans. It is usually defined as that proteinaceous material remaining after all other connective tissue components have been removed. Essentially all methods of purification of elastin adhere to these two basic principles. When isolated in relatively pure form, fibers of elastin behave as true rubberlike elastomers. The method by which nonelastin components are removed from a connective tissue has been the only significant difference between the purification procedures used for insoluble elastin at the end of the 19th century and today.

According to Richards and Gies (1902), Tilamus in 1840 is probably to be credited with describing the first purification of elastin from elastic tissues. The method employed cold-water extraction to remove traces of blood and inorganic matter, followed by dehydration with alcohol and ether. If, in addition, the residue was treated with boiling dilute acetic acid, it was found to be free of sulfur. This material according to Tilamus was pure elastin. Muller (1861) improved on the procedure of Tilamus by adding two additional steps: treatment with boiling dilute alkali (KOH) and treatment with cold mineral acid (dilute HCl).

Richards and Gies (1902) probably were the first to note the relative ease with which elastin from bovine ligamentum nuchae could be studied. These authors used cold limewater instead of boiling KOH, followed by successive treatment of the residue with boiling water, boiling 10% acetic acid, and 5% HCl at room temperature. Instead of the boiling water, the use of the autoclave was introduced in 1928 by Schneider and Hajek (1928). Lowry et *al.* (1941) employed alternating autoclave and hot 0.1 N NaOH treatments to determine the concentration of elastin and collagen in several tissues. Lansing et *al.* (1951) described a purification of elastin from aortic tissue that also included 0.1 N NaOH. After washing with 0.9% NaCl, the authors heated the aortic material in 0.1 N NaOH for 45 min at 95°C, the alkali removed by washing with water, and the residue finally treated with alcohol and ether to effect the removal of lipid and water from the purified elastin.

Partridge et *al.* (1955) utilized an exhaustive autoclave procedure to obtain pure elastin from ligamentum nuchae of the ox. The latter is the most commonly used source of tissue for the preparation and purification of large quantities of insoluble elastin. One is usually required to autoclave the previ-

ously milled and saline-extracted material for successive periods of 1 hr until no further protein appears in the supernatant. This treatment, although harsh, yields a preparation of elastin with an amino acid composition that remains constant through further treatment in the autoclave. Elastin purification from aortic tissue cannot be accomplished by autoclaving alone. One is still required to treat the washed aortic material with cold alkali. As pointed out so frequently, exposure to hot alkali and/or exhaustive autoclaving may lead to extensive peptide-bond cleavage, which may not be readily detected in insoluble elastin preparations.

Attempts have been made to avoid the use of such drastic treatments. Hospelhorn and Fitzpatrick (1961) first proposed the use of enzymes to purify elastin. According to their procedure, elastic tissue is extracted with 1 N NaCl, dried, defatted, and then digested alternately with trypsin and collagenase. The product that results has an amino acid composition somewhat different from alkali-extracted elastin. Miller and Fullmer (1966) extracted tissue at neutral pH and 5°C for successive 48-hr periods with 3% NaH_2PO_4, 25% KCl, and 5 M guanidine-HCl. These treatments were followed by incubation of the residue with a purified collagenase. Comparison of the elastin prepared in this manner with that prepared by the Lansing *et al.* (1951) procedure revealed some slight differences in amino acid content.

Ross and Bornstein (1969) reported the isolation of fetal bovine ligamentum nuchae elastin by extraction with 5 M guanidine followed by digestion with bacterial collagenase. The preparation of elastic fibers obtained by this method showed two morphologically different constituents in the electron microscope, a central amorphous region and the surrounding microfibrillar component. The latter was removed by proteolytic enzymes or by reduction of disulfide bonds with dithioerythritol in 5 M guanidine.

A more recent procedure for the preparation of insoluble elastin has employed CNBr to remove all of the nonelastin components. This is possible because elastin does not contain any methionine residues. Rasmussen *et al.* (1975) treated tissues with 5 M guanidine-HCl containing 1% mercaptoethanol, then with a 5-hr autoclaving treatment, and finally with CNBr after the residue from the autoclave had been suspended in 97% formic acid. An important consideration in all of these approaches is what materials are being removed and what remain. For example, great care must be taken in the characterization of the collagenase that is used in the various purification procedures. If there are impurities that might catalyze the hydrolysis of elastin, preparations may well vary from one laboratory to another.

Any method for the preparation of insoluble elastin must approach the problem by removal of all of those materials that are not elastin. It should be clear to the reader that the choice of method of preparation must be made carefully, as the removal of elastin components that are not completely insoluble is possible. Thus, the definition of insoluble elastin is strictly an operational definition that is dependent upon describing the method of purification.

3. Solubilization of Elastin

3.1. Chemical

As elastin is an insoluble polymer of soluble elastin molecules that have been covalently cross-linked *in vivo*, solubilization *in vivo* or *in vitro* can be achieved only by the disruption of peptide bonds or the cross-links themselves, either by the action of the proteolytic enzymes discussed below or by the use of relatively harsh chemicals such as weak acids and bases or oxidizing agents. By successive 1-hr treatments of elastin from bovine ligamentum nuchae with 0.25 N oxalic acid at 100°C, Partridge and Davis (1955) were able to solubilize bovine elastin. This treatment resulted in the formation of two soluble components designated α elastin and β elastin. α-Elastin was found to be polydisperse with an average molecular weight between 60,000 and 84,000, while β elastin was monodisperse and had an average molecular weight of approximately 5500. Examination of the NH_2-terminal residues of these two fractions by the use of fluorodinitrobenzene (FDNB) suggested that the α protein contained approximately 17 chains with an average of 35 amino acid residues per chain, while the β protein was comprised of two chains with an average of 27 amino acids per chain. It was postulated from these data that a significant number of covalent cross-links must be present in elastin. Hall and Czerkawski (1961) have reported the preparation of similar α elastins by digesting elastin with purified pancreatic elastase in the presence of sodium dodecyl sulfate or by treatment with hot ethanolic-HCl or 40% urea. These latter α-elastin preparations as well as that of Partridge *et al.* (1955) are all capable of forming a coacervate at elevated temperatures. The phenomenon is pH dependent, but the optimum varies with the solubilization procedure. For example, the oxalic acid-prepared α elastin yields a coacervate optimally at pH 5.5, while an α elastin prepared with ethanolic-HCl forms a coacervate optimally at pH 9.0. The latter when treated with mild alkali at room temperature resulted in a shift of the maximum coacervation to pH 4.0. It will be seen later that this ability to form a coacervate from soluble elastin preparations was an important tool in the isolation of the soluble precursor to the insoluble amorphous elastin protein.

Robert and Poullain (1963) have found that elastin can be solubilized in 0.1 N KOH in 80% ethanol at room temperature. This solubilized preparation has been designated κ elastin.

Circular dichroism studies of α elastin (Mammi *et al.*, 1968; Urry *et al.*, 1969) have suggested that some parts of the protein chains in water-swollen insoluble elastin, probably less than 10%, exist in the α-helix form. A more complete discussion of the ordered regions of elastin will be described below in the section on tropoelastin.

3.2. Enzymatic

The digestion of elastin by proteolytic enzymes has been examined in many laboratories. Balo and Banga (1949) isolated an enzyme from pancreatic

tissue capable of solubilizing elastin. This enzyme, which was subsequently crystallized, was thought to be specific for elastin and was therefore designated an elastase. Although this elastase does indeed dissolve elastin, it has been shown to attack other proteins as well. Examination of synthetic peptides with highly purified preparations of elastase indicated a strong specificity for alanyl peptide bonds. It is also of interest to note that elastase has esterolytic activity, and very simple procedures can be employed to detect elastase activity in various tissue preparations (Ardelt *et al.*, 1970; Clark *et al.*, 1980; Kagan *et al.*, 1972; Quinn and Blout, 1970; Senior *et al.*, 1971; Stone *et al.*, 1977). Much has been learned of the mechanism of pancreatic elastase solubilization of elastin (Jordan *et al.*, 1974; Visser and Blout, 1969).

More recently, mammalian elastases from polymorphonuclear leukocytes, alveolar macrophages, and platelets have been isolated and characterized (Baugh and Travis, 1976; Hinman *et al.*, 1980; Legrand *et al.*, 1973). They have properties that are quite different from the pancreatic enzyme and appear to have a specificity for leucyl peptide bonds. In cell cultures, elastaselike activities have been detected in rat smooth muscle cells and human fibroblasts (Bourdillon *et al.*, 1980). Pepsin and chymotrypsin have both been shown to attack insoluble elastin slowly (Foster *et al.*, 1976). It should be pointed out that conflicting data have been reported in the latter two cases. Partridge (1962) pointed out that differences in such results may be attributed to differences in the preparations of elastin used. Of the nonmammalian enzymes possessing elastolytic activity, Pronase, papain, ficin, bromelain, and Nagarse are included (Partridge, 1962; Franzblau, 1971). These enzymes are generally considered nonspecific, being capable of hydrolyzing peptide bonds between many different amino acid residues.

A complete description of the degradation of elastin in regard to the turnover of elastin is beyond the scope of this chapter. Suffice it to say that there have been several interesting possible molecular mechanisms of disease proposed that include as a significant event the elastolysis of elastin. The nature of the interaction of elastin and elastase has been described in detail, and its implications in pulmonary emphysema (Karlinsky and Snider, 1978; Stone *et al.*, 1979), atherosclerosis (Robert *et al.*, 1974; Sandberg, 1976), and pancreatitis (Geokas, 1968) have been amply documented.

4. Mechanical Properties

It should be obvious to the reader that studies on the properties of elastin are hampered in interpretation by the problems inherent in the purification procedures mentioned above. Several recent reviews on the subject serve well to outline the details of the findings from several laboratories (Gosline, 1976; Sandberg, 1976).

In his review on the properties of blood vessels, Burton (1954) explained in detail and with great clarity the several parameters that enable blood vessels to resist deformation by the development of a resisting force or "tension." All coefficients of elasticity are defined as the ratio of this resisting force to the

measure of the deformation produced. Accordingly, Burton pointed out that a material possessing "high elasticity" is one that resists deformation, while a material of "low elasticity" does not resist deformation, and a small force to the latter will produce large deformation. Thus, glass or steel has a much higher elasticity than does rubber. Another parameter pointed out by Burton is related to the degree of deformation or stretch that is possible before there is an irreversible change. This characteristic encompasses "tensile strength," "breaking strength," and "yield point." "Tensile strength" refers to a load that produces an irreversible change, while "breaking strength" is the load required to produce actual rupture. The "maximum extension" based on the percentage of the original length before the yield point is reached is also of interest when describing elasticity.

When discussing biological fibers and their elastic properties, one almost certainly encounters Young's modulus, which is defined as

$$F = \frac{Y \cdot \Delta l}{l_0} \cdot A$$

where F is the force in dynes exerted on the material, Δl is the extension produced beyond the nonstretched length l_0, and A is the cross-sectional area in square centimeters. Y, the elastic modulus, is given in dynes per square centimeter for 100% elongation.

The constant (Y) obtained for elastic fibers indicates that the fibers are quite extensible compared to most substances, actually more extensible than rubber (Burton, 1954). A rubber band with a cross-sectional area of 0.04 cm^2 and a 50-g load attached to it can be stretched approximately 7%, while an elastic fiber from aortic tissues of the same cross-sectional area and load can be stretched 40% (Burton, 1954). In summary we can say that elastin possesses a high extensibility combined with a low modulus, which is very similar to the elastic properties of rubber.

It had been shown by Wohlisch *et al.* (1943) and Meyer and Ferri (1936) that the stress–strain curve for elastin, like that of rubber, swings upward at high extensions. However, the rise is more abrupt and occurs at somewhat lower elongations than that of rubber. To explain this, Wohlisch and co-workers suggested that in the case of elastin, crystallization occurs on stretching. However, Astbury (1940) could find no evidence for crystallization based on an examination of the X-ray diffraction patterns of stretched elastin.

Hoeve and Flory (1958) reexamined the elastic properties of unpurified ox ligamentum nuchae and concluded that no crystallization occurs when elastin is stretched. Furthermore, they concluded that the stress–strain curve is explained by volume changes in the insoluble elastic fiber; the abrupt rise in stress at high elongations is attributed to straightening out of the initially curled fibers of collagen that are associated with native elastin in the unpurified ligament. The erroneous results of earlier works were attributed to the fact that they did not take into account the deswelling of elastin in water at elevated temperatures. The data of Hoeve and Flory (1958) were obtained in 30% glycerol, where

the degree of elastin swelling in this solvent is independent of temperature. Thus, thermoelastic measurements carried out in this solvent indicated that all elastic energy is stored as a decrease in the entropy of the system.

Mukherjee *et al.* (1974) reexamined the stress–strain properties of purified ligamentum nuchae elastin. The specimens studied contained two kinds of void spaces. "Macrovoids" resulted from the removal of collagen and proteoglycans, while "microvoids" referred to those spaces between the peptide chains within the bulk elastin molecular regions.

The stress–strain properties of purified elastin immersed in deionized water exhibit three distinct components. At low loads (0–200 g/cm) there is an initial slack in the nonwoven fabriclike structure, which results in an initial low-modulus, linear stress–strain curve. At intermediate loads (200–1000 g/cm) the stress–strain curve is also linear but with a higher modulus, and it was suggested that in this region the stress is now carried by the peptide chains on a molecular level. At both low and intermediate loads, the stress–strain behavior is reversible. At higher loads (1000 g/cm) the fibers begin to slip irreversibly and to rupture. By application of the kinetic theory of rubber elasticity (Flory, 1953), the slopes of the stress–strain curves are related to the molecular weight (M_c) between cross-links. Data were obtained for elastin preparations immersed in both aqueous and nonaqueous solutions. The M_c value of 3400 obtained in deionized water increases with increasing concentration of formamide, reaching a maximum of 7900 at a formamide/H_2O concentration of 44% (v/v). The increase in M_c was shown to be irreversible, and was explained by the fact that the peptide chains in elastin assume a new equilibrium conformation. Similar findings have been obtained in ethanol and butanol solutions. These results are compatible with those of Robert and Poullain (1966) who examined the effect of alcohols on the rate of hydrolysis of elastin in alkali.

Weis-Fogh and Andersen (1970) reported that the internal energy change in stretched elastin was due to the exposure of nonpolar amino acid side chains to the solvent during the stretching itself. They proposed a liquid drop model that attributed part of the elastic force to an interfacial mechanism involving the exposure of hydrophobic groups. Although more recent studies do not appear to support this supposition, it nonetheless is frequently encountered in the literature (Sandberg, 1976).

On the basis of stress–temperature measurements in suitable solvent mixtures, assuming that elastin is an assembly of randomly coiled polypeptide chains, several groups proposed that the molecular basis for its elasticity is the retroactive force resulting from the deformation of the network (entropic elasticity). In this model (Gosline, 1976; Hoeve, 1977) the chains are in rapid random Brownian motion in the swelling medium in which they are dispersed. This is quite different from the liquid drop elastomer and appears to be the more acceptable interpretation of the physical measurements.

Plots of the heat capacity measurements of elastin preparations containing various amounts of water display S-shaped curves, suggesting that elastin forms a glasslike structure at low temperatures. The transition from rubberlike

to glasslike structure occurs over a relatively narrow temperature range. The midpoint of the range is called the glass point. Below the glass point the material cannot be deformed without rupture. Under physiological conditions at 37°C, elastin is rubberlike, which is significantly above the temperature of the glass point. If one assumes that elastin *in vivo* contains 50 g H_2O/100 g elastin, the glass point is below the freezing point of water, while at 25 g H_2O/100 g, the glass point is approximately 20°C. An interesting speculation by Hoeve (1977) suggests that the glass transition of elastin may be likened to the hardening of arteries. Lipid deposition may render elastin more hydrophobic and thus more dehydrated, possibly approaching the glass point.

Gosline (1976), studying the role of hydrophobic interactions in the swelling of elastin, suggested that elastic energy is stored in the reversible exposure of hydrophobic groups to water with stretching. The idea of conformational changes causing the reversible exposure of various hydrophobic groups of elastin is accepted by several laboratories studying the physicochemical properties of elastin.

5. Morphology and Distribution

Early work on elastic tissue was based primarily on the unique staining properties of elastic fibers. These histological properties, together with an ability to stretch when wet, rendered elastic tissue relatively easy to identify. Orcein and Weigert's resorcin–fuchsin, the most widely used dyes, have been shown to stain elastin selectively. Other histological stains that have been used include Verhoeff's iron hematoxylin, Gomori's aldehyde fuchsin, and orcinol–new fuchsin to a lesser extent. Newer fluorescent stains that focus on the aldehyde contents of elastin have been described as well (Keith *et al.*, 1977).

Of the more pronounced characteristics of elastin, its yellow color and strong blue-white fluorescence have been known for many years. Although the compound or compounds responsible for these properties have still not been completely characterized, it is thought that the fluorescent chromophore may be bound to the polypeptide backbone.

Available evidence indicates that there is a sparsity of elastin in skin, tendon, and loose connective tissue. It has been reported that elastin comprises only 2–5% of the dry weight of skin (Varadi and Hall, 1965). On the other hand, relatively large amounts of elastin are found in ligaments and large blood vessel walls. The content of elastin in aorta (Neuman and Logan, 1950; Harkness *et al.*, 1957) is 30–57% and in ligamentum nuchae (Seifter and Gallop, 1966) 78–80%. Recently, elastin fibers have been demonstrated in bovine ear, epiglottis, and laryngeal cartilages. Several studies are presently focusing on the elastin from cartilaginous tissue (Quintarelli *et al.*, 1979).

Morphologically, the fibers of elastin are not similar in all tissues. The tunica media of aorta accounts for the majority of the elastic tissue of aortic vessels. This fiber usually takes the form of 50–60 concentric elastic membranes about 2.5 μm thick that are separated by layers of tissue 6–18 μm thick,

consisting of collagen fibers and elastic fibers, lipid material, smooth muscle cells, and fibroblasts embedded in ground substance. In ligamentum nuchae of the ox, the elastic fibers are thick; under the light microscope, preparations of this elastin appear as short, smooth rodlike fibers of almost circular cross section. The thickness of such fibers appears to be uniform and gives a measure of 6.7 μm for the mean value of the diameter.

Ultrastructurally, elastin is unstained by the cations generally used for enhancing contrast in electron microscopy. When tissues are stained by phosphotungstic acid, a density is revealed on the growing surfaces of elastic membranes and fibers probably due to the deposition of new elastin. The faint inhomogeneities of amorphous elastin are believed to be due to the incorporation of microfibrils within the elastin, as they are readily stained by commonly used uranyl and lead salts. More recently, Cotta-Pereira and his colleagues (Cotta-Pereira *et al.*, 1977) have employed a tannic acid–glutaraldehyde fixative that tends to enhance the stains of the elastic fiber components. In addition, the staining of collagen in the same tissues is preserved. This procedure, which was originally described by Fullmer and Lillie (1958), allowed the identification of three ultrastructurally distinct components of the elastic fibers. The three have been designated oxytalan, elaunin, and elastic fiber. The oxytalan first described by Fullmer and Lillie (1958) was originally thought to be a new connective tissue fiber, but later it was suggested by the same authors that oxytalan may represent an immature or specifically modified elastic fiber, possibly a preelastic fiber. More recently, they suggested that the oxytalan is synonymous with the elastic tissue microfibril.

Gawlik (1965) has demonstrated another fibrous component of connective tissue in cartilage and tendon that were stained by classical methods for elastic tissues but not by Verhoeff's iron hematoxylin or orcinol–new fuchsin. He suggested from studies on elastogenesis in aortae of human fetuses and newborns that the fibers that appear initially in the aorta have the tinctorial characteristics of oxytalan fibers, which progressively acquire the staining properties of elastic fibers and finally appear as elastic laminae. Cotta-Pereira *et al.* (1977) observed the three components in human skin as well. They proposed that a unit, the microfibril or oxytalan, is common to all three components and that the latter appear in sequence. When the amorphous material is present in patches with the microfibrillar component, one has elaunin, and when the amorphous material is abundant and homogeneous, one has elastic fibers. Thus, elastogenesis may be described morphologically as the oxytalan → elaunin → elastic fiber system.

Morris *et al.* (1978) have found that palladium chloride ($PdCl_2$) in aqueous solution stains elastic fibers in thin sections of Epon-embedded tissues. When $PdCl_2$ is used with a lead citrate counterstain, high-contrast sections with gray to black elastic fibers are obtained. Figures 3-1, 3-2, and 3-3 are typical electron micrographs illustrating this technique and the nature of the ultrastructure of elastin. Shown in Figure 3-1 is a section from adult mouse lung, and Fig. 3-2 is from adult mouse trachea stained en bloc. Figures 3-3a,b are extracted preparations of bovine ligamentum nuchae unstained and stained with

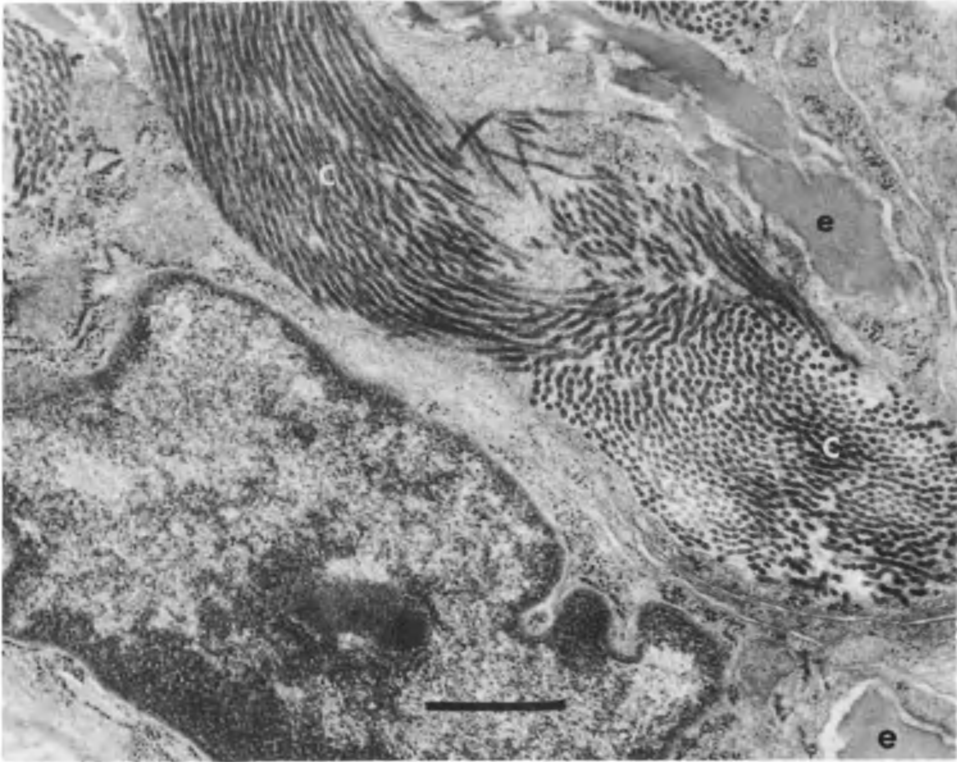

Figure 3-1. Electron micrograph of a section of adult mouse lung, stained with palladium chloride and lead citrate. Elastic fibers (e) show the grayish electron density typical of elastic fiber in adult tissues. C, collagen. Bar = 0.5 μm.

$PdCl_2$ for 15 min, respectively. Chemical studies indicate that palladium binds directly to the purified elastin and that this binding is not affected by glutaraldehyde fixation. Osmium postfixation of glutaraldehyde-fixed elastin did not significantly lower the amount of palladium bound.

Evidence for order in the amorphous matrix of ligamentum nuchae has come from high-resolution electron microscopy. Gotte *et al.* (1974) have demonstrated a filamentous structure in alkali-purified, ultrasonicated elastin by negative staining. The parallel filaments 3–4 nm in diameter displayed an axial periodicity of about 4–4.5 nm corresponding to a "beaded" structure. Regions of the relaxed elastic fibers suggest that the fiber is composed of slender filamentous ropes arranged approximately parallel to the fiber axis but with Y-shaped bifurcations. Every rope consists of a pair of filaments of about 1.5-nm diameter that appear to be cross bound with a periodicity of 3.5–4 nm along the axis. Gotte *et al.* (1974) have proposed that the 1.5-nm filaments are the basic morphological component of elastin in the relaxed state. That they appear as

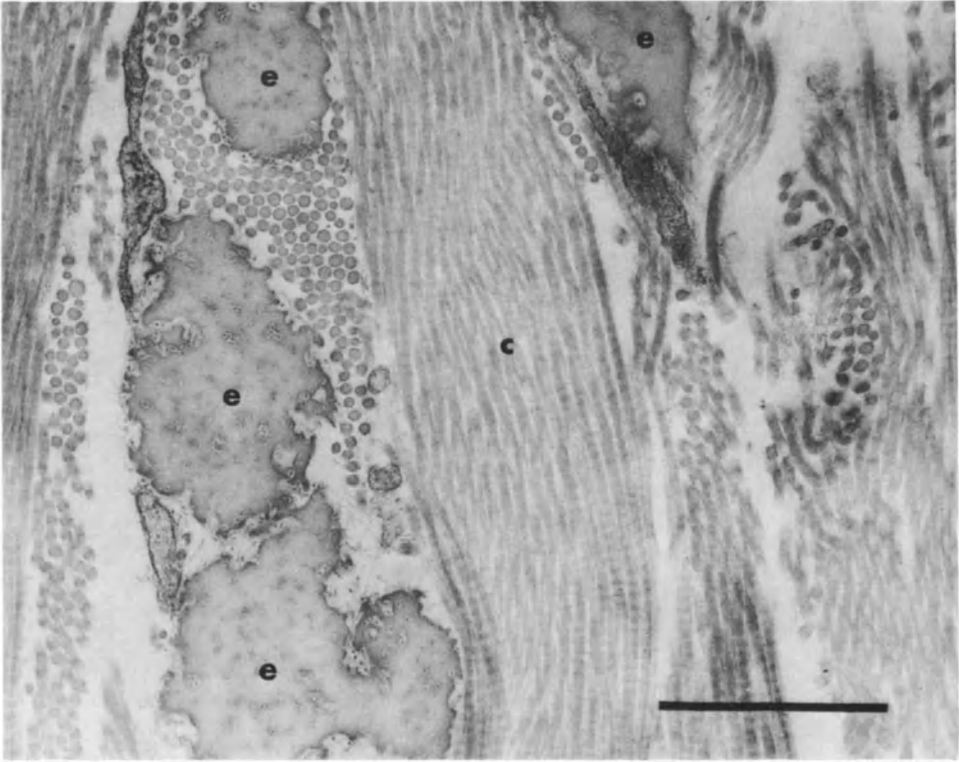

Figure 3-2. Electron micrograph of trachea of adult mouse, stained en bloc with palladium chloride and counterstained in section with lead citrate. e, elastic fiber; c, collagen. Bar = 1.0 μm.

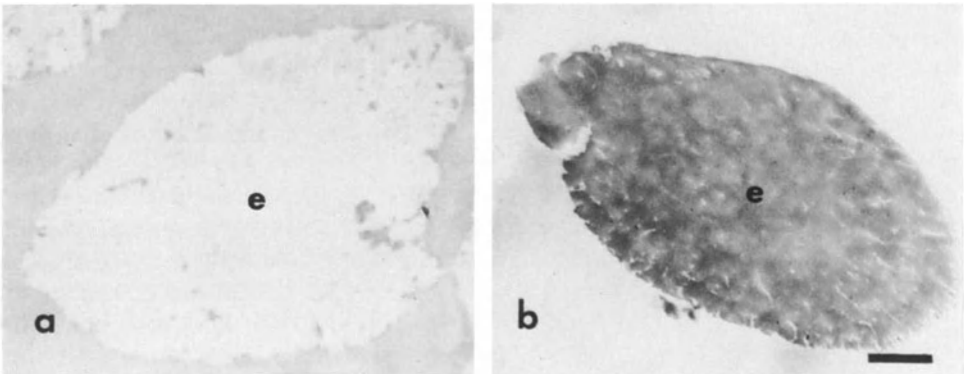

Figure 3-3. Electron micrographs of extracted bovine ligamentum nuchae elastin: (a) unstained; (b) stained with palladium chloride for 15 min. e, elastin. Bar = 1.0 μm.

associated pairs or double helices also suggests that they are periodically interlinked by covalent bonds that do not act as links between adjacent pairs of helices.

6. Amino Acid Composition

Regardless of the method of preparation, the amino acid composition of purified elastin is unique and confirms the hydrophobicity of amorphous elastin. As in collagen, one-third of the amino acid residues in elastin are glycine. One-ninth of the residues are proline, but in contrast to collagen, elastin contains very little hydroxyproline, no hydroxylysine, and a preponderance of the nonpolar amino acids alanine, valine, leucine, and isoleucine. There are very few residues of aspartic acid, glutamic acid, lysine, or arginine. As shown in Table I, the amino acid compositions of elastins from rabbit aorta, bovine ligamentum nuchae, and cultured rabbit smooth muscle cells are similar to one another but considerably different from rat skin collagen. There is no methionine in elastin, and it had been thought that cysteine was also absent. More recently, however, the discovery of two different tropoelastin units (see below) has revealed the presence of cysteine in one of them. Well over 90% of all the amino acid residues of elastin are nonpolar. Because of the preponder-

Table I. Amino Acid Analyses of Various Elastin Preparations[a]

Amino Acid	Bovine ligamentum nuchae	Rabbit aorta[b]	Cultured rabbit aortic smooth muscle cells[c]
Hydroxyproline	7	15	4
Aspartic acid	7	3	13
Threonine	10	10	15
Serine	10	12	17
Glutamic acid	17	17	26
Proline	125	119	125
Glycine	316	345	319
Alanine	213	243	223
Valine	134	99	104
Isoleucine	27	21	23
Leucine	65	54	60
Tyrosine	6	23	27
Phenylalanine	34	19	25
Lysine	4	5	6
Histidine	1	1	5
Arginine	7	6	12
Isodesmosine	1.1	0.7	0.5
Desmosine	1.7	1.1	0.4
Lysinonorleucine	0.9	1.2	

[a] Values expressed as residues per 1000 residues.
[b] Weanling rabbit aorta.
[c] Cells in culture for 20 days after second subcultivation. The source of the cells in culture was the rabbit aorta shown in the third column (Faris et al., 1976).

ance of glycine and alanine in the protein, the average residue weight is approximately 87.

The amino acid that appears to have the greatest variability when one examines preparations of elastin from several species or from different organs in the same species is tyrosine. The latter varies from 6–10 residues per 1000 residues in bovine ligamentum nuchae to approximately 40 residues per 1000 residues in elastin obtained from the swim bladder of the carp. Human aortic elastin, for example, contains 25 residues of tyrosine per 1000 residues. Of special interest is the unusually high content of valine residues in elastin. Approximately 100–140 residues of valine are present per 1000 amino acids.

Of course, insoluble elastin is unique in that it contains several unusual lysine-derived cross-linking amino acids. Desmosine and isodesmosine described by Partridge and his collaborators can be utilized to determine the content of elastin in a tissue in the same manner that hydroxyproline is used for collagen. The nature of the cross-links is described below. Although the amino acid sequence of elastin has not been completely elucidated, much is known of the sequence of the soluble precursor of elastin, tropoelastin (Section 8).

7. Cross-Linking

Because elastic fibers behave as rubberlike elastomers (Hoeve and Flory, 1958), certain key cross-links should be present between the various polypeptide chains that make up the elastic fiber. These cross-links, as Baumann (1959) pointed out, impose a necessary restriction on the elastic fiber such that, upon stretching, the individual chains are constrained so that they do not slip past one another. Release of the tension allows the individual chains to snap back to their original conformation. (In the manufacturing of rubber, the process of vulcanization is used to create cross-linking between chains.) The cross-links in elastin are unique, and the origin of their structure was initially described by Partridge and his collaborators (Partridge *et al.*, 1963) as two unusual amino acids isolated from strong acid hydrolysates of purified elastin. Both were found to be polyfunctional amino acids containing a pyridine nucleus and alkylated in four positions including the ring nitrogen. These two compounds were found to be geometric isomers differing only in the position of ring substitution. The structures of these amino acids are shown in Fig. 3-4 and have

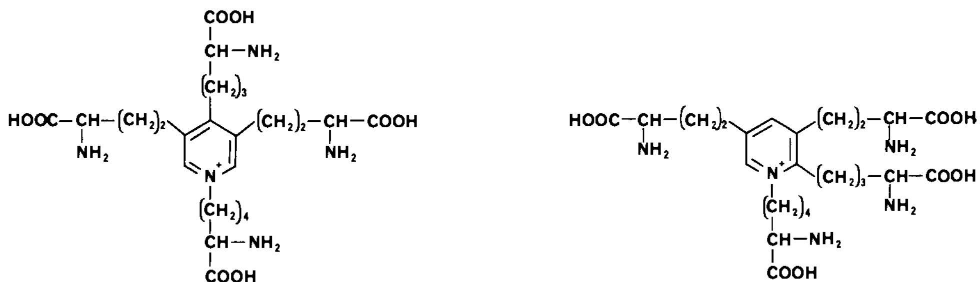

Figure 3-4. Structure of desmosine and isodesmosine.

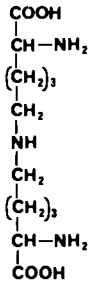

Figure 3-5. Structure of lysinonorleucine.

been designated by Thomas et *al.* (1963) as desmosine and isodesmosine, respectively.

Franzblau et *al.* (1965, 1969) found, in addition to desmosine and isodesmosine, another unusual compound. Isolation and chemical characterization led to the identification of still another amino acid, which was assigned the trivial name of lysinonorleucine. The structure of lysinonorleucine, shown in Fig. 3-5, suggests that it, too, probably serves a cross-linking function in elastin. Still another structure described as the aldol condensation product of two residues of α-aminoadipic acid δ-semialdehyde (allysine) was isolated after reduction with $NaBH_4$ and hydrolysis in alkali (Lent et *al.*, 1969). It structure is shown in Fig. 3-6.

The discovery of these amino acids provided chemical markers for elastin, just as hydroxylysine and hydroxyproline are markers for collagen. Studies by Miller et *al.* (1964) on chick embryo aorta and Partridge et *al.* (1966) on rat aorta suggested that lysine is the precursor of both desmosine and isodesmosine. That lysine is also the precursor of lysinonorleucine and the aldol condensation product was proposed by Franzblau et *al.* (1965, 1969) and Lent et *al.* (1969). Repeating the studies of Miller et *al.* (1964) on developing chick embryos, it was found that the concentration of lysinonorleucine in aortic elastin increased at the same rate as the desmosines.

It is now well established that the covalent cross-links in insoluble elastin are derived from lysine. The deamination of lysyl residues of a precursor soluble elastin molecule is catalyzed by the enzyme lysyl oxidase, a copper-requiring enzyme or family of enzymes specific for the connective tissue proteins collagen and elastin. The resulting aldehydes condense, as described

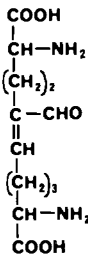

Figure 3-6. Structure of the aldol condensation product of two residues of allysine.

below and illustrated in Fig. 3-7, to form the various cross-links. As the cross-linking process is quite slow, it is not surprising to find significant amounts of intermediate components not yet completely converted into the final cross-links. The use of $NaBH_4$ has aided immensely in describing the structures of these intermediates (Franzblau, 1971). The most common intermediate seen is the aldol condensation product of two allysine moieties. Although schematically illustrated in Fig. 3-7, the biosynthesis of the cross-links will be described briefly in the section on organ and cell culture studies.

8. Soluble Elastins

8.1. Tropoelastin

A major advance in elastin research occurred with the isolation of an elastinlike soluble component from the aortae of copper-deficient swine (Sandberg *et al.*, 1969, 1971). Further purification and characterization of this soluble protein revealed that it is the precursor to insoluble elastin, and thus it was designated tropoelastin.

Soluble elastin, as well as the mature insoluble elastin, is rich in glycine (33%) and the nonpolar amino acids alanine, valine, and proline. As expected,

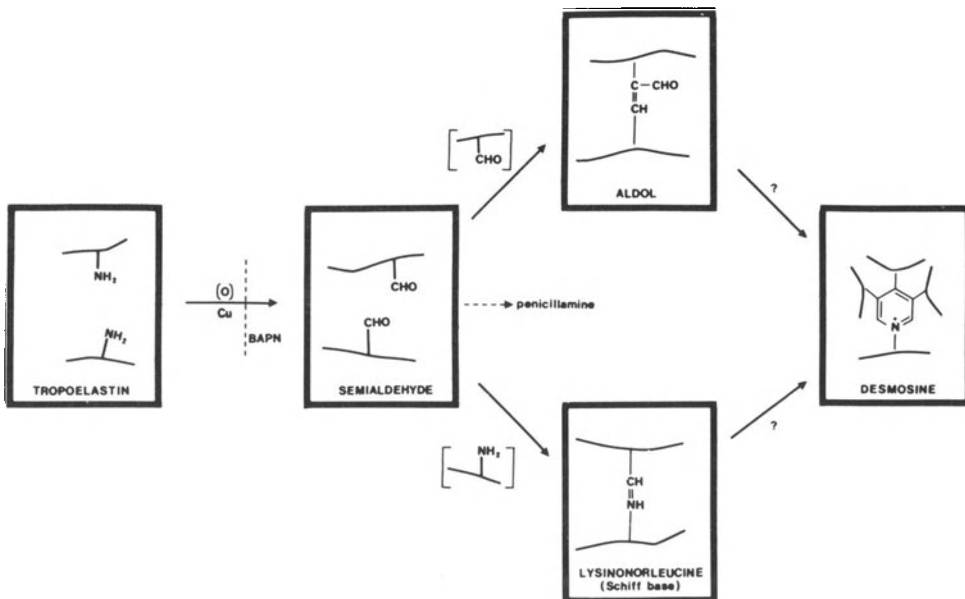

Figure 3-7. A schematic of the formation of the cross-links of elastin. Starting with the precursor soluble elastin (tropoelastin), the lysine residues contained therein undergo a deamination and condensation leading to the cross-links. Also shown is the effect of β-aminopropionitrile (BAPN), Cu^{2+}, and penicillamine.

it differs from the insoluble protein in its high content of lysine residues and lack of any cross-links.

The isolation of sufficient quantities of tropoelastin from either lathyritic or copper-deficient animals has been an obstacle to both structural and metabolic studies of elastin. Recently, Foster *et al.* (1980a) have reported the use of α-aminoacetonitrile in place of β-aminopropionitrile as the lathyrogen. The yields, which were significantly increased, were attributed not only to the ability of α-aminoacetonitrile to inhibit lysyl oxidase but also to inhibit anti-trypsin activity.

Sandberg (1976) has reported the presence of significant clustering of alanine and lysine residues in the soluble protein, as evidenced by the structures of the small tryptic peptides and of the COOH-terminal fragments of the large tryptic peptides. Peptides such as Ala-Ala-Ala-Lys and Ala-Ala-Lys are quite abundant in the tryptic digests of tropoelastin. This clustering of alanine near the lysine residues of the molecule is in agreement with the isolation of alanine-enriched, cross-linked peptides from mature elastin. Desmosine-containing peptides have been shown to be consistent with the Ala-Ala-Ala-Lys sequence as indicated in Fig. 3-8. Through the characterization of other large tryptic peptides, Foster *et al.* (1973) have been able to sequence over 400 residues, i.e., approximately half of the residues present in the tropoelastin molecule.

To date, the sequences obtained reveal a tropoelastin primary structure quite distinct from that of tropocollagen. Tropoelastin possesses repeat units of a tetrapeptide, Gly-Gly-Val-Pro, a pentapeptide, Pro-Gly-Val-Gly-Val, and a hexapeptide, Pro-Gly-Val-Gly-Val-Ala. In addition, as noted above, alanine residues are close to the lysyl residues of elastin. Several of the peptides contain a partial substitution of hydroxyproline for proline, especially in the sequences

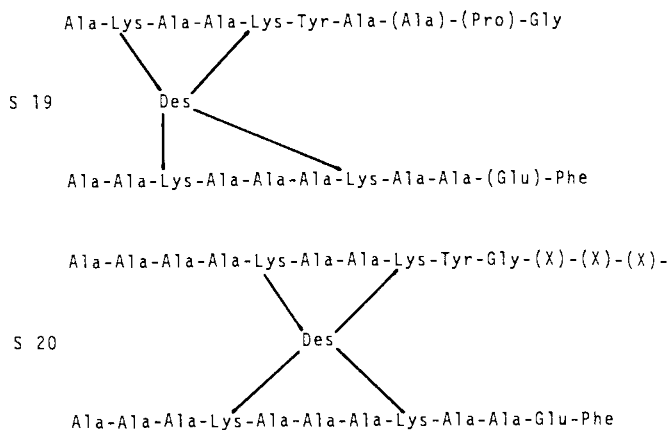

Figure 3-8. Illustration of sequence data obtained from solubilized peptides of elastin. The connections shown between Lys and Des denote the arms of the desmosine cross-links, which are not detected as phenylthiohydantoin derivatives. X, unknown residue. (From Franzblau *et al.*, 1977.)

Gly-Leu-Pro-Gly and Gly-Ile-Pro-Gly. As the sequences of these peptides resemble those of other tropoelastin peptides and also lack glycine in every third position (contrary to collagen), this is definitive evidence for the existence of hydroxyproline in elastin. The role of hydroxyproline in soluble elastin is unknown. *In vitro*, Schein *et al.* (1977) have been able to hydroxylate 44% of all the available proline in tropoelastin. The combined data of the sequences from the soluble elastin have led to a model of elastin that resembles a huge extension of coiled springs with the desmosines located in alanine-rich areas (Gray *et al.*, 1973). These have been noted as "oiled coils."

8.2 Relationship of Tropoelastin to Insoluble Elastin

The sequence findings are consistent with respect to studies of insoluble bovine ligamentum nuchae elastin digested with elastase and Nagarse. A fraction was isolated by ion-exchange chromatography that proved to contain the tetrapeptide Gly-Val-Pro-Gly. This peptide accounts for 9–11% of the glycine, valine, and proline residues in bovine ligamentum nuchae elastin. However, the authors (Crombie *et al.*, 1973) point out that these values are minimal and assume that 100% recovery from the chromatographic procedures utilized was accomplished. These findings are in agreement with those from tropoelastin in that the Gly-Val-Pro-Gly sequence occurs 10 times in the sequences thus far determined. If one looks for other tetrapeptide sequences containing at least three of these four amino acids in that given primary order, the number increases many times more so that the sequence Gly-Val-Pro-Gly or a minor variation of it appears to account for a large part, perhaps as much as 25%, of the extensible portion of the molecule. In addition to this, nearest-neighbor relationships indicate that in the extensible portion of the molecule, glycine is usually preceded by valine, leucine, and isoleucine (79%) as well as often being followed by these (58%). These findings lend credence to the generalization that the tetrapeptide Gly-Val-Pro-Gly or a variation of it has significance with respect to the overall structure of the molecule, perhaps akin to the Gly-X-Y repeats of collagen.

8.3. Peptide Models of Tropoelastin

On raising the temperature of aqueous solutions of tropoelastin, or of α elastin as noted earlier, a phase separation (coacervation) occurs in which the molecules order themselves into a filamentous structure. Urry and his collaborators (Cox *et al.*, 1973, 1974; Volpin *et al.*, 1976) have studied the nature of repeat peptide sequences similar to those found in tropoelastin and found that they behave similarly. The polypentapeptide (Val-Pro-Gly-Val-Gly)$_n$ and the polytetrapeptide (Val-Pro-Gly-Gly)$_n$ undergo similar phase transitions and

have been extremely interesting polymers to study as models of elastogenesis (Volpin et *al.*, 1976). When these peptides as well as tropoelastin and α elastin undergo the phase transition or coacervation the molecules order themselves into filamentous structures with periodicities verified by optical diffraction of electron micrographs (Volpin et *al.*, 1976). The synthetic polypentapeptide fibers, when cross-linked with flow orientation to form a matrix, are elastomeric and can, with the appropriate water content, have the same elastic modulus (Young's modulus) as fibrous aortic elastin (Urry et *al.*, 1976). Most recently, Kagan and his collaborators have shown (Kagan et *al.*, 1980) that this same polypentapeptide is a substrate for lysyl oxidase when valine is replaced by a lysine residue.

8.4. Other Soluble Elastins

Recent advances in the study of soluble elastin precursor components have been somewhat confusing. A high-molecular-weight (100,000–140,000) soluble precursor of elastin, termed proelastin, was reported (Foster et *al.*, 1978), and evidence from organ culture studies indicated that this molecule was the gene product in elastin biosynthesis *in vivo*. However, more recent studies using cell-free translation systems with RNA preparations from several sources have not confirmed the appearance of this high-molecular-weight substance. It is now felt that this high-molecular-weight species is possibly a posttranslational product of two different but closely related soluble tropoelastins designated tropoelastin A and tropoelastin B. Tropoelastin B is the classic tropoelastin that had been originally detected as described in Section 8.1. This component has a molecular weight of approximately 70,000. Tropoelastin A has been observed in cultures producing soluble elastin and has been estimated to have a molecular weight of 73,000. The distribution of tropoelastins A and B appears to vary from tissue source to tissue source. It is felt by some investigators that these two soluble precursors may be distributed in different proportions to impart different biologic properties. It is tempting to speculate that the 140,000-molecular-weight proelastin species may be a dimer of tropoelastin A and tropoelastin B (Foster et *al.*, 1980b).

8.5. Sequencing of Tropoelastin

Although much is known of the sequence of tropoelastin, its amino acid sequence is not truly known. Approximately 30–40 residues from the NH_2 terminal have been sequenced from several species. Most of these studies were carried out before it was discovered that there are two different tropoelastins. Although many peptide sequences have been described, the sequence of the molecule (~ 800 residues) has not been determined, and much work remains to be done. It is most likely that this will be most readily accomplished by sequencing the complementary DNA of the mRNA of the tropoelastins.

8.6. Antigenicity of Elastin and Its Derivatives

It should be noted that antibodies have been produced to tropoelastin, α elastin, purified insoluble elastin, and desmosines conjugated to albumin (Mecham and Lange, 1981). Significant cross-reactivity among these antibodies has been shown to occur in these systems. Although some difficulties are inherent, RIA and ELISA methods have recently been developed and should aid significantly in studies on the biosynthesis of elastin *in vivo* and in cell cultures as well.

8.7. Cell-Free Translations

Recently, several laboratories have reported studies employing cell-free translation systems (Foster et *al.*, 1980b). Translation of chick aortic mRNA in an mRNA-dependent reticulocyte lysate resulted in the synthesis of two major proteins of molecular weight 70,000 and 73,000. Both proteins were shown to be soluble forms of elastin by isotope incorporation, immunoprecipitation, collagenase, and CNBr. As noted earlier, there was no evidence for a high-molecular-weight form of soluble elastin. Proteins of the same molecular weight were also seen in organ cultures of chick embryonic aorta labeled with [^{3}H]valine. The authors concluded that elastin is first synthesized as two distinct polypeptide chains that differ slightly in size and overall charge.

9. Microfibrils

Several investigators have described the appearance of microfibrillar material in association with developing elastin (see the excellent papers by Ross and Bornstein, 1969; Robert et *al.*, 1971). These fibrils are susceptible to digestion by proteolytic enzymes such as trypsin, chymotrypsin, and pepsin. With the use of dithiothreitol, a disulfide-bond reducing agent, solubilization of the microfibrillar material is possible. In intact young elastic fibers, this component represents about 8% of the fiber, and amorphous elastin the remaining 92%. The microfibrils are rich in polar amino acids and lack hydroxyproline, hydroxylysine, and the lysine-derived cross-links. They have a very high cystine content (70–80 residues per 1000 residues), resulting in the numerous disulfide linkages responsible for its relative insolubility. Sherr et *al.* (1973) have pointed out that these microfibrils have an amino acid composition resembling that of the NH_2- and COOH-terminal extensions of the procollagens.

Because tropoelastin appears to contain no sugar moiety, the distinction between it and the microfibrils is quite clear, as the latter are composed of glycoprotein. The microfibrils may help align the tropoelastin molecules in a precise array for appropriate cross-linking. During elastogenesis, microfibrils appear in the form of aggregates that gradually assume the shape and direction

of the future elastic fiber. With increasing maturity, insoluble elastin begins to form between each bundle of microfibrillar material. By prolonged osmium tetroxide fixation, Cliff (1971) was able to reveal an underlying fibrillar structure in mature elastin, presumably representing penetration of the microfibrillar material into the elastin structure. Thus, the microfibrils can be visualized as a scaffolding for the alignment of tropoelastin molecules prior to the oxidation of their peptide-bound lysine. Kadar *et al.* (1973) have observed the microfilaments of the elastin of pig aorta as a beaded structure of 120-Å diameter on which larger granules of 180-Å material can be seen. They propose that these large granules represent noncross-linked tropoelastin molecules aligned on the microfibrils. As noted earlier, the detection of these microfibrils by electron microscopy is facilitated by procedures employing tannic acid.

10. Formation of Elastin in Cell and Organ Cultures

As elastic fibers are extracellular connective tissue matrix components, the biosynthesis of the insoluble protein component, elastin, has to have its origin in the intracellular protein synthetic machinery. Following its secretion from the cell, elastin is incorporated through cross-linking into the fiber. By examining the end product, the insoluble elastin, one is able to learn something of a cell's or tissue's ability to (1) synthesize protein, (2) hydroxylate prolyl residues, (3) transport macromolecules to the extracellular milieu, (4) assemble larger protein aggregates, and (5) form connective tissue cross-links. Studies of this type can yield insight into the mechanisms of elastin turnover.

The study of elastin synthesis in cell and organ cultures has been limited because very few culture systems are capable of producing insoluble elastin. Thus far, the only cells that have been reported capable of producing insoluble elastin in culture are vascular smooth muscle cells, human endothelial cells from umbilical cord veins (although there is not uniform agreement on this), chondroblasts, and, indirectly, fibroblasts from bovine ligamentum nuchae.

As noted by several laboratories, aortic smooth muscle cells in culture are capable of synthesizing both insoluble collagen and elastin. The soluble precursors to these connective tissue proteins have also been identified in these same cell cultures, while insoluble collagen and elastin fibers have been shown to accumulate over long periods of time. More recently, these cultures have been shown to accumulate glycosaminoglycans in exactly the same proportion as found in the donor rabbit aorta (Namiki *et al.*, 1980). It should be pointed out, however, that vascular smooth muscle cells from many species do not produce elastin in culture even under the same conditions. For example, it has been shown that calf smooth muscle cells do not produce insoluble elastin. Addition of different agents at various times during the life of a culture has pronounced effects on insoluble elastin formation in culture. A recent report (DeClerk and Jones, 1980) suggests that addition of ascorbate to rat smooth muscle cells stimulates the formation of insoluble collagen fibers, and at the same time inhibits the accumulation of insoluble elastin. These results have now been

confirmed in rabbit smooth muscle cell cultures. The mechanisms involved in insoluble elastin formation and accumulation are quite important and may well relate to the collagen and proteoglycan components of the connective tissue matrix. Having a cell culture that can predictably produce insoluble elastin allows one to evaluate in a systematic manner elastin biosynthesis and turnover.

There are relatively few criteria that are relied upon to substantiate the formation of insoluble elastin in a cell culture. Taking care that no preexisting insoluble elastin is present in the culture system, these include (1) evaluation of the culture by ultrastructural analyses, (2) evaluation of the insoluble elastin formed by amino acid composition of the product obtained after purification of the elastin, (3) detection and possible quantification of the formation of the unique lysine-derived cross-links and (4) detection and quantification of the precursor molecules of insoluble elastin. It is suggested that no one criterion is sufficient for providing evidence that a cell culture is producing insoluble elastin.

The sequence of appearance of the two components, the amorphous elastin and the microfibrillar component, of the elastic tissue in primary 5-day-old rat aorta smooth muscle cells grown in culture for 28 days has been reported by Hinek and Thyberg (1977). Initially, the tissue is comprised of small bundles of microfibrils that become associated with small conglomerates of a dense amorphous material. Later, in the more mature elastic fibers (older cultures), such material appears to form confluent amorphous areas in which it is difficult to detect microfibrils.

The extracellular ultrastructure of smooth muscle cells in culture can be examined for several weeks from the time of seeding. Fig. 3-9a is a typical micrograph of a pulmonary artery smooth muscle cell culture at 14 days after the second passage. Little amorphous elastin is detected, but there are significant quantities of the microfibrillar component. As the cell cultures become older, there is an increasing amount of insoluble elastin fibers (Fig. 3-9b). If ultrastructural analysis indicates the presence of insoluble elastin in a cell culture, the results should be corroborated, if possible, by chemical studies. The exact time in culture at which insoluble amorphous elastin can be detected by electron microscopy may vary in different culture preparations.

Insoluble elastin fractions can be prepared from cultures of rabbit aortic smooth muscle cells (Faris *et al.*, 1976) by treating the cell layer with hot alkali as described by Lansing *et al.* (1951). The amino acid composition of the insoluble elastin is quite consistent throughout the age of the culture, except for very early and late in the second passage. Early in the passage the amino acid composition of the insoluble elastin preparation from the cultures contains more acidic amino acids than normal. This most likely reflects a higher proportion of the microfibrillar component in the early developing elastic fiber. Older cultures also contain higher contents of polar amino acids suggesting accumulation or association with other glycoprotein components, similar to the observation made by Lansing *et al.* (1952) in aged human tissues. However, the total elastin content from these cell layers was found to increase with time in culture.

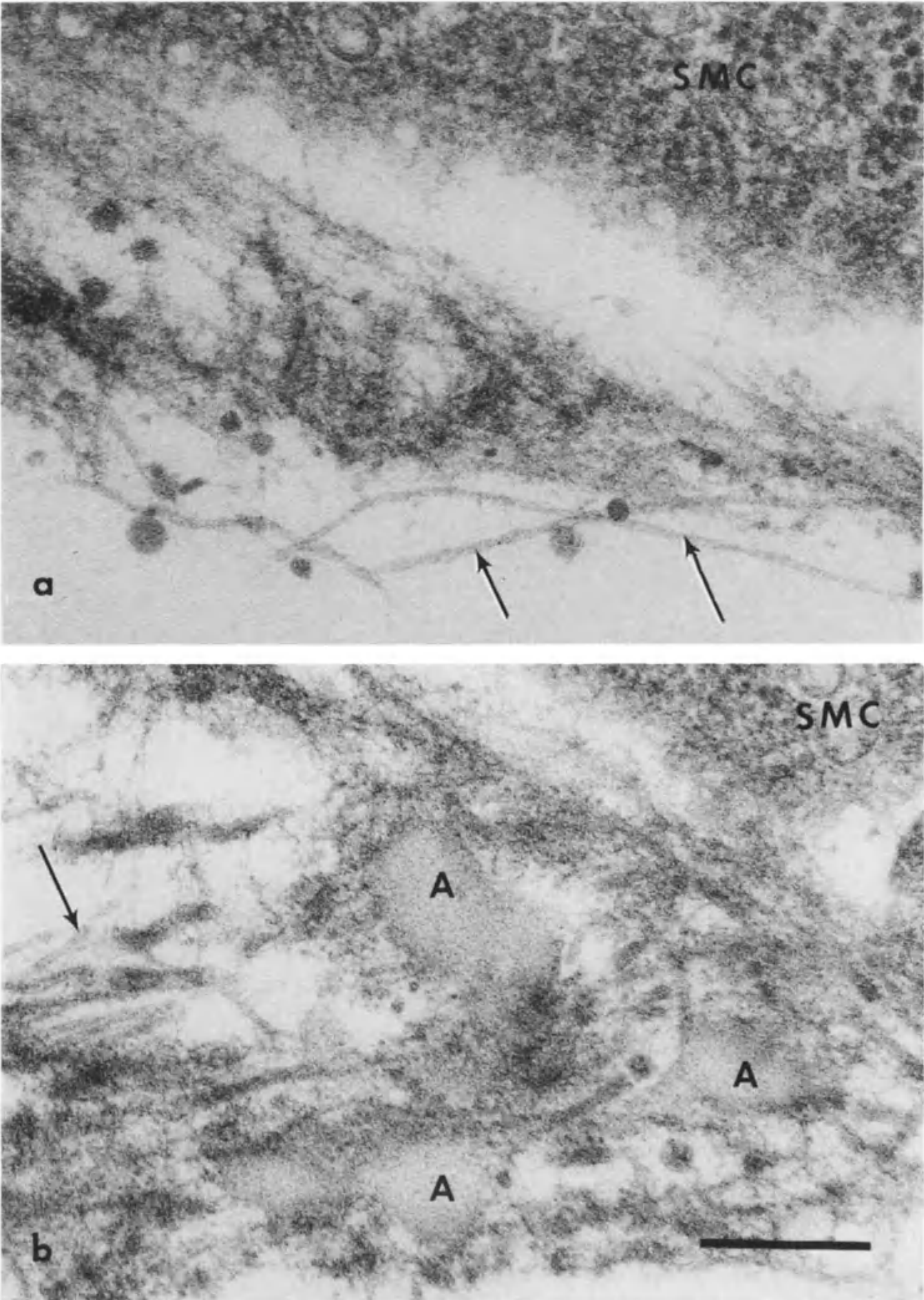

Figure 3-9. Electron micrographs of sections of pulmonary smooth muscle cells in culture for (a) 14 days and (b) 53 days after second subcultivation. SMC, smooth muscle cell; arrows, microfibrillar component; A, insoluble amorphous elastin. Bar = 0.2 μm.

It should be noted at this time that the desmosine content in the NaOH-insoluble elastin from the cell cultures, while significant, is less than that found in either the aortic or the ligament elastin (see Table I).

Knowing that lysine is the precursor of the unusual cross-linking amino acids, one may then ask how they are synthesized or incorporated into the structure of elastic fibers. Recent studies have yielded some insights into the cell or cell type responsible for the biosynthesis of elastin. It may well be that several cell types in culture have the capacity to produce elastin but because of the culture conditions do not accumulate insoluble elastin.

No matter which cell or cell types are responsible for the synthesis of elastic fibers, the first step in the biosynthesis of elastin requires the formation of a soluble precursor of elastin (recognizable by its amino acid composition). Compared to mature elastin, these precursor molecules contain little or no desmosine, isodesmosine, or lysinonorleucine residues, but relatively more lysine. As noted earlier, there may be more than one type of tropoelastin, just as there are different types of collagen. The tropoelastins must then be extruded from the cell and incorporated into the insoluble elastic fiber in a ratio commensurate with the tissue structure and mechanical needs. The cross-linking of the tropoelastins to the insoluble developing elastic fiber probably occurs via the following sequence of events (see Fig. 3-7).

1. A lysyl oxidase-catalyzed deamination of the ϵ-amino groups of specific lysine residues along the polypeptide chain occurs; this leads to the formation of residues of the corresponding aldehyde of lysine (allysine). Any reagents that interfere with this step will prohibit cross-linking. Substances such as β-aminopropionitrile, a known lathyrogen, are routinely employed in cultured systems when inhibition of cross-linking is desired. Substances such as D-penicillamine also interfere with cross-linking at this step, but the mechanism is not related to inhibition of the enzyme but rather to the interaction of the thiolamine groups of the penicillamine with the aldehyde group of the allysine moiety (Franzblau, 1971).
2. These aldehyde residues are then capable of interacting with other aldehyde residues on other tropoelastin molecules or other aldehyde residues on the same molecule. When two aldehydes combine, an aldol condensation occurs.
3. Alternatively, one aldehyde residue may combine with an ϵ-amino group of a lysine residue that has not been deaminated; this would result in the formation of a Schiff base.
4. One aldol condensate and one Schiff base may combine to form a desmosine or isodesmosine. The reaction involves an additional oxidation, the origin of which is still unknown.

If, in any of the steps outlined, the aldehyde component(s) or the ϵ-amino group of the lysine originates from a lysyl residue already incorporated in the fiber, then incorporation of the soluble elastin molecule into the fiber would occur. Note that at one step a dehydrolysinonorleucine is formed.

Data from cells pulsed with precursor amino acids, such as proline and lysine, and then chased for various times corroborate that there is an accumulation of insoluble elastin in rabbit aortic smooth muscle cell cultures (Snider *et al.*, 1981). The data also suggest that desmosine formation is quite slow although cross-link formation (lysine-derived aldehydes) begins rapidly. The protein-bound [^{14}C]proline associated with the cell layer was seen to decrease with time, whereas the [^{14}C]hydroxyproline in this same fraction remained constant throughout the first 2 weeks of the chase period. These observations would suggest that the turnover of collagen and elastin is much slower than the overall protein turnover in these cultures. However, the radioactive hydroxyproline which remains insoluble after the hot alkali treatment (elastin fraction) increased continually during the chase period, indicating that more of the elastin synthesized during the pulse period becomes insoluble during the chase period.

From those cells pulsed with lysine, one is able to examine the synthesis of both the desmosine and the precursor aldehyde. The desmosine content is determined by standard acid hydrolysis of proteins followed by amino acid analysis or by HPLC (Faris *et al.*, 1981).

An interesting point to be made relates to the definition of insoluble elastin presented earlier (Section 2). The formation of insoluble elastin in *in vitro* cell culture systems has been the focus of several laboratories in recent years. *In vivo*, the disruption and/or lack of synthesis of the amorphous elastin component of elastic fibers leads to devastating diseases, such as pulmonary emphysema and perhaps atherosclerosis. Studies on the biosynthesis of the insoluble elastin fiber in cell culture can provide valuable information to our understanding of the formation and turnover of this important connective tissue component. Before such studies can be fully appreciated, the nature of the precursor soluble molecules of elastin, the mechanism of secretion from the cell and incorporation into a functional fibrous entity must all be accomplished.

11. Concluding Remarks

It is evident that elastic fibers are important components of several but not all extracellular matrices. This chapter was designed to give the reader an overview of the important components of elastic fibers and to illustrate through their molecular and ultrastructural properties the nature of their biologic function. There are still many gaps in our knowledge of this system. Most assuredly, the workers in the field are hampered by the insolubility of the protein elastin and the almost infinite molecular weight that elastin bears when it is present as a highly cross-linked elastomer of tropoelastin molecules enmeshed in a framework of microfibrils. Only when the two components have been fully dissected and described on a molecular level will we be able to understand the mechanisms of elastic fiber formation.

It should also be clear from the scope of this treatise that elastic fibers are not formed in a vacuum. The influence that the other extracellular matrix

components have on the formation of elastic fibers and vice versa is completely unknown. Yet, if we are to fully understand the assembly of an extracellular matrix, these questions must be answered. One surely has come a long way since 1902 and the definitive study by Richards and Geis, but complete understanding of the structure and function of elastic fibers is still not within our grasp.

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Chapter 4

Fibronectin and Other Structural Proteins

KENNETH M. YAMADA

1. Introduction

Extracellular matrices and basement membranes contain several other important proteins in addition to the well-known macromolecules collagen, elastin, and proteoglycans. Until these additional proteins were identified by high-resolution biochemical techniques, they were described collectively as "noncollagenous structural proteins" (e.g., Robert et *al.*, 1970).

An exciting recent advance has been the purification and functional analysis of several of these glycoproteins, which are now known as fibronectin, laminin, and chondronectin. It is possible that more glycoproteins in this class will be described in the near future. The proteins that have been characterized have interesting properties that include roles in cell adhesion and behavior, as well as binding interactions with other matrix constituents such as collagen.

2. Fibronectin

2.1. Types of Fibronectin

The glycoprotein fibronectin [*fibre* = fiber + *nectere* = to bind, tie] exists in at least two forms, *plasma* fibronectin and *cellular* fibronectin (Yamada and Olden, 1978; Vaheri and Mosher, 1978; Hynes et *al.*, 1979; Mosher, 1980; Pearlstein et *al.*, 1980). These two types of fibronectin have very similar molecular properties (Table I). They have nearly identical amino acid and carbohydrate compositions, as well as indistinguishable secondary and tertiary polypeptide structures according to spectrophotometric studies (Yamada et *al.*, 1977; Vuento et *al.*, 1977; Alexander et *al.*, 1979). Moreover, antibodies raised against either type of fibronectin reportedly cross-react completely with the

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other type within the same species, and each antigen completely absorbs out reactivity with the other (Ruoslahti and Vaheri, 1975).

Yet, these types of fibronectin do differ in certain biological activities (Chapter 10, this volume; Yamada and Kennedy, 1979) and, moreover, plasma fibronectin is slightly smaller than cellular fibronectin (Table I). The difference in apparent molecular weight is preserved in proteolytic fragments of the proteins, suggesting that there may be structural differences greater than simple proteolytic processing of one molecule to yield the other (e.g., Hahn and Yamada, 1979a). Unlike plasma fibronectin, cellular fibronectin is relatively insoluble unless maintained at a highly alkaline pH. The purified molecule readily self-aggregates and adheres tenaciously to a variety of substrates (Hynes et *al.*, 1976; Yamada et *al.*, 1977). Recent monoclonal antibody and proteolytic structural analyses have identified at least three sites of apparent polypeptide difference between cellular and plasma fibronectins (Atherton and Hynes, 1981; Hayashi and Yamada, 1981).

Plasma fibronectin is a soluble glycoprotein that circulates in blood at a concentration of approximately 0.3 mg/ml. Molecules of a similar size that share the property of binding to denatured collagen have been reported in mammals, birds, and fish (Mosesson et *al.*, 1975; Engvall et *al.*, 1978). Cellular fibronectin is produced by a number of cell types when cultured *in vitro*, and is present on the cell surface, in extracellular matrices, and as a secreted protein in culture media (Chapter 10).

2.2. Location of Fibronectin

Fibronectin *in vivo* has been localized by immunohistochemistry in a variety of mesenchymal tissues, in extracellular spaces, and in association with certain basement membranes (Linder et *al.*, 1975; Stenman and Vaheri, 1978; Chapter 10, this volume). One uncertainty with immunological localization is that the form of fibronectin that is present in each tissue or structure is unknown, because the antibodies generally used to detect fibronectin could not distinguish between plasma and cellular fibronectin (Chapter 10). During embryonic development, quantities of fibronectin change in specific tissues; these developmentally regulated alterations and their possible significance in differentiation are reviewed elsewhere (Chapter 10, this volume; Yamada, 1980).

Fibronectin is associated with most, but not all, basement membranes (basal laminae). Although reportedly present in basement membranes of blood vessels and the kidney mesangium, fibronectin is apparently present only in low amounts in the normal kidney glomerular basement membrane (Stenman and Vaheri, 1978; Bray, 1978; Weiss et *al.*, 1979; Courtoy et *al.*, 1980). Cultured endothelial cells maintain fibronectin on their basal surfaces, in a structure that resembles a basement membrane (see Gospodarowicz and Tauber, 1980). Ultrastructural immunohistochemistry indicates that fibronectin is present in both the lamina externa and the lamina interna of the corneal basal lamina (Fig. 4-1) and glomerular basement membrane

Table I. Molecular Properties of Fibronectin[a]

	Cellular fibronectin	Plasma fibronectin
Molecular weight	450,000 220,000–240,000 (M)	450,000 215,000–220,000
Subunit structure	Disulfide-linked dimers and multimers	Disulfide-linked dimers
Sedimentation constant	8.5 (pH 8) 7.6 (pH 11)	12.3–13.0 (pH 7) 8.0 (pH 11)
Partial specific volume	0.717	0.72
Frictional ratio	2.9 or 2.8 (M) at pH 11	2.8 (pH 11); 1.7 (pH 7)
Stokes radius	110 Å (M) at pH 11	
Extinction coefficient at 280 nm ($E^{1\,cm}_{mg/ml}$)	1.2	1.28
Circular dichroism (far-UV) minimum/maximum	212 nm/227 nm	213 nm/227 nm

[a] Values for fibronectin monomer are indicated by (M) within the table.

(Chapter 11). *In vivo*, fibronectin is associated with striated collagen fibrils (Fig. 4-1), unstriated fibrils 10 nm in diameter, and amorphous interstitial bodies (Chapter 10).

Studies using tissue culture have shown interesting variations in the location of fibronectin in different cell types. The cellular fibronectin of fibroblasts is initially located underneath and in between cells, in locations suggesting a role in cell adhesion (Chen *et al.*, 1976; Culp, 1976; Mautner and Hynes, 1977; Yamada, 1978; Hedman *et al.*, 1978). As cells become confluent, the fibronectin forms an extracellular matrix that completely surrounds the cells (Fig. 4-2). In chick fibroblast cultures, formation of this matrix does not appear to require collagen, although these two proteins are often codistributed (Chen *et al.*, 1978; Vaheri *et al.*, 1978).

In all of these locations *in vitro*, cellular fibronectin exists as aggregates and fibrils; this supramolecular organization might be expected from its known insolubility under physiological conditions. Interestingly, plasma fibronectin from other species that is present in serum used in culture media can also be found in fibrillar arrangements (Hayman and Ruoslahti, 1979). It is not known whether such plasma fibronectin is forming fibrils due to interactions with other molecules such as collagen, or due to formation of complexes with preexisting cellular fibronectin. The latter mechanism of fibronectin binding to the cell surface has been demonstrated by adding fibronectin labeled with a

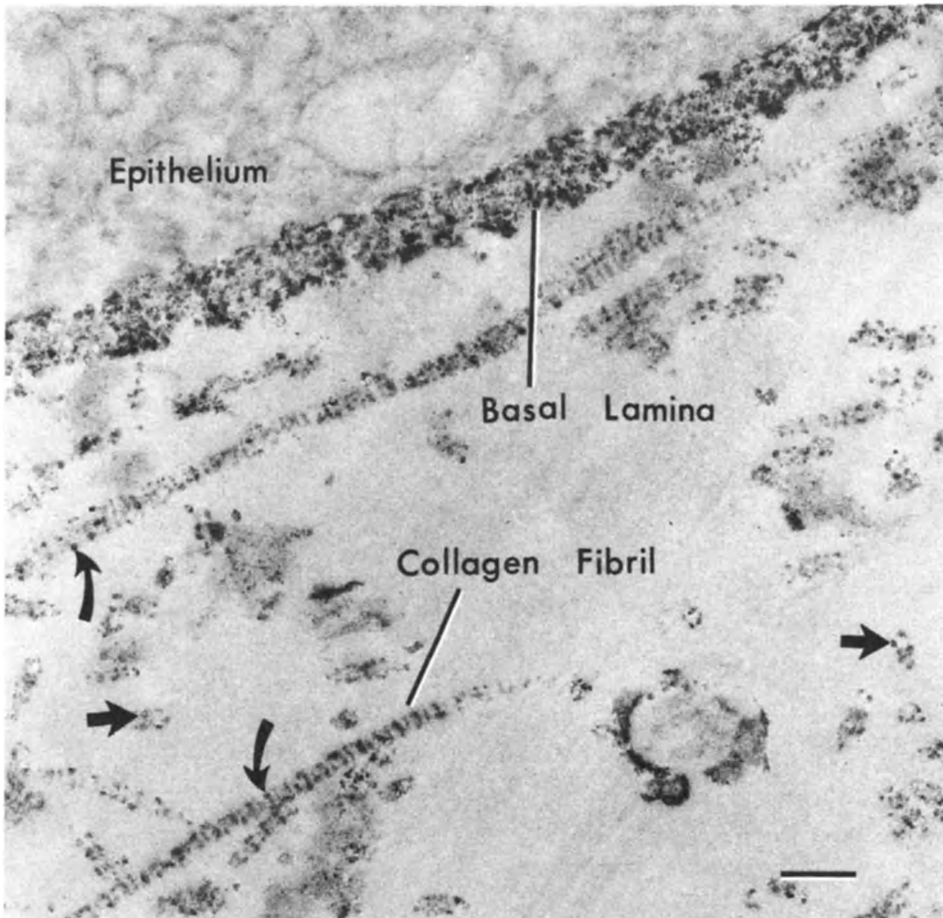


Figure 4-1. This electron micrograph of the embryonic avian cornea at 10 days of incubation shows the distribution of fibronectin in the basal lamina (basement membrane) and along the collagen fibrils. The tissue was stained en bloc with anti-fibronectin followed by link antibody and peroxidase–anti-peroxidase as described by Mayer *et al.* (1981). The peroxidase aggregates localizing the tissue antigen appear granular (arrows). The layer of the basal lamina next to the epithelium (external lamina rara) and the layer next to the stroma (internal lamina rara) are more densely stained for fibronectin than is the lamina densa. These same laminae rarae appear electron-lucent in routine electron micrographs, but in ruthenium red-fixed material are seen to contain glycosaminoglycans (see Fig. 2-12). The distribution of fibronectin along the striated collagen fibrils is also similar to the distribution of glycosaminoglycan, and appears periodic. In other regions of the embryo, fibronectin may occur in nonstriated fibrils. Bar = 500 nm. (Courtesy of B. W. Mayer, Jr., E. D. Hay, and R. O. Hynes.)

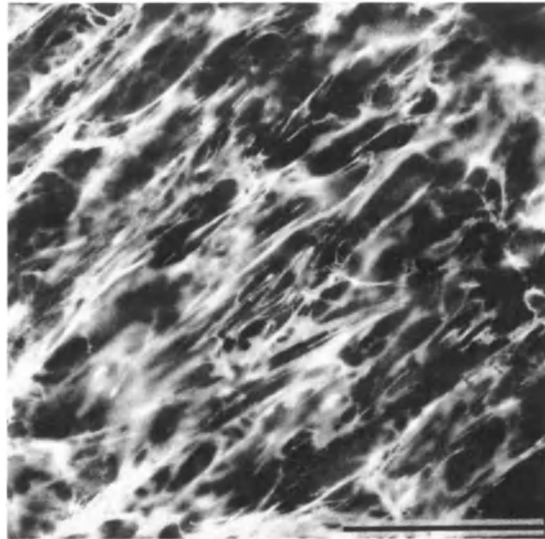


Figure 4-2. Fibronectin in the extracellular matrix. Fibronectin was localized by immunofluorescence using anti-fibronectin antibodies labeled with fluorescein. Cellular fibronectin is found to exist as fibrils on the cell surface and in the extracellular matrix of this densely confluent culture of chick embryo fibroblasts. Bar = 50 μm.

fluorescent dye to cells. It binds to preexisting fibronectin fibrils rather than to new sites (Schlessinger *et al.*, 1977).

2.3. Molecular Properties of Fibronectin

Fibronectin's structure has been elucidated by a number of physical techniques. Its molecular properties are listed in Table I. Both cellular and plasma fibronectin are composed of disulfide-linked subunits. Plasma fibronectin consists of dimers, whereas cellular fibronectin exists as a mixture of dimers, larger polymers, and small amounts of monomer. The subunits of fibronectin may not be identical, as subunits of plasma fibronectin are found to differ in apparent size according to polyacrylamide gel electrophoresis (reviewed by Mosher, 1980).

The disulfide bonds linking fibronectin's subunits are unusual in that they are confined to only one end of the molecule (Fig. 4-3). Both cellular and plasma fibronectin possess a protease-susceptible region close to these COOH-terminal interchain disulfide bonds, and cleavage at these points will separate the two or more subunit chains into monomeric fragments (see Wagner and Hynes, 1979, for references). To speculate, these disulfide bonds may be placed asymmetrically in order to increase the capacity of the molecule to stretch between distant points in mediating adhesive interactions.

In addition, the subunits of fibronectin themselves are highly asymmetric or unfolded (Fig. 4-3). Circular dichroism studies show little evidence for regular secondary structure, such as α helix or β sheet, even though the molecule is known to have an elongated shape with a large Stokes radius and an unusually

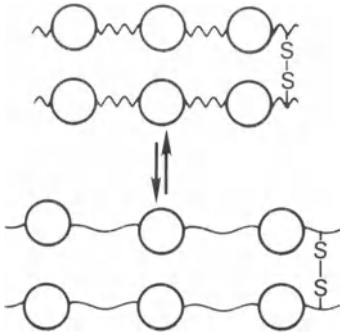


Figure 4-3. Hypothetical model of the overall structure of fibronectin. Fibronectin is thought to be comprised of disulfide-linked subunits containing domains of polypeptide structure. These possibly globular domains are postulated to be separated by regions of unfolded polypeptide that can reversibly stretch or contract depending on ionic conditions.

low sedimentation coefficient for a protein of its size. These findings suggest that fibronectin is asymmetric because the polypeptide backbone is unfolded, rather than helical and rigid as in collagen (Yamada et *al.*, 1977; Alexander et *al.*, 1978, 1979).

Although there are no major regions of ordered secondary structure, the molecule does appear to have tertiary structure. Fluorescence studies indicate that the tryptophan residues are buried within the protein, and become exposed to water only after denaturation of the molecule by heat or by strong denaturing agents. The simplest interpretation of these results is that the parts of the molecule containing tryptophan residues are folded so as to exclude water (Alexander et *al.*, 1978; Colonna et *al.*, 1978). In addition, ultracentrifugation experiments conducted at elevated pH reveal reductions in sedimentation constants, suggesting that the molecule is flexible and can expand or stretch in an altered environment. Neutralization results in a return to a more compact configuration that sediments more rapidly (Alexander et *al.*, 1978, 1979).

A general model that explains all of these results is that the molecule is composed of structured polypeptide domains that are separated by regions of flexible polypeptide chain that can expand or shorten depending upon ionic conditions (Fig. 4-3).

2.4. Specific Structural and Functional Domains

Besides mediating a number of biological effects (discussed in Chapter 10), fibronectin can bind directly to a number of important targets, some of which are listed in Table II. It is thought that by means of these specific binding interactions, fibronectin can mediate a variety of adhesive or binding events. A major advance has been the realization that fibronectin contains a series of functional as well as structural domains (Fig. 4-4). In this model, fibronectin is viewed as more than a simple, nonspecifically adhesive or gluelike molecule; it is instead thought to act via specialized domains that are specific for particular biological activities. For example, there appear to be separate sites for binding to collagen or to cells.

Table II. Materials Bound by Fibronectin

Fibrinogen/fibrin
Collagen/gelatin
Heparin and heparan sulfate
Transglutaminase substrates
Cells
Bacteria (*S. aureus*)
Actin
DNA
Hyaluronic acid

A valuable way to dissect apart these functional domains is to use proteases such as trypsin or chymotrypsin. Proteases tend to cleave proteins preferentially in regions of the molecule where the polypeptide chain is unfolded, and they tend to spare folded regions. This property permits the isolation of intact binding sites of molecules, *if* they are located in globular regions in which the polypeptide backbone is folded (Fig. 4-4).

A useful strategy is indicated in Fig. 4-5, which is a general procedure for isolating any type of active-site region that binds to a protease-resistant molecule or ligand; it may also prove useful in evaluating the biochemical basis of binding of other extracellular matrix components to each other. In this procedure, the molecule of interest, e.g., fibronectin, is permitted to bind to a ligand, e.g., collagen, that is covalently attached to agarose beads. The complex is digested with a protease, which cleaves unfolded regions and spares structured domains. After extensive washing, the only part of the fibronectin molecule that remains bound to the beads will be the active-binding site. A popular alternative to this approach is to digest the fibronectin first with the protease, treat with protease inhibitors, and then purify the binding domain by affinity chromatography using the ligand bound to beads (Fig. 4-5, part II).

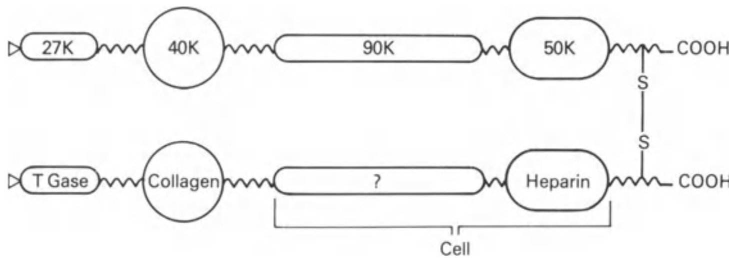


Figure 4-4. Current model of the specific functional and structural domains of fibronectin. The molecular weights of protease-resistant structural domains that have been isolated are indicated along the upper fibronectin subunit (K = 1000). Functional interaction or binding activities of each of these fragments are indicated along the lower subunit. The shapes shown are current, tentative approximations of the shapes of the domains. The triangles on the left represent the blocked NH_2 terminals; for example, human plasma fibronectin contains an NH_2-terminal pyrrolidonecarboxylic acid. Sites such as the binding regions for hyaluronic acid and actin are not shown; the relative positions of the 50K and 90K fragments relative to one another are not yet certain.

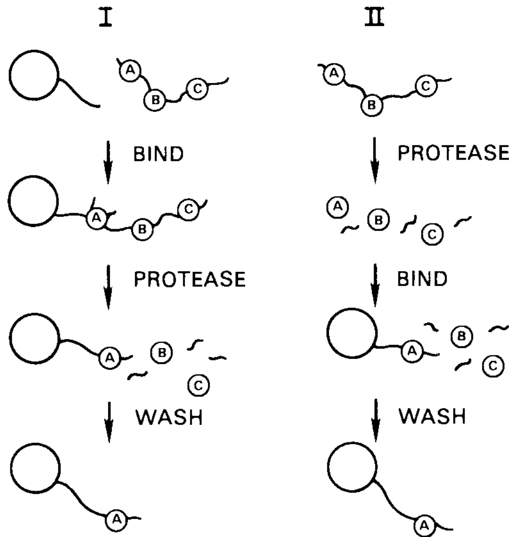


Figure 4-5. General strategies for purifying active-site domains of binding molecules. The binding molecule, e.g., fibronectin, contains structural domains labeled A, B, and C. The molecule that is bound, such as collagen, is called the ligand. The ligand is first coupled covalently to agarose beads. In strategy I, the molecule is permitted to bind to the ligand, which occurs via specific binding site "A." It remains bound after protease treatment and washing of the beads, and can be recovered in relatively pure form. In strategy II, the isolated binding molecule is first cleaved by a protease. Site A retains its binding capacity and can subsequently bind to the ligand-beads. These procedures may prove valuable in dissecting the binding interactions of other extracellular matrix components.

2.4.1. Collagen-Binding Domain

It is known that both cellular and plasma fibronectin can mediate the attachment of cells to collagen, and that both molecules can bind directly to collagen. The first target of several laboratories therefore has been to purify this interesting collagen-binding portion of fibronectin. Using proteases such as chymotrypsin or trypsin to cleave the molecule into fragments, and affinity columns containing denatured collagen (which is found to have an especially high capacity to bind fibronectin), the collagen-binding domain of fibronectin was recovered in fragments of 30,000–40,000 daltons (Balian *et al.*, 1979; Ruoslahti *et al.*, 1979; Hahn and Yamada, 1979a,b; Gold *et al.*, 1979; Furie *et al.*, 1980). For example, chymotrypsin cleavage yields a fragment of 40,000 daltons that is, not surprisingly, relatively globular in shape.

These collagen-binding domains are characterized by unusually large numbers of intramolecular disulfide bonds, which appear to be necessary for maintaining collagen-binding activity (Balian *et al.*, 1979; Wagner and Hynes, 1979). This purified protein domain cannot by itself mimic the attachment of cells to collagen substrates mediated by intact fibronectin, but this domain is the functionally important site for fibronectin binding to collagen (Hahn and Yamada, 1979b). The sites on collagen to which fibronectin binds are known. A major binding site overlaps the site attacked by animal collagenases, which may also be a region of unusual instability of the collagen triple helix (Kleinman *et al.*, 1978; Dessau *et al.*, 1978).

Although fibrin and collagen have been reported to compete with each other for binding to fibronectin (Engvall *et al.*, 1978), these molecules bind to different domains on fibronectin [Sekiguchi and Hakomori, 1980b].

2.4.2. Heparin-Binding Domain

Fibronectin binds to the glycosaminoglycan heparin, and heparin appears to strengthen the binding of fibronectin to collagen (Stathakis and Mosesson, 1977; Jilek and Hörmann, 1979; Perkins et *al.*, 1979; Ruoslahti and Engvall, 1980; Johansson and Höök, 1980; K. M. Yamada et *al.*, 1980). The binding is relatively strong, as indicated by a moderately high binding affinity ($K_D = 10^{-7}$–10^{-9} M; K. M. Yamada et *al.*, 1980). A heparin-binding fragment of 50,000 daltons can be isolated after digestion of fibronectin with Pronase, which is a broad-spectrum protease. Like the collagen-binding domain, this domain is relatively globular in shape as compared to the highly asymmetric shape of the fibronectin molecule itself. It is nearly devoid of cysteine residues and carbohydrate (Hayashi et *al.*, 1980). The resistance of this fragment to degradation by moderate concentrations of Pronase is strong evidence for the existence of a tightly folded polypeptide domain containing no regions of exposed polypeptide accessible to proteolysis. As expected, the heparin- and collagen-binding domains can be readily separated, for only the heparin-binding domain binds to heparin affinity columns.

2.4.3. Hyaluronic Acid-Binding Site

Hyaluronic acid may have important regulatory effects on cells during embryonic development (discussed in Chapter 9). One of the molecules with which this glycosaminoglycan may interact is fibronectin (Jilek and Hörmann, 1979; K. M. Yamada et *al.*, 1980; Ruoslahti and Engvall, 1980). Purified preparations of fibronectin will bind to hyaluronic acid, and the binding site is kinetically separate from the site for binding to heparin. Binding of one molecule does not affect the binding of the other, even though each site is readily saturated by its appropriate ligand (K. M. Yamada et *al.*, 1980).

A structural domain for binding hyaluronic acid has not been identified as yet. However, the site appears to have considerable specificity regarding the number of repeating disaccharide units of hyaluronic acid that it can recognize. In competitive inhibition experiments, the hyaluronic acid recognition site is found to be between 8 and 10 monosaccharide units in size. The site has a moderately high binding affinity of 10^{-7} M (K. M. Yamada et *al.*, 1980). To speculate, this binding site might provide one of the means by which hyaluronic acid interacts with cells, or it might possibly play a role in organizing hyaluronic acid chains in extracellular spaces.

The existence of separate binding sites on fibronectin for collagen, heparin, and hyaluronic acid suggests that fibronectin may be a central molecule in the organization and cellular binding of a variety of extracellular or secreted molecules.

2.4.4. Binding to Actin and DNA

Other macromolecules are known to interact with fibronectin, but the specificity of these interactions is not yet clear. Actin and various types of DNA have

been shown to bind to fibronectin (Keski-Oja et *al.*, 1980; Zardi et *al.*, 1979). The actin-binding domain of fibronectin is approximately 27,000 daltons in size, and appears to be separate from the collagen- and heparin-binding sites (Keski-Oja and Yamada, 1981). The functions of these sites that bind important *intracellular* molecules are not yet known (Chapter 10). One speculation is that they could assist the reticuloendothelial system in the removal from blood of actin and DNA liberated by trauma, by means of an "opsonic" activity of fibronectin (Saba et *al.*, 1978; Molnar et *al.*, 1977; Chapter 10, this volume).

2.4.5. Plasma Membrane-Binding Domain

An interaction of fibronectin that is of obvious importance is its binding to the cell surface, for the molecule is known to have biological effects on cells, e.g., to mediate cell adhesive events. A proteolytic fragment of 160,000 daltons has recently been purified from fibronectin that is capable of binding to cells (Hahn and Yamada, 1979; see also Ruoslahti and Hayman, 1979; Sekiguchi and Hakomori, 1980a; McDonald and Kelley, 1980). This fragment lacks the collagen-binding site, and is therefore unable to mediate the attachment of cells to collagen. It can, however, bind to cells and competitively inhibit the binding of intact fibronectin, thereby inhibiting cell attachment to collagen. It can also mediate other adhesive events that do not involve collagen, such as permitting the attachment and spreading of cells on a plastic tissue culture substratum (Hahn and Yamada, 1979b; McDonald and Kelley, 1980). This 160,000-dalton fragment is relatively elongated, even though part of it contains the more globular heparin-binding site (Fig. 4-4).

A very large fragment of fibronectin with a molecular weight of 205,000 contains collagen-, heparin-, and cell-binding sites. It readily mediates cell attachment to collagen (Hahn and Yamada, 1979b; Wagner and Hynes, 1979; K. M. Yamada et *al.*, 1980; Sekiguchi and Hakomori, 1980).

2.4.6. Carbohydrates

In addition to binding sites, certain structural features of fibronectin are also localized to a limited part of the molecule. The carbohydrate on fibronectin is localized to the collagen-binding and cell-binding parts of the molecule (Wagner and Hynes, 1979; Sekiguchi and Hakomori, 1980; McDonald and Kelley, 1980). The functional importance, if any, of this localization is not yet known.

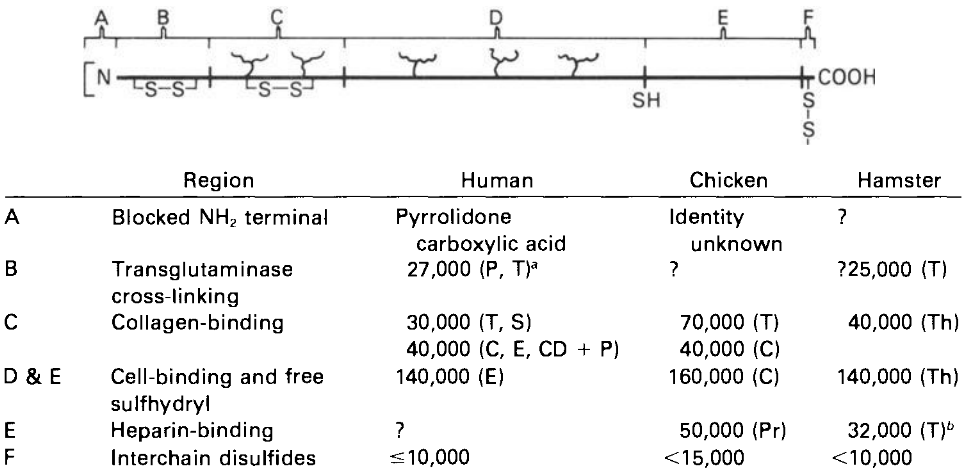
Both cellular and plasma fibronectin contain only one type of oligosaccharide unit (Yamada et *al.*, 1977; Fukuda and Hakomori, 1979b; Takasaki et *al.*, 1979). There is an average of five of these "complex" asparagine-linked oligosaccharides per subunit, each containing the sugars *N*-acetylglucosamine, mannose, and galactose, with additional sialic acid or fucose residues. This structural finding has proven useful recently. A new inhibitor of glycoprotein glycosylation has been discovered (tunicamycin), and it has been shown to be a specific inhibitor of the formation of only this type of oligosaccharide linkage.

It is therefore possible to synthesize fibronectin that is free of carbohydrate residues, in order to evaluate their structural or biological roles.

The carbohydrate moiety of fibronectin has no known role in its function, for unglycosylated fibronectin is as active as the native molecule. Instead, the carbohydrate appears to stabilize the molecule against proteolytic attack and abnormal rate of turnover. Carbohydrate-free fibronectin is substantially more easily degraded by Pronase, and has a severalfold higher rate of protein turnover (Olden *et al.*, 1978, 1979).

2.4.7. Sulfhydryls

One or two free sulfhydryl groups are located at specific regions of fibronectin (Fig. 4-6), and it has been suggested that these groups may be important in permitting the molecule to form higher-order polymers on the cell surface (Wagner and Hynes, 1979, 1980; Fukuda and Hakomori, 1979a; McDonald and Kelley, 1980). Intramolecular disulfide bonds are concentrated in a limited region of the molecule that is 40,000–70,000 daltons in size (Balian *et al.*, 1979; Ruoslahti *et al.*, 1979; McDonald and Kelley, 1980). As intact intrachain disulfide bonds are required for fibronectin to bind collagen, and as the collagen-binding domain is highly enriched in cysteine, these bonds may help to maintain a specific conformation of this domain that is necessary to recognize and to bind to collagen. In striking contrast, the heparin-binding domain appears to lack enough cysteine for even one disulfide bond (Hayashi *et al.*, 1980).



	Region	Human	Chicken	Hamster
A	Blocked NH_2 terminal	Pyrrolidone carboxylic acid	Identity unknown	?
B	Transglutaminase cross-linking	27,000 (P, T)[a]	?	?25,000 (T)
C	Collagen-binding	30,000 (T, S) 40,000 (C, E, CD + P)	70,000 (T) 40,000 (C)	40,000 (Th)
D & E	Cell-binding and free sulfhydryl	140,000 (E)	160,000 (C)	140,000 (Th)
E	Heparin-binding	?	50,000 (Pr)	32,000 (T)[b]
F	Interchain disulfides	≤10,000	<15,000	<10,000

[a] P, plasmin; T, trypsin; S, subtilisin; C, chymotrypsin; E, leukocyte elastase; CD, cathepsin D; Th, thermolysin; Pr, Pronase.
[b] Site on molecule not yet proven.

Figure 4-6. Summary of structural data on fibronectin from different species.

2.4.8. Transglutaminase Reaction Site

Fibronectin can be covalently cross-linked by the enzyme transglutaminase to form complexes with itself, fibrinogen, or collagen. This cross-linking occurs at a specific site on fibronectin involving glutamine residues of a 27,000-dalton region of the molecule (Jilek and Hörmann, 1977; Mosher *et al.*, 1980; Figs. 4-4, 4-6). This reaction-site domain is distinct from the site for fibronectin binding to collagen, and appears to be the domain closest to the NH_2 terminal of the molecule (Furie and Rifkin, 1980; Wagner and Hynes, 1980; McDonald and Kelley, 1980). This domain is also reported to be the site by which fibronectin binds to the bacterium *Staphylococcus aureus* (Kuusela, 1978; Mosher and Proctor, 1980).

2.5. Cell Receptor for Fibronectin

To what constituent of the plasma membrane does the cell-binding domain of fibronectin bind? One experimental approach to answering this question is to examine for competitive inhibitors of fibronectin in biological assays. If a molecule is the natural receptor for fibronectin, then adding excess amounts of free receptor to assays should result in a competition for binding to fibronectin between the receptor on cells and the added free receptor. The result should be an increasing inhibition of fibronectin's activity as its cell-binding domain becomes saturated with larger amounts of added free ligand.

From preliminary experiments of this type, excellent current candidates for the fibronectin receptor are negatively charged lipids such as glycolipids containing several sialic acid residues, e.g., the ganglioside GD_{1a} (Kleinman *et al.*, 1979). These lipids can bind to fibronectin and can effectively block many of the fibronectin-mediated biological events described in Chapter 10, including cell attachment to collagen, hemagglutination, restoration of a normal morphology to transformed cells, and cell spreading (Kleinman *et al.*, 1979; Yamada *et al.*, 1981). As expected, the cell-spreading activity of the 160,000-dalton cell-binding domain itself is also inhibited by the exogenously added gangliosides.

The part of the ganglioside that is recognized by fibronectin appears to be the carbohydrate moiety, as the purified oligosaccharide portion will also inhibit cell attachment to collagen, cell spreading, and other activities; in contrast, the lipid portion is without effect (Kleinman *et al.*, 1979; Yamada *et al.*, 1981). Phospholipids that have been tested to date are at least one order of magnitude less effective. A current hypothesis regarding the receptor for fibronectin is therefore that fibronectin binds to a specific cell-surface receptor consisting of specific charged molecules such as gangliosides or glycoproteins containing ganglioside-type oligosaccharides. Evidence for a protein component of the receptor for fibronectin has recently been provided by Grinnell (1980); under certain conditions, protease pretreatment of cells also inhibits the activity of fibronectin.

2.6. Biosynthesis of Fibronectin

Fibronectin is synthesized in the rough endoplasmic reticulum. It can be localized to this site as well as to the Golgi apparatus by immunofluorescence and immunoelectron microscopy procedures (Yamada, 1978; S. S. Yamada *et al.*, 1980). The rate of synthesis of fibronectin appears to be regulated by the amounts of mRNA for this protein, and its characteristic decrease after malignant transformation of cells can be partially explained by a major decrease in levels of fibronectin mRNA (Adams *et al.*, 1977). The mRNA for fibronectin has been isolated, and DNA complementary to fibronectin mRNA has been cloned in bacteria recently. These tools should prove useful in analyzing the regulation of this glycoprotein in malignancy and development (Fagan *et al.*, 1981).

During biosynthesis, the carbohydrate moiety is added to fibronectin as an immature oligosaccharide unit similar to that described for other *N*-asparagine-linked oligosaccharides. As the protein proceeds to the cell surface, it loses two-thirds of its original mannose residues and gains the *N*-acetylglucosamine, galactose, sialic acid, and fucose residues characteristic of the carbohydrate of the mature protein (Choi and Hynes, 1979; Olden *et al.*, 1980). It also becomes disulfide-linked into multimers near the time of secretion (Choi and Hynes, 1979). Surprisingly, the secretion of fibronectin does not appear to require the presence of carbohydrate, as secretion occurs normally even when glycosylation is prevented by tunicamycin (Olden *et al.*, 1978). The fibronectin is thought to be secreted either directly onto the cell surface or into culture media.

How secreted fibronectin becomes organized into its characteristic fibrillar patterns is still not clear at the molecular level. Purified cellular fibronectin will readily self-aggregate, and a fraction of plasma fibronectin has been reported to form fibrillar structures under certain *in vitro* conditions. Whether these fibrils are identical to those found *in vivo* is not yet known. It is also of interest that heparin can induce plasma fibronectin to form fibrillar complexes, suggesting that other extracellular components might possibly be involved in normal fibronectin fibrillogenesis (Iwanaga *et al.*, 1978; Jilek and Hörmann, 1979; Vuento *et al.*, 1980).

Once fibronectin is in place on the cell surface, it can be lost by sloughing from cells or by degradation by proteases. The rate of turnover of fibronectin on the cell surface is relatively slow, with a half-life of 30–36 hr, which is similar to that of total protein turnover (Olden and Yamada, 1977). This rate of turnover is accelerated in malignantly transformed cells, or if the fibronectin is synthesized without the oligosaccharide moiety (Hynes and Wyke, 1975; Olden and Yamada, 1977; Olden *et al.*, 1978).

3. Laminin

Laminin [lamina = layer] is a newly discovered glycoprotein that appears to be an important structural constituent of the basal lamina or so-called basement membrane. It has been isolated from tumor cells known to secrete other

basement membrane constitutents (Chung et *al.*, 1977, 1979; Timpl et *al.*, 1979). For example, it can be isolated from basal lamina-like material thought to be analogous to Reichert's membrane, produced by embryonic carcinoma cells (Chung et *al.*, 1979).

Laminin is distinct from the collagens and fibronectins by criteria that include different amino acid compositions, lack of immunological cross-reactivity, and differences in electrophoretic mobility. Laminin contains a subunit of 220,000 daltons, which is disulfide-linked into larger complexes or even into the structural scaffolding of the basal lamina itself. In one tumor line studied, laminin exists as molecules of roughly 800,000 daltons containing the 220,000-dalton subunit as well as subunits of 440,000 daltons that remain intact even after reduction of disulfide bonds (Chung et *al.*, 1979; Timpl et *al.*, 1979; Timpl and Martin, 1981). Electron microscopy reveals long and short arms that cross each other (Fig. 4-7).

Laminin is somewhat enriched in cysteine, which may contribute to its extensive cross-linking by disulfide bonds. A large, pepsin-resistant fragment of laminin (P1) has been isolated that is rich in cysteine; there is thus a concentration of disulfide bonds in a domain constituting a quarter of the original protein (Timpl et *al.*, 1979; Chung et *al.*, 1979). Laminin has relatively large amounts of carbohydrate (12–15%) with a significant amount of sialic acid (4–6%). Protease cleavage has identified a fragment that shows affinity for heparin (see Timpl and Martin, 1981). Laminin also interacts with type IV collagen and cells (see below). The cell-binding activity may be in the center of the cross (Fig. 4-7; Höök, Rohde, and Timpl, unpublished; see also Engel et *al.*, 1981).

Immunofluorescence studies with antibodies specific for laminin have shown that it is localized to a variety of basement membranes, including Reichert's membrane and kidney glomerular and tubular basement membranes (Chung et *al.*, 1979; Rohde et *al.*, 1979; Timpl et *al.*, 1979; Ekblom et *al.*, 1980; Wartiovaara et *al.*, 1980) and the corneal basement membrane (Fig. 4-1). Recent electron microscopic studies have localized laminin in the lamina rara externa of the glomerular basement membrane (Foidart et *al.*, 1980) and in the lamina rara interna (Chapter 11). Interestingly, laminin is not produced by fibroblasts; it is produced by epithelial cells, endothelial cells, and myotubes (Chung et *al.*, 1979; Timpl et *al.*, 1979; Foidart et *al.*, 1980).

Current research suggests that laminin is an adhesive glycoprotein that can mediate the adhesion to basement membrane collagen of certain epithelial cells (epidermal cells, carcinoma cells) that cannot utilize fibronectin for attachment to type IV collagen (Terranova et *al.*, 1980; Vlodavsky and Gospodarowicz, 1980). This glycoprotein may therefore be an important adhesive glycoprotein for epithelial cells. In addition, laminin is known to be cross-linked by disulfide bonds, and might also thereby form a heavily cross-linked structural framework within basement membranes (Chung et *al.*, 1979). A structural role is further suggested by the finding that laminin binds to heparan sulfate and heparin, as heparan sulfate proteoglycan is a prominent constitutent of basement membranes (Sakashita et *al.*, 1980; Chapter 12, this volume).

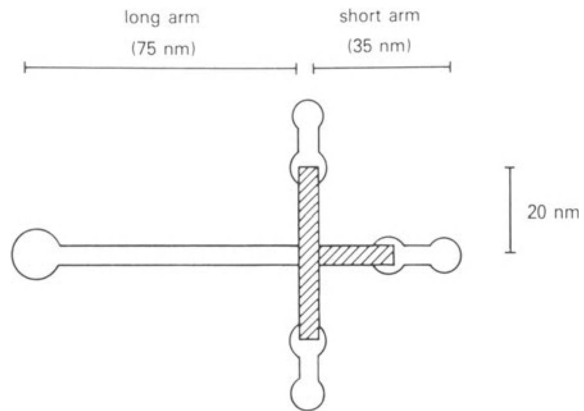


Figure 4-7. Structural model of laminin based on electron micrographs of isolated molecules viewed after rotary shadowing and negative staining. The hatched area denotes the possible position of the P1 fragment (290,000 daltons) obtained by pepsin digestion. The two chains (220,000 and 440,000 daltons) obtained by reduction of disulfide bonds lie within the long and short arms. The length of the long arm is 75 nm and that of the three short arms, 35 nm. Arms are 2 nm in diameter and contain larger globules (5–7 nm) at each end. (From Timpl and Martin, 1981.)

It is therefore possible that laminin complements the biological and adhesive activities attributed to fibronectin, and it may also be critical to the internal organization and function of basement membranes.

4. Chondronectin

Chondronectin [*chondros* = cartilage + *nectere* = to bind, tie] is a newly described adhesion factor that is present in serum and in cartilage. It can mediate the attachment of chondrocytes to collagen, especially to the type II collagen that is characteristic of cartilage (Hewitt et *al.*, 1980; Kleinman et *al.*, 1980). It is therefore different from fibronectin, which does not show such strong specificity for collagen types and which cannot mediate the attachment of chondrocytes to collagen. It should be noted parenthetically that fibronectin is not specific in origin or in attachment activity for fibroblasts alone, whereas chondronectin seems to be specific for chondrocytes.

Chondronectin has been purified recently from serum. It is not bound by affinity chromatography on a type I collagen affinity column, and can also be separated from fibronectin by ion-exchange chromatography (Hewitt et *al.*, 1980). The molecule has been tentatively identified as a 180,000-dalton molecule with subunits of apparent molecular weight 80,000 after reduction of disulfide bonds (Hewitt et *al.*, 1981). A similar factor that is found in extracts of cartilage has not yet been purified for molecular comparisons.

The discovery of chondronectin suggests that there could be a class of several different adhesive molecules that can mediate attachment of cells to extracellular substrates. However, it must be emphasized that the mechanism of action of chondronectin may differ from that of fibronectin, as its binding to type II collagen is unusually slow and temperature dependent (Hewitt et *al.*, 1980).

5. Concluding Remarks

The noncollagenous structural proteins of the extracellular matrix include the proteins fibronectin, laminin, and chondronectin. These proteins may have a common denominator of adhesiveness for cells and for extracellular materials, although the latter two proteins are not yet fully characterized. Their existence suggests that different tissues and structures may contain multiple glycoproteins that play roles in cell–cell, cell–matrix, and matrix-organizing interactions. Characterization of these proteins and their active-site domains provides an exciting opportunity to unravel the ways in which cells interact with their microenvironment and develop and maintain tissue and extracellular matrix organization.

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Chapter 5

Proteoglycan Biosynthesis

ALBERT DORFMAN

1. Introduction

Proteoglycans of cartilage are glycoproteins that contain a protein core to which are attached large numbers of two types of glycosaminoglycan chains and a smaller number of mannose-containing oligosaccharides. Current concepts of the structure of the proteoglycan molecule are discussed in Chapter 2 of this volume and have been reviewed elsewhere (Hascall and Heinegård, 1979). The discussion in Chapter 2 makes it clear that although great progress has been made in the elucidation of the structure of cartilage chondroitin sulfate proteoglycan (CSPG), important questions remain, some of which must be resolved before a complete understanding of biosynthesis will become possible. Nevertheless, studies on biosynthesis have made considerable progress and may, in turn, contribute to the understanding of both structure and function.

Investigations of the biosynthesis of proteoglycans have been primarily concerned with cartilage CSPG although there is now considerable information regarding the mechanism of heparin biosynthesis (Lindahl et *al.*, 1977; Rodén, 1980); this, however, pertains to the synthesis of carbohydrate chains rather than to the synthesis of the entire proteoglycan molecule and will not be reviewed here. There is still no definitive answer as to whether hyaluronic acid is synthesized as part of a proteoglycan; the bulk of available evidence suggests that this glycosaminoglycan is not covalently linked to protein.

Earlier studies were concerned almost exclusively with definition of the pathway of synthesis of the carbohydrate chains of chondroitin sulfate. However, it is apparent that comprehension of the physiology of matrix requires an understanding of the synthesis of the entire proteoglycan molecule. Elucidation of the intricate mechanisms that lead to the coordination of all of the reactions necessary for formation of a molecule as complex as cartilage proteoglycan represents an exciting challenge. Fabrication of this molecule requires the formation of a high-molecular-weight protein, probably processing of this protein in the endoplasmic reticulum and addition of two or three

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different types of complex carbohydrate side chains, beginning in the endoplasmic reticulum and terminating in the Golgi apparatus. The synthesis of a proteoglycan monomer requires the formation of approximately 25,000 covalent bonds. This number does not include the synthesis of nucleotide precursors, amino acids, tRNAs, and mRNA. All of these reactions must be coordinated to produce the CSPG subunit that in turn interacts with hyaluronic acid (HA) and link protein to form the characteristic aggregates of cartilage. In cartilage, the production of large amounts of proteoglycan is a measure of differentiation.

The coordination of synthesis of CSPG with other products characteristic of the cartilage phenotype, such as type II collagen, link protein, and perhaps the recently discovered chondronectin (Hewitt *et al.*, 1980), is fundamental to the process of chondrogenesis. Collagen and glycosaminoglycan synthesis occur simultaneously in the same cell (Vertel and Dorfman, 1979), but it is not known whether the protein components are produced in different compartments of the endoplasmic reticulum. Concomitant with the onset of synthesis of CSPG and type II collagen is the reciprocal inhibition of synthesis of type I collagen and fibronectin by the chondrocytes *in vitro* (Dessau *et al.*, 1978).

We do not understand all of the events involved in coordination of these reactions, especially with respect to their localization within the cell. An attempt will be made in this chapter to summarize the pertinent information available to date, with the expectation that in the next decade cell biologists will unravel the exact sites of the many steps involved in the manufacture of proteoglycans and collagens by the cell.

2. Synthesis of Core Protein

2.1. Variation in Structure of Core Protein

Available information indicates that chondroitin sulfate is present in a variety of tissues and in all cases appears to be linked covalently to protein. It should be noted that because dermatan sulfate is formed from chondroitin sulfate (Malström *et al.*, 1975), the core protein of proteoglycans containing dermatan sulfate must be classified as a variety of CSPG. It is beyond the scope of this chapter to consider the nature of the various CSPGs, which has been reviewed in detail elsewhere (Dorfman *et al.*, 1980b) and is considered in Chapter 2. Whether or not the protein cores of different proteoglycans are the products of closely related genes originating by gene duplication is at present unknown. Such questions will undoubtedly be answered as modern methods of molecular biology are applied to this problem. At present, there is good reason to believe that proteins of different CSPGs differ in structure and represent different gene products. Clearly, the core protein of heparin proteoglycan differs from that of CSPG (see Chapter 2). In the cornea, keratan sulfate is linked to a different protein than is chondroitin sulfate (Axelsson and Heinegård, 1975; Hassell *et al.*, 1979), although in cartilage keratan and chondroitin sulfates are linked to the same protein. Of considerable interest are the small

proteoglycans containing chondroitin sulfate present in undifferentiated limb buds, calvarium, and other tissues. The structural relationship of these substances to core protein of CSPG of cartilage is not clear, although immunological cross-reactivity of calvarial CSPG and cartilage CSPG has been demonstrated (Sugahara and Dorfman, unpublished results).

In view of the recent studies of the genetic mechanism involved in immunoglobulin synthesis (Brack *et al.*, 1978), it is possible that the smaller proteoglycan represents part of the molecule that is part of the characteristic large CSPG of cartilage. This may result from gene fusions that could account for both the variety of proteoglycans in different tissues and the appearance of large amounts of cartilage CSPG during differentiation.

2.2. Cell-Free Synthesis of Core Protein

Upholt *et al.* (1979) have succeeded in obtaining mRNA from embryonic chick sternal cartilage and differentiated limb bud cultures that when translated in a wheat germ or reticulocyte cell-free system gives rise to a protein of approximately 340,000 daltons. The molecular weight determined solely by SDS-PAGE must be considered approximate. Treadwell *et al.* (1980) have also recently reported the synthesis of a high-molecular-weight protein by cell-free translation of RNA isolated from calf articular cartilage. Figure 5-1 illustrates the SDS-PAGE pattern of products translated from sternal RNA and the reactivity of the 340,000-dalton protein with a clonal antibody to CSPG (Dorfman *et al.*, 1980a,b). The following findings suggest that this large, unique protein represents core protein of CSPG:

1. The protein was not found when mRNA of calvarium, liver, or limb bud mesenchyme was translated. The amount formed by translation of mRNA from BrdUrd-inhibited limb bud cultures is markedly decreased as compared to that obtained by translation of mRNA from differentiated cultures.
2. The protein reacts with a clonal antibody secreted by a clone of a hybridoma prepared by fusion of a mouse myeloma with spleen cells of a rat immunized with hyaluronidase-treated CSPG derived from embryonic chick cartilage.
3. The product is insensitive to collagenase.

The mRNA responsible for synthesis of core protein sediments at approximately 27 S in 70% formamide or sucrose gradients. No definitive evidence is yet available concerning the NH_2- or COOH-terminal end of this molecule. On the basis of pulse chase experiments in developing chick limb bud cultures, De Luca *et al.* (1978) concluded that the portion of the proteoglycan containing chondroitin sulfate represents the NH_2-terminal end of the molecule.

How this large protein is converted to the proteoglycan molecule is not clear. Several important questions may be enumerated as follows. (1) Is the translated protein inserted into the membrane of the rough endoplasmic re-

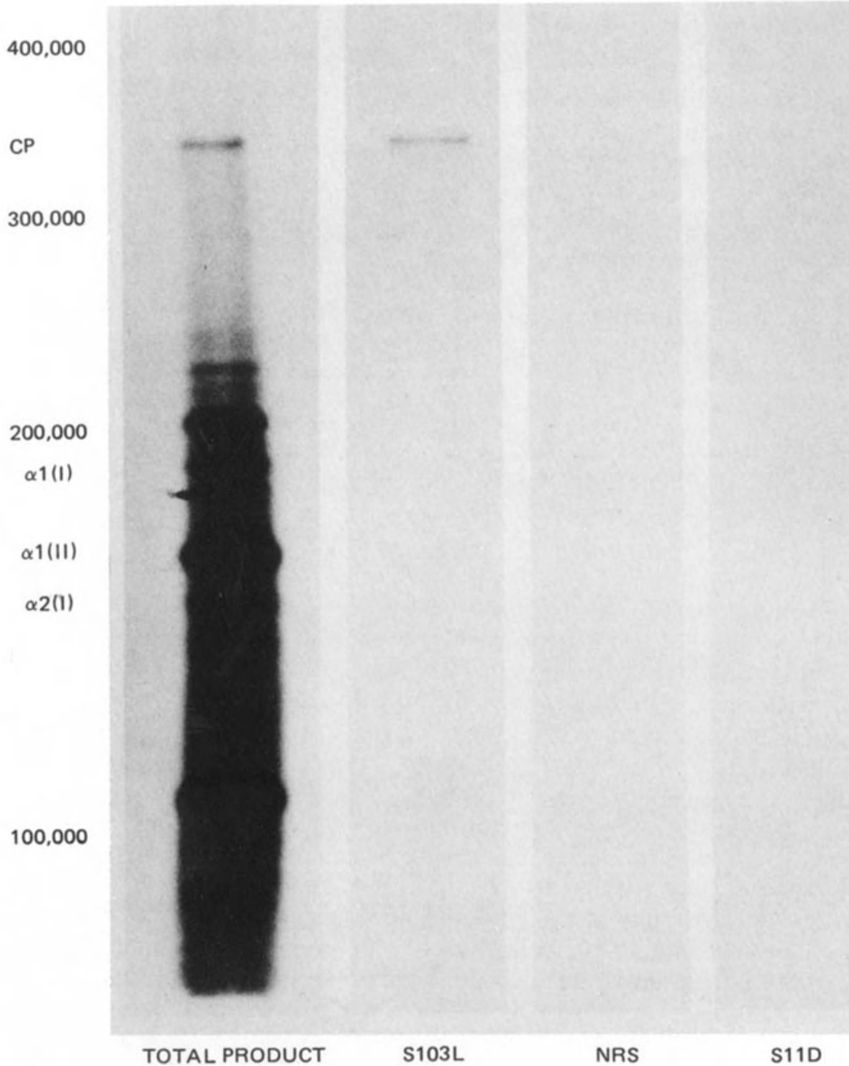


Figure 5-1. SDS-PAGE pattern of cell-free translation products of total RNA of 17-day chick embryo sterna. S103L and S11D are clonal antibodies.

ticulum by a leader sequence? (2) If such insertion occurs, is carbohydrate added to nascent chains? (3) What is the order of addition of the various types of polysaccharide chains, and where does this occur in the cell? (4) Is a portion of the protein removed in the course of processing?

In view of firm adherence to membranes of the enzymes involved in chondroitin sulfate synthesis and the established mechanism of synthesis of other glycoproteins, insertion into a membrane of the rough endoplasmic reticulum seems likely. No definitive information is yet available as to whether carbohy-

drate chain initiation occurs on nascent chains. In the synthesis of the glycoproteins involving N-glycoside linkages, carbohydrate addition appears to occur before completion of peptide synthesis (Struck and Lennarz, 1980). In these cases, carbohydrate side chains are added en bloc from dolichol pyrophosphate intermediate. Indeed, there is evidence that carbohydrate addition may not be possible after completion and folding of peptide chains (Pless and Lennarz, 1977). In the cell, autoradiographic studies suggest that carbohydrate incorporation can occur in the Golgi zone as well as the endoplasmic reticulum (see Section 7.2).

Recent studies (De Luca et *al.*, 1980; Lohmander et *al.*, 1980) indicate that CSPG of chick cartilage and rat chondrosarcoma CSPG contain approximately 15 N-glycoside oligosaccharides per monomer unit located in the HA-binding region. It is somewhat difficult to be absolutely certain that these oligosaccharides are not attached to contaminating proteins.

Schwartz and Miller (1978) found evidence of phosphorylation of CSPG monomer in human cartilage, and Peters et *al.* (1979) have reported phosphorylation of proteoglycan in bovine nasal cartilage. It is possible this phosphorylation occurs on mannose residues of N-glycosidically linked oligosaccharides as has been found for a variety of lysosomal enzymes (Kaplan et *al.*, 1977; Sando and Neulfeld, 1977; Bach et *al.*, 1979). Thonar and Sweet (1979) discovered that proteoglycans of articular cartilage contain oligosaccharides composed of galactosamine, glucosamine, galactose, and sialic acid. Studies of Lohmander et *al.* (1980) indicate that Swarm chondrosarcoma CSPG contains O-glycosidically linked oligosaccharides that are similar in structure to the linkage region of keratan sulfate, even though this proteoglycan does not contain keratan sulfate. Similar oligosaccharides were found by De Luca et *al.* (1980) in CSPG monomer isolated from differentiated chick limb bud cultures. Vertel et *al.* (unpublished results) have demonstrated that when putative core protein mRNA is translated in the presence of dog pancreas smooth membranes, an immunoreactive band on SDS-PAGE gels appears that is slightly larger than that that is formed in the absence of dog pancreas membranes. A protein of similar size was found in cell extracts (Vertel et *al.*, unpublished results). It is possible that this band may represent core protein to which N-glycoside oligosaccharides have been added.

Whether xylosylation occurs on nascent chains to initiate chondroitin sulfate chains remains unknown. Recent studies by Strouss (1979) on the synthesis of intestinal mucin indicates that GalNAc residues in O-glycoside linkage are added to nascent chains. As little is known regarding the biosynthesis of the keratan sulfate linkage region, it is impossible at present to decide how and when in the course of biosynthesis initiation of keratan sulfate chains occurs.

The large size of the cell-free product suggests that processing of the translated protein may involve removal of a portion of the molecule, as the size of core protein has usually been estimated to be approximately 200,000 daltons. Faltynek and Silbert (1978a) have found evidence of the presence on microsomes of a proteoglycan larger than the usual CSPG monomer. All preparations of CSPG so far studied are polydisperse. Heinegård (1977) has proposed that

polydispersity is due to the existence of core proteins containing an HA-binding region and progressively larger peptide segments bearing chondroitin sulfate chains. It seems unreasonable that such a variation of molecular size occurs in the course of biosynthesis of the protein unless there is either a variation in processing or more than one gene product is involved. More recently, Heinegård and Hascall (1979) have indicated that nonaggregating proteoglycans of bovine septal cartilage are structurally different from aggregating proteoglycans and are unlikely to result from a simple degradation process.

3. Synthesis of Chondroitin Sulfate

Chondroitin sulfate synthesis involves the participation of six specific glycosyltransferases and the two sulfate transferases. The pathway of synthesis is illustrated in Fig. 5-2.

3.1. Chain Initiation

As already indicated, it is not yet certain whether or not initiation of chondroitin sulfate chain synthesis occurs on nascent chains or after polypep-

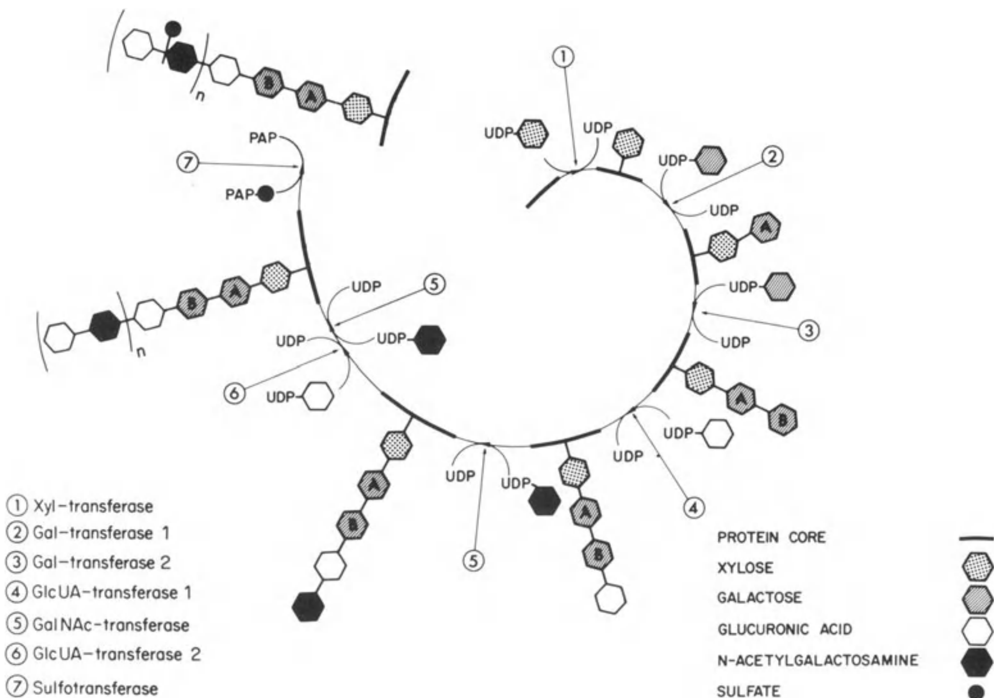


Figure 5-2. Pathway of biosynthesis of chondroitin sulfate.

tide completion. In any case it is quite clear that the transfer of xylose from UDP-xylose to hydroxyl groups of serine in the core protein is responsible for chain initiation. Whether the same xylosyltransferase is involved in synthesis of heparin and heparan sulfate proteoglycans as in CSPG synthesis is unknown. All serine residues of core protein are not xylosylated. The factors that determine which hydroxyl groups are xylosylated have not yet been determined. When chondroitin sulfate chains are removed by Smith degradation (Baker *et al.*, 1972) or by hydrofluoric acid (Coudron *et al.*, 1980a), the resultant protein core serves as a xylose acceptor. Synthetic peptides containing serine and glycine also serve as acceptors (Coudron *et al.*, 1980b).

UDP-D-xylose core protein:β-D-xylosyltransferase has been purified to homogeneity from embryonic chick cartilage (Schwartz and Rodén, 1974) and from rat chondrosarcoma (Schwartz and Dorfman, 1975a). The enzyme has a molecular weight of 95,000–100,000 and consists of two pairs of nonidentical subunits of molecular weight 23,000 and 27,000.

Several studies have been concerned with the possible role of a lipid intermediate in chondroitin sulfate synthesis. These have been facilitated by the use of tunicamycin, an inhibitor of the formation of *N*-acetylglycosaminylpyrophosphorylisoprenol. Takatsuki and Tamura (1977) have reported that tunicamycin inhibits the biosynthesis of glycosaminoglycans in cultured chick embryo fibroblasts. Similar findings have been reported by Pratt *et al.* (1979), but the latter workers found little or no inhibition of synthesis of sulfated glycosaminoglycans by chondrocytes.

Hart and Lennarz (1978) found almost complete inhibition of synthesis of corneal keratan sulfate by tunicamycin. Corneal keratan sulfate, unlike chondroitin sulfate or cartilage keratan sulfate, is covalently linked to protein by an *N*-glycoside linkage; accordingly, inhibition by tunicamycin is not surprising. In the same study, moderate inhibition of chondroitin sulfate synthesis was observed but was attributed as possibly due to secondary inhibition of protein synthesis. The partial inhibition of chondroitin sulfate as measured by $^{35}SO_4^{2-}$ incorporation may result from one of the following effects: (1) a general inhibition of protein synthesis resulting in diminished production of core protein; (2) an inhibition of xylosyltransferase synthesis, as this enzyme is itself a glycoprotein; or (3) failure to form core protein adequate for initiation of chondroitin sulfate or keratan sulfate chains and as a result inhibition of addition of *N*-glycosidically linked oligosaccharides.

Two different experimental findings suggest that the xylosyltransferase step may play an important role in the regulation of CSPG synthesis. Neufeld and Hall (1965) found that UDP-xylose is an inhibitor of UDP-glucose dehydrogenase. Accordingly, a decrease of synthesis of core protein, which has been shown to be essential for chondroitin sulfate synthesis (Telser *et al.*, 1965), would result in accumulation of UDP-xylose, which might in turn inhibit synthesis of UDP-glucuronic acid and consequently further synthesis of UDP-xylose and of chondroitin sulfate. UDP-xylose is formed from UDP-glucuronic acid by action of UDP-glucuronate carboxylyase.

Perhaps of even greater importance are certain properties of xylosyltransferase that differ from those of the other glycosyltransferases involved in chon-

droitin sulfate synthesis. Xylosyltransferase is a relatively soluble enzyme, readily obtained free of membranes, which interacts specifically with galactosyltransferase I, the enzyme involved in the next step requisite for chondroitin sulfate synthesis (Schwartz, 1975). Schwartz (1976b) has shown that xylosyltransferase turns over in tissue at a much more rapid rate than do the other glycosyltransferases involved in chondroitin sulfate synthesis. Taken together, these findings suggest that xylosyltransferase may play a key role in regulating proteoglycan synthesis.

The interaction of xylosyltransferase with its substrate, core protein, and with the next enzyme in series may serve as an efficient mechanism for the coordination of core protein and chondroitin sulfate chain synthesis.

Figure 5-3 illustrates the mechanism by which xylosyltransferase may serve to couple core protein to the enzyme complex involved in subsequent

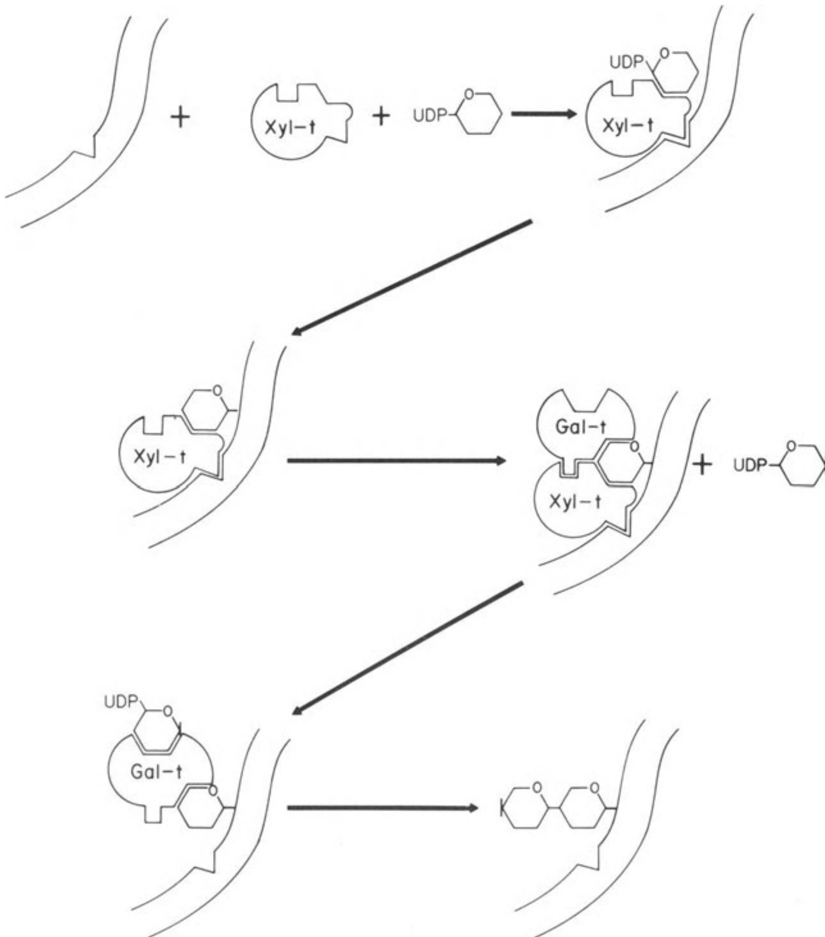


Figure 5-3. Interaction of xylosyltransferase (Xyl-t) with core protein and galactosyltransferase (Gal-t) to initiate chondroitin sulfate synthesis.

synthetic steps. Xylosyltransferase is considered to contain sites for binding to UDP-xylose, core protein, and galactosyltransferase I. If galactosyltransferase I is in turn bound to the other required glycosyltransferases, xylosyltransferase may serve as the effective link in initiation of chondroitin sulfate synthesis.

3.2. Synthesis of Galactosyl–Xylosyl Protein Linkage

Following xylosylation of serine groups in core protein, the linkage region is extended by transfer of galactose from UDP-Gal. The enzyme, galactosyltransferase I, that catalyzes this reaction is bound to membranes, but like the other glycosyltransferases responsible for subsequent reactions, it may be solubilized by treatment with the nonionic detergent Nonidet-P40 in the presence of 0.5 M KCl (Schwartz and Rodén, 1975). This enzyme has been purified to give a preparation yielding two bands on SDS-PAGE (Schwartz and Rodén, 1975). The full activity of this enzyme appears to be dependent on the presence of phospholipids (Schwartz, 1976a,b).

Galactosyltransferase I transfers galactose to free D-xylose and β-D-xylosides such as O-β-D-xylosyl-L-serine, methyl-β-D-xyloside, and p-nitrophenyl-β-D-xyloside (Helting and Rodén, 1969a). Not only is this enzyme capable of transferring galactose to the xylosides, but such xylosides serve to initiate synthesis of complete glycosaminoglycan chains when added to a variety of cell types. This phenomenon, which has been widely used in the studies of connective tissue cells, will be reviewed in Section 6.4.

3.3. Completion of Linkage Region

The linkage region is completed by the addition of a second galactose residue catalyzed by galactosyltransferase II and the addition of the first glucuronic acid residue as a result of the reaction catalyzed by glucuronic acid transferase I. The latter enzyme is distinct from that involved in chain elongation.

Both of these enzymes are tightly bound to membranes and have not as yet been isolated. Galactosyltransferase II transfers galactose to 4-O-β-D-xylose but not to other galactose-containing disaccharides (Helting and Rodén, 1969b). It is interesting to note that in contrast to β-D-xylosides, β-D-galactosides will not serve as glycosaminoglycan chain initiators in the experience of the author (Galligani et *al*., 1975), although contrary results have been reported by Robinson et *al*. (1975).

3.4. Chain Elongation

Elongation of the chondroitin chains results from the alternate transfer of N-acetylgalactosamine and glucuronic acid residues from their respective nucleotides by two distinct transferases (Perlman et *al*., 1964; Silbert, 1964).

The specificity of these enzymes has been studied in some detail utilizing low-molecular-weight acceptors (Telser *et al.*, 1966). These studies contributed to the understanding of the mechanism by which precision of polysaccharide synthesis is achieved. Certain general characteristics of these glycosyltransferases have been deduced and are summarized as follows:

1. Glycosyltransferases are specific for the monosaccharide unit transferred.
2. Glycosyltransferases are specific for the nonreducing terminal-accepting residue including the configuration of its glycoside linkage.
3. Glycosyltransferases are not always specific for the penultimate nonspecific sugar.

These principles are important in the understanding of the mechanism by which sugar sequences are determined in complex carbohydrates. Unlike the template mechanisms utilized for specification of protein and nucleic acid structure, the defined structures of complex polysaccharides result from the specificity of the action of the appropriate glycosyltransferases.

The two enzymes involved in chain elongation appear to be tightly bound to membranes. Although they have now been successfully solubilized, they have not yet been isolated (Schwartz and Rodén, 1975). Silbert and Reppucci (1976) have confirmed the alternate addition of monosaccharides and showed that with oligosaccharide acceptors, as many as 14–16 monosaccharide units may be added. With endogenous primers in the microsomal preparations, more extensive chain elongation occurs.

The supply of UDP-GlcNAc for conversion to UDP-GalNAc utilized for chain elongation may be controlled by the inhibition of fructose 6-phosphate glutamine transamidase by increased quantities of UDP-GlcNAc (Kornfeld *et al.*, 1964).

3.5. Sulfation

Sulfation of chondroitin sulfate chains may occur in the 4- or 6-position of the GalNAc residue. Specific sulfotransferases transfer sulfate from phosphoadenylylsulfate to acceptor chondroitin sulfate. The extent of 4- and 6-sulfation varies with age (Mathews, 1975). Hybrid molecules occur in which 4- and 6-sulfates may occur in the same molecule (Seno *et al.*, 1975; Faltynek and Silbert, 1978b). In addition, residues containing both 4- and 6-sulfate have been demonstrated by Suzuki *et al.* (1968).

The basis of choice of position of sulfation remains unknown. Whether it depends on the relative activities of the two sulfotransferases or is controlled by the nature of the acceptor is unclear. The extensive purification of chondroitin 6-sulfate transferase by Greiling *et al.* (1972) clearly indicates the existence of separate 4- and 6-sulfate transferases. The studies of De Luca *et al.* (1973) indicate that sulfation occurs as chain formation is continuing.

Decreased sulfation of chondroitin sulfate has been observed by Orkin *et al.* (1976) in brachymorphic mice. This defect is an autosomal recessive trait that

does not interfere with reproduction of the homozygous affected individuals. Schwartz *et al.* (1978) have shown that defective sulfation is not due to lack of sulfotransferases but rather is due to defective synthesis of phosphoadenylylsulfate. More recently, Sugahara and Schwartz (1980) have localized the defect to adenosylphosphosulfate kinase, although some diminution of ATP sulfurylase activity was also observed. It is of interest that diminished enzyme activity was observed in cartilage and liver but not in brain or skin. Intermediate levels were found in kidney.

3.6. Chain Termination

Because polysaccharide formation apparently does not involve a template mechanism, the signals for chain termination would not be expected to be analogous to those involved in protein or nucleic acid synthesis. As all chondroitin sulfate preparations so far examined appear to be polydisperse, chain length does not seem to be uniquely determined. What then results in chain termination? One possible mechanism was suggested by the finding by Telser *et al.* (1966) that oligosaccharides containing nonreducing terminal N-acetylgalactosamine 4-sulfate residues do not serve as acceptors for glucuronic acid. However, oligosaccharides containing N-acetylgalactosamine 6-sulfate residues are excellent acceptors. Silbert (1978) has recently studied the end groups of chondroitin sulfate synthesized by microsomal preparations of embryonic chick cartilage. In a mixture of chains terminating in N-acetylgalactosamine and glucuronic acid, 1.5 to 3.0 times as many chains were found to terminate in the former monosaccharide.

It is possible that chain termination does not occur when a specific length is reached, but rather depends on the relative affinity of the elongation enzymes for acceptors of specific size.

4. Keratan Sulfate Synthesis

As extensive evidence indicates that keratan sulfate chains in cartilage are covalently linked to the same protein as are chondroitin sulfate chains (Mathews, 1975), the mechanism of synthesis of keratan sulfate is an integral part of the problem of CSPG synthesis. Only a limited amount of information concerning this problem is so far available. As indicated above, the synthesis of corneal keratan sulfate (keratan sulfate I) is inhibited by tunicamycin (Hart and Lennarz, 1978). However, no information is available regarding synthesis of the O-glycoside linkages of keratan sulfate II in CSPG. Keratan sulfate of cornea appears to be present in a proteoglycan that does not contain chondroitin sulfate chains (Hassell *et al.*, 1979; Chapter 2).

Christner *et al.* (1979) have purified a galactosyltransferase from bovine cornea that transfers galactose to bovine septum agalactokeratan sulfate, as well as to GlcNAc, N-acetylglucosaminides, and glycoproteins containing nonre-

ducing N-acetylglucosaminyl terminal units. This enzyme appears to be relatively nonspecific and appears to be similar or identical to milk A protein.

No information is yet available as to the specific determinants of the location of keratan sulfate chains as compared to chondroitin sulfate chains on the core protein or the order in which the two different types of chains are added on the core protein during biosynthesis.

5. Hyaluronic Acid Synthesis

The demonstration of the cell-free synthesis of HA in a preparation obtained from group A streptococci represented the first unequivocal example of a cell-free system of a complex polysaccharide (Markovitz et *al.*, 1959). Subsequently, systems for the study of HA in preparations from eukaryotic cells have been developed (Hopwood et *al.*, 1974; Appel et *al.*, 1979).

The mechanism of HA synthesis remains poorly understood, particularly with respect to chain initiation. Unlike chondroitin sulfate synthesis, HA formation is insensitive to inhibition of protein synthesis in streptococci (Stoolmiller and Dorfman, 1969) and is only slightly inhibited in eukaryotic systems (Matalon and Dorfman, 1968). Dolichol pyrophosphate derivatives isolated by Turco and Heath (1977) and Hopwood and Dorfman (1977) do not seem to be involved in HA synthesis, which is not inhibited by tunicamycin (Sugahara et *al.*, 1979). Attempts at transferring individual monosaccharides to oligosaccharides have so far been unsuccessful.

As HA is requisite for formation of the aggregates of proteoglycan characteristic of cartilage, it is to be expected that some coordination of HA and proteoglycan biosynthesis must exist.

Following transformation of chondrocytes by Rous sarcoma virus (Pacifici et *al.*, 1977; Muto et *al.*, 1977), a marked decrease in chondroitin sulfate synthesis occurs concomitant with a striking increase in HA synthesis, suggesting a reciprocal relationship in their syntheses. What is probably a simplistic view of this phenomenon is the possibility that transformation results in an inhibition of core protein synthesis with resultant increase in supply of UDP-GlcNAc and UDP-GlcUA for hyaluronic acid synthesis.

6. Study of Proteoglycan Synthesis *In Vivo*

Whereas definition of metabolic pathways *in vitro* is both of intrinsic interest and requisite to an understanding of the physiology of matrix, it is also of importance to assess the *in vivo* activity of connective tissue cells in forming matrix. Recent progress has permitted considerable advances in this direction, largely as a result of the use of xylosides to study chondroitin sulfate synthesis and the availability of a sensitive radioimmune assay for the study of core protein synthesis (Ho et *al.*, 1977). More recently, a radioimmune assay for the study of link protein has also been developed (Caterson et *al.*, 1979).

By the use of four determinations, it is possible to assess various steps in

the synthesis of proteoglycans and their possible differences in various tissues and under different conditions. These may be enumerated as follows: (1) sulfate incorporation into glycosaminoglycans; (2) measurement of core protein synthesis by radioimmune inhibition assay; (3) assay for xylosyltransferase activity; and (4) capacity to form chondroitin sulfate chains in the presence of β-D-xylosides.

6.1. Sulfate Incorporation

The measurement of incorporation of $^{35}SO_4^{2-}$ to assess glycosaminoglycan synthesis has been in use for many years and has furnished valuable information both when employed with specific chemical isolation and with radioautography. In recent years, information regarding biosynthesis has been augmented as a result of improved methods of separation and identification of glycosaminoglycans, particularly those using specific enzymes. Isotopic precursors other than SO_4^{2-} such as labeled glucose, galactose, GlcNAc, and acetate have also been widely used. However, interpretation of results obtained with these precursors must be cautious because of pool size effects, purity of isolated glycosaminoglycans, and other metabolic pathways involving these precursors.

6.2. Measurement of Core Protein

A number of studies of proteoglycan biosynthesis have been based on the determination of proteoglycans by cesium chloride gradients and gel filtration. A more specific micromeasure of core protein is now possible utilizing a radioimmune inhibition assay introduced by Ho et *al*. (1977). This assay permits the distinction between core protein synthesis and the subsequent steps involved in proteoglycan synthesis. The availability of clonal antibodies holds promise of further refinement of this method (Dorfman et *al*., 1980a).

6.3. Measurement of Xylosyltransferase

As the radioimmune assay does not distinguish between core protein and xylosylated core protein, a detailed assessment of biosynthesis requires specific assay for xylosyltransferase. At present, this is most easily accomplished by the use of artificial acceptors (see Section 3.1).

6.4. Use of Xylosides

The use of free xylose on β-D-xylosides as initiators of chondroitin sulfate synthesis (Robinson et *al*., 1975; Levitt and Dorfman, 1973; Galligani et *al*., 1975) has made available a potent tool for the dissection of the pathway of proteoglycan synthesis. Earlier studies had indicated that xylosides, while in-

creasing the synthesis of free chondroitin sulfate chains (attached to xylosides), inhibited incorporation of SO_4^{2-} into proteoglycans. Kato et *al*. (1978) reported that xylosides inhibit the synthesis of a large proteoglycan (PCS-H). However, because inhibition of core protein synthesis was not distinguished from inhibition of chondroitin sulfate chain formation, it was not possible to interpret the significance of these findings.

The mechanism of this phenomenon has been explored by Schwartz (1977, 1979). She has demonstrated that β-D-xylosides compete with xylosylated core protein as substrates for galactosyltransferase I. As a result, in the presence of xylosides, although there was no decrease in synthesis of xylosylated core protein, there was a decrease in the number of chondroitin sulfate chains per core protein molecule. A small decrease in the length of chondroitin sulfate chains occurred.

In the presence of xylosides, chondroitin sulfate chain synthesis proceeds independently of synthesis of xylosylated core protein. It has been suggested previously (Horwitz and Dorfman, 1968) that the enzymes involved in chondroitin sulfate synthesis may exist as a multienzyme system attached to membranes and that xylosyltransferase serves to bind this enzyme system to core protein (Schwartz, 1975). Formation of free chondroitin sulfate chains in the presence of xylosides may then serve as a measure of the activity of such a multienzyme system independent of synthesis of xylosylated core protein.

This technique was used by Levitt and Dorfman (1973) to demonstrate that the primary effect of BrdUrd inhibition on differentiation of chick limb bud cells is on the capacity to form xylosylated core protein rather than on the capacity to synthesize chondroitin sulfate chains. Stearns and Goetinck (1979) used this technique to demonstrate that the cartilage of a proteoglycan-deficient chick mutant, leading to nanomelia, retains the capacity to synthesize xyloside-stimulated free chondroitin sulfate chains. The lesion in this genetic mutant must therefore result from defective xylosylated core protein synthesis. Xylosides have also been used by Johnston and Keller (1979) to study the synthesis of heparan sulfate by SV40-transformed Swiss mouse 3T3 cells. As in the case of chondroitin sulfate, β-D-xylosides served as chain initiators for heparin sulfate.

7. Control and Localization of Synthesis of Matrix Components

Thus far, attention has been focused on the biochemical mechanisms involved in the synthesis of CSPG. However, a complete understanding of matrix biology requires a delineation of the factors that regulate these processes and their localization with the cell.

7.1 Control of Synthesis of Matrix Components

The nature of extracellular matrix components appears to be specific for various developmental stages and tissues. In the case of cartilage there is good

evidence that differentiation of mesenchyme to cartilage is characterized by augmented synthesis of CSPG, type II collagen, and link proteins (Lash and Vasan, 1978) and decreased synthesis of type I collagen and fibronectin. The recently described chondronectin (Hewitt *et al.*, 1980) appears to be yet another cartilage-specific product (Chapter 4).

Vertel and Dorfman (1979) have studied the coordination of the synthesis of proteoglycan and types I and II collagen in cultured chondrocytes using simultaneous indirect immunofluorescence. In order to do this, cells were fixed and treated to permit intracellular antibody access. Initially, double staining with antibodies directed against core protein and type II collagen demonstrated the extracellular accumulation of proteoglycan and the intracellular accumulation of type II collagen for cells with characteristic chondrocyte morphology. This extracellular immunoreactive proteoglycan material could be removed by brief digestion with purified testicular hyaluronidase prior to fixation and intracellular reaction product could then be revealed. As shown in Fig. 5-4, core protein and type II collagen were observed within distinct, sometimes identical cytoplasmic regions of the same cartilage cells. In preliminary studies (Vertel and Dorfman, unpublished results), the distribution of link protein was found to be similar to proteoglycan subunit. Flattened fibroblastlike cells did not react with antibodies directed against the cartilage matrix components, but instead localized antibodies to type I collagen. Only rarely were cells found that contained type I collagen and core protein or type II collagen. Although these studies do not solve the problem of mechanism, they do appear to indicate that the synthesis of cartilage matrix components occurs concomitantly and that the switch from synthesis of type II collagen to type I collagen involves a switch from synthesis of other cartilage products such as core protein as well.

There is yet little information regarding the factors that govern the extent of synthesis of matrix components in the differentiated cell. There are indications that various hormones control the rate of synthesis of chondroitin sulfate and presumably proteoglycan. Older studies (Dorfman, 1974) showed decreased synthesis of sulfate containing polysaccharides in skin as a result of hypophysectomy, thyroidectomy, and alloxan diabetes.

Nevo and Dorfman (1972) studied the effect of large concentrations of chondroitin sulfate and proteoglycan on proteoglycan synthesis by suspensions of chondrocytes. Contrary to feedback inhibition that might be expected, a marked stimulation of proteoglycan synthesis was observed. Similar results were obtained by Huang (1974) and Schwartz and Dorfman (1975b) on chondrocytes cultured in monolayers. Nevo and Dorfman (1972) found the stimulation was not specific for chondroitin sulfate, but was observed with other highly charged cationic polyelectrolytes. In contrast to stimulation by chondroitin sulfate, Toole *et al.* (1972) have reported the inhibition of proteoglycan synthesis by HA (see Chapter 9 for further discussion).

As proteoglycan in cartilage exists in aggregates that contain CSPG, link protein, and HA, the coordination of synthesis of these three components is of importance in producing a physiologically normal matrix. Kimura *et al.* (1978, 1979, 1980) have studied the formation of aggregates by short-term cultures of Swarm chondrosarcoma. They concluded that under the conditions of their

experiments, chondrocytes synthesize more link protein than is required for binding of all endogenously synthesized CSPG monomer. They suggest that the assembly of aggregates occurs as a sequential process in which monomer–link complex is formed first and subsequently associates with HA to form the ternary complex with HA (see Chapter 2).

7.2. Localization of Proteoglycan Synthesis, Secretion, and Aggregation

In the rough endoplasmic reticulum, synthesis of core protein occurs on membrane-bound polyribosomes. As already indicated, there is as yet no definitive evidence as to whether chain initiation by transfer of xylose groups occurs before or after peptide chain completion.* By analogy with formation of other glycoprotein macromolecules, it is probable that chain initiation and perhaps completion of the linkage region may occur on nascent peptides before the protein is relayed to the cisternae. Figure 5-5 illustrates such a model and suggests that once xylosylation occurs, binding to a membrane-associated multienzyme complex promotes chain elongation. In Fig. 5-5, no distinction is made between smooth endoplasmic reticulum contiguous with rough endoplasmic reticulum and Golgi vesicles. By analogy with the pathway of synthesis of other *N*-glycoside glycoproteins, it is to be expected that the mannose-rich oligosaccharides would be added from a dolichol pyrophosphate intermediate to appropriate aspartic acid residues on nascent peptides. It is probable that at the same time keratan sulfate chains are initiated by transfer of GalNAc from UDP-GalNAc. Little is known about the sequence of the events leading to the initiation of the different types of polysaccharide chains or the factors that determine their localization on the core protein.

The site of elongation of chondroitin sulfate chains has been the subject of a number of investigations since the original studies of Godman and Lane (1964), who observed in radioautographs that SO_4^{2-} was concentrated in the juxtanuclear Golgi apparatus of secreting chondrocytes within 3 min of exposure to SO_4^{2-}. From this area, vacuoles migrate peripherally to the subcortex from whence the contents are discharged to the matrix (Chapter 2). Neutra and Leblond (1966) conducted similar studies utilizing [^{3}H]galactose and [^{3}H]glucose. In chondrocytes, labels derived from both galactose and glucose appeared first in the Golgi and subsequently in the matrix. In order to obtain more precise resolution, Revel (1970) utilized [^{3}H]acetate and again found appearance of the label in the Golgi, in this case 1 min after exposure to the precursor. It was pointed out that the radioactivity from carbohydrate precursors appeared in the Golgi much more rapidly than did radioactivity from amino acid precursors. Apparently, sulfation occurs rapidly, for when chondrocytes in culture are exposed to SO_4^{2-} for only 1–2 min, the labeled

*Recently, however, Sugahara *et al.* (1981) showed that nascent peptides of core protein isolated from polysomes contain xylose, indicating that xylosylation occurs before peptide completion.

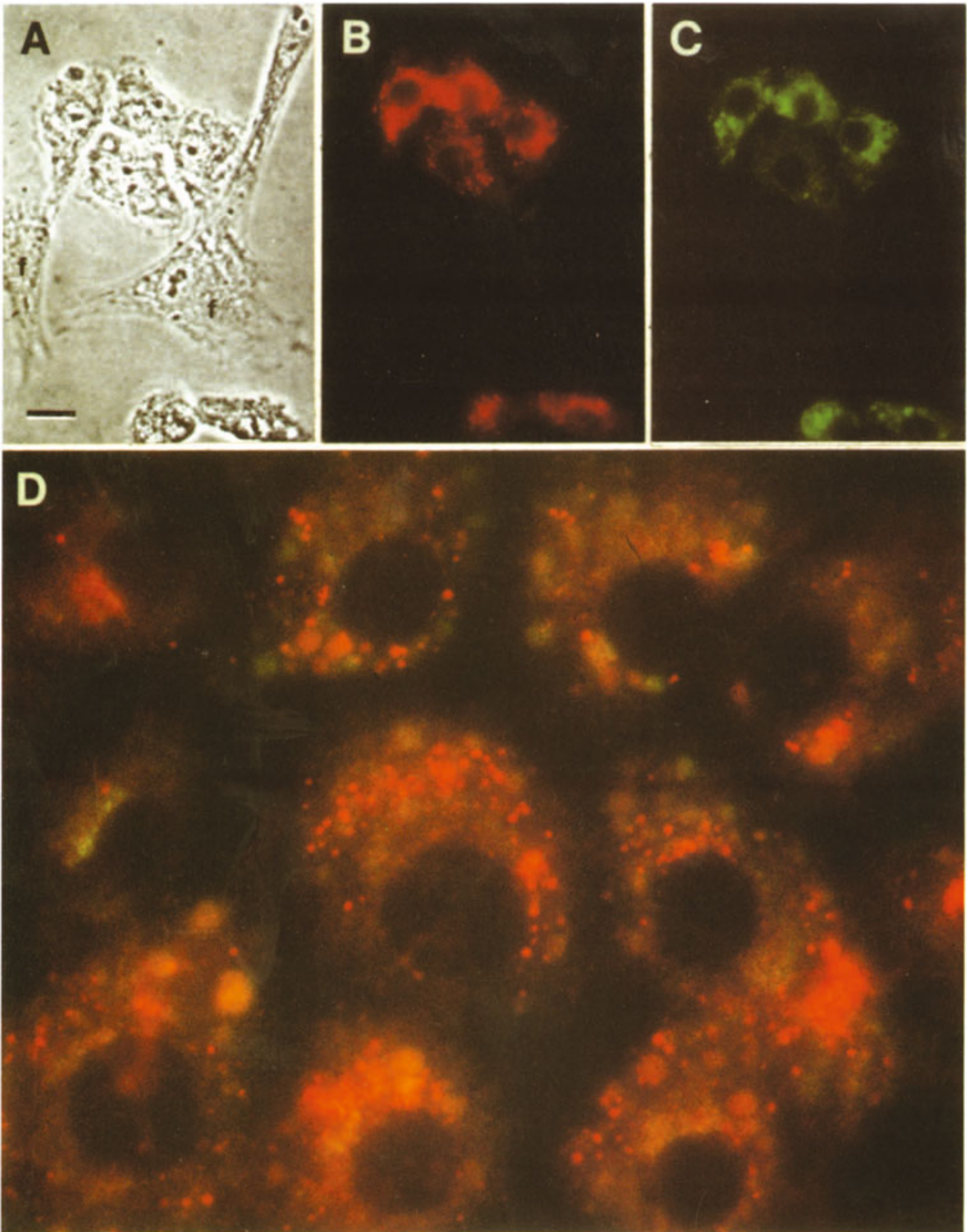


Figure 5-4. Simultaneous immunofluorescence localization of CSPG core protein (rhodamine-labeled) and type II collagen (fluorescein-labeled) in sternal chondrocytes cultured for 4 days and digested with purified testicular hyaluronidase prior to fixation. (A) Phase micrograph of two groups of chondrocytes and three fibroblastlike cells (f). (B) Localization of core protein. (C) Localization of type II collagen over the same cells. Note absence of reaction of fibroblastlike cells. (From Vertel and Dorfman, 1979.) (D) Double-exposed micrograph of another preparation similarly treated reveals that some of the core protein and type II collagen antibodies are localized in the same intracellular areas (yellow), while other discrete cytoplasmic regions contain primarily core protein (red) or type II collagen (green). Bar = 10 μm. (Courtesy of Barbara M. Vertel.)

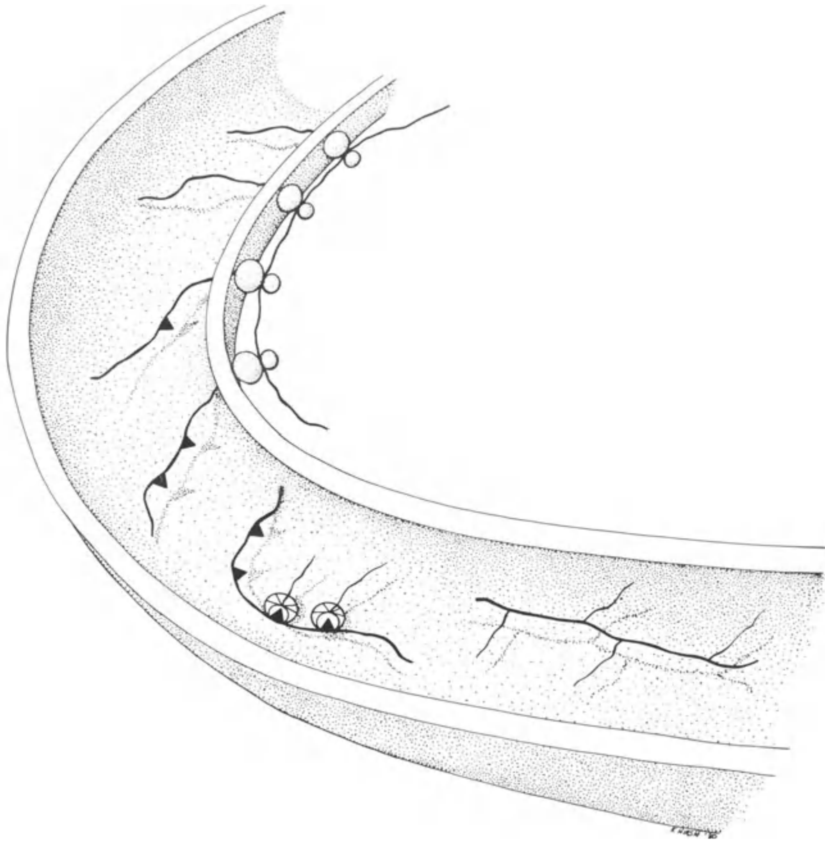


Figure 5-5. Model for biosynthesis of chondroitin sulfate in endoplasmic reticulum.

proteoglycan has a molecular size distribution indistinguishable from proteoglycans isolated after much longer labeling times (see Chapter 2).

Attempts to study localization of enzymes responsible for chondroitin sulfate synthesis in cell fractions were first made by Horwitz and Dorfman (1968). In these studies, no attempt was made to specifically isolate Golgi membranes. The enzymes responsible for synthesis of the proximal portion of chondroitin sulfate chains were concentrated in the rough endoplasmic reticulum while sulfotransferases were concentrated in smooth membranes. Enzymes responsible for chain elongation were found in both fractions. These studies were carried out before artificial substrates were available and consequently the activities measured were a function of both enzyme activity and acceptor concentration.

Freilich *et al.* (1975) have studied the biosynthesis of chondroitin sulfate in purified Golgi preparations obtained from mouse mastocytoma cells. On the basis of elongation of chains of endogenous acceptors and transfer of sugars to a pentasaccharide, it was found that the enzyme activity for elongation of chondroitin sulfate chains was concentrated in the Golgi. The available information indicates that elongation of chondroitin sulfate chains occurs in the

Golgi. By analogy, it might be expected that elongation of keratan sulfate chains also occurs in this cell organelle.

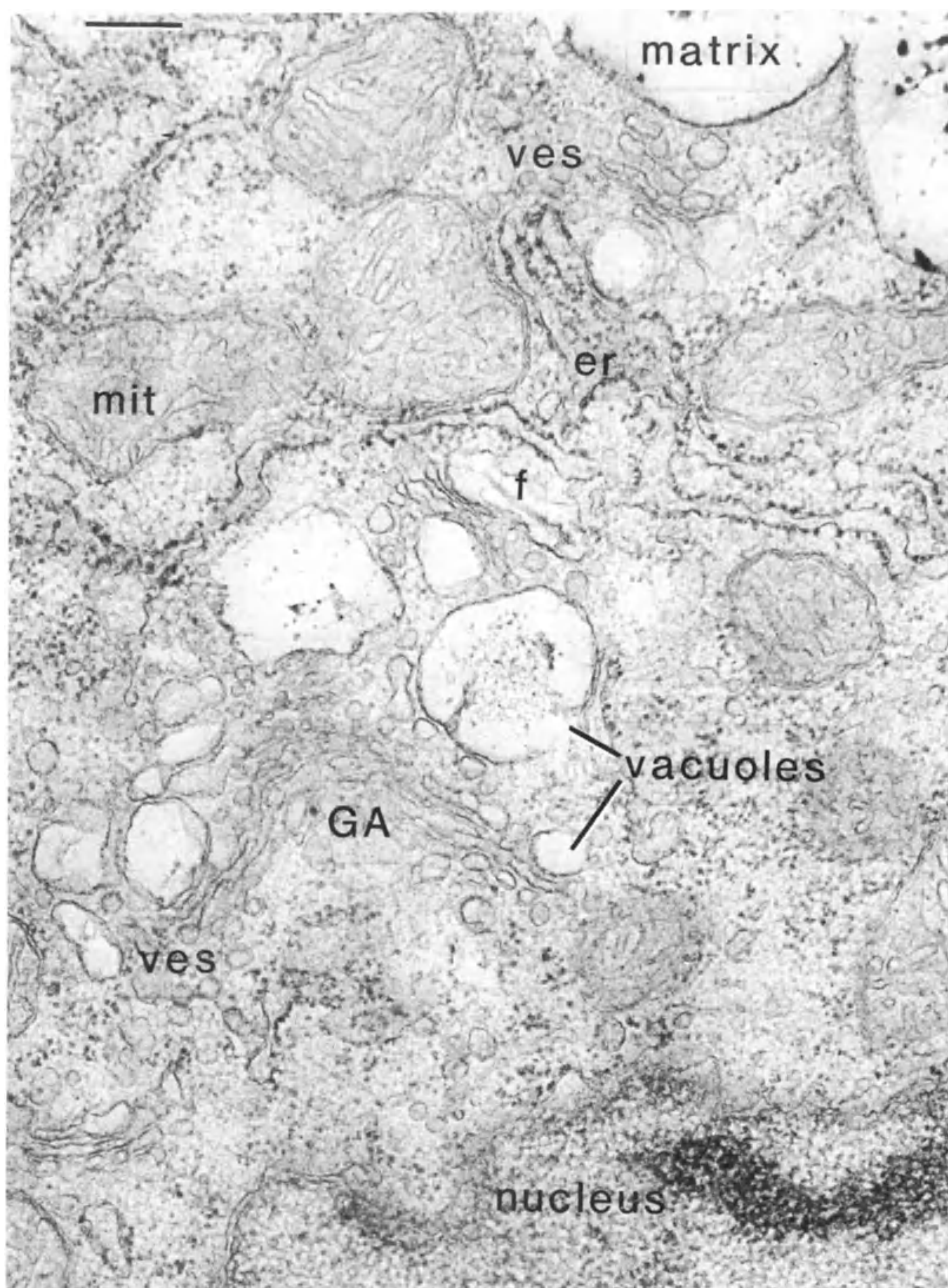
There is a considerable body of evidence that in other *N*-glycoside glycoproteins, the mannose-rich oligosaccharides chains are modified by removal of glucose and mannose residues. Subsequently, GlcNAc, galactose, sialic acid, and possibly fucose residues are added in the Golgi (Struck and Lennarz, 1980). It seems likely that such a mechanism obtains in the synthesis of CSPG.

The completed molecules are collected in secretory vacuoles, which appear to contain collagen or procollagen in fibrillar (f) (Fig. 5-6) or amorphous form, together with dense granules that are believed to be proteoglycan molecules (Chapter 2). Presumably, the contents of the granular endoplasmic reticulum (er) are relayed to Golgi cisternae (GA) by small vesicles (ves) where they accumulate with collagen in vacuoles of increasing size. The contents of the vacuoles are secreted to the matrix over a period of 10–30 min (Kimura *et al.*, 1980). Kimura *et al.* (1980) have proposed that at least a portion of the aggregation of CPSG, link protein, and HA occurs extracellularly (see Chapter 2).

8. Concluding Remarks

The biosynthesis of the large proteoglycan characteristic of cartilage matrix appears to be an extremely complex process. Initially, a large polypeptide is translated by conventional mechanisms. To this peptide must be added two or three types of polysaccharide chains. It is probable that the peptide is initially inserted into the membrane of the rough endoplasmic reticulum by a signal peptide. Preliminary evidence suggests that initial processing may involve addition of glycopeptides presumably via dolichol pyrophosphate mechanism. The site and time of addition of keratan sulfate chains are unknown. Chondroitin sulfate chain initiation occurs by xylosylation of core protein. Whether or not chain initiation occurs on nascent peptides or after peptide completion remains to be determined, but it is clear that the enzyme complex required for chondroitin sulfate synthesis is membrane bound. It is postulated that xylosyltransferase may serve not only to transfer xylose groups, but to bring core protein in juxtaposition with the multienzyme system required for chondroitin sulfate synthesis. This may occur by the demonstrated specific interaction

Figure 5-6. Electron micrograph of part of the cytoplasm and nucleus of a chondrocyte showing the Golgi apparatus (GA) and granular endoplasmic reticulum (er). The secretory product visible in the lumen of the reticulum is filamentous or amorphous in appearance. It is presumably transported to the Golgi zone in smooth-surfaced vesicles (ves) that fuse with the Golgi cisternae (GA) and give rise to Golgi vacuoles of various sizes. The smaller vacuoles probably fuse to form the larger vacuoles. The material in the larger vacuoles is similar to the matrix in appearance and probably represents a combination of proteoglycan and collagen. Some of the material in the Golgi vacuoles is fibrillar (f) in form, and the remainder is granular or filamentous. These vacuoles can be seen to fuse with the surface of the cell (Chapter 2). mit, mitochondrion. Larval *Ambystoma* limb, osmium fixation, uranyl acetate and lead hydroxide stain. Bar = 250 nm. (Courtesy of J.-P. Revel and E. D. Hay.) ⟶


matrix
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between xylosyltransferase and galactosyltransferase I. Sulfation occurs as polysaccharide chain elongation progresses in the Golgi. No specific mechanism of chain termination for chondroitin sulfate has yet been described.

Recent work has increased our insight into processes required for the synthesis of this proteoglycan. Yet, important questions remain. How does the cell coordinate the machinery required for the formation of 25,000 covalent bonds? What is the genetic program that sets these processes in motion when a mesenchyme cell differentiates to a cartilage cell? Why do noncartilage cells of many types have the machinery for making chondroitin sulfate? What is the structural relationship of the relatively ubiquitous CSPG to the proteoglycan of cartilage? With available techniques and newer knowledge of the structure of the eukaryotic genome, it seems likely that answers to these questions will be forthcoming. Such answers should not only contribute to our understanding of matrix biology, but perhaps further our general understanding of differentiation of eukaryotic cells.

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Chapter 6

Collagen Biosynthesis

BJORN REINO OLSEN

1. Introduction

The biosynthesis of the collagen component of connective tissues involves the regulated expression of gene members of the large collagen gene family (Chapter 1) as well as complex interactions between primary collagen translation products and several cotranslational and posttranslational modifying enzymes (for review, see Bornstein and Traub, 1979). Although the total number of genes that code for collagen polypeptides has not yet been established, it is probably between 10 and 20. Nothing is known about the linkage between the different genes in the family, but it is likely that they evolved by duplication and divergence of a small ancestral gene (Ohkubo et *al*., 1980; Yamada et *al*., 1980).

The biosynthesis of a fully functional collagen molecule requires the concerted expression of collagen structural genes and genes coding for posttranslational modification enzymes. As yet, nothing is known about the molecular basis for this interaction. Recent work using recombinant DNA techniques has allowed isolation of structural collagen genes. The complete chicken proα2 gene (Ohkubo et *al*., 1980; Wozney et *al*., 1981) and a fragment of the sheep proα2 gene (Boyd et *al*., 1980) have been isolated. In addition, a portion of the mouse proα1(I) gene has been characterized (Monson and McCarthy, 1980). We are thus beginning to understand the structure of collagen genes, and we can expect to obtain definitive information about the chromosomal location of the genes in the near future. On the basis of the limited information now available, it appears that the collagen genes are very large (about 40,000 bases long) and that they contain a large number (more than 50) of intervening noncoding sequences (Ohkubo et *al*., 1980). Therefore, synthesis of collagen mRNA molecules involves a large number of RNA splicing events.

The primary products of translation of collagen mRNA molecules are polypeptides, preproα chains, that contain hydrophobic signal sequences found in most other secretory polypeptides (Palmiter et *al*., 1979; Olsen and Berg, 1970; Graves et *al*., 1981). During or after ribosomal synthesis of the preproα chains,

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the chains are extensively modified by cotranslational and posttranslational modifying enzymes. These modification reactions include conversion of preproα chains to proα chains by removal of signal sequences, as well as hydroxylation, glycosylation, and disulfide-bond formation (for review, see Prockop et *al*., 1979). In the rough endoplasmic reticulum (RER) or in the Golgi region (see below), the hydroxylated and glycosylated proα chains fold into a characteristic triple helix. Following secretion of procollagen from cells, the procollagen molecule is converted to collagen by the action of specific proteolytic enzymes called procollagen proteases.

In the present chapter we will examine the structure of procollagens and their genes and the transcription of these genes. We will then discuss the translation of mRNA molecules into collagen polypeptides and the different posttranslational modification reactions that lead from the primary translation products to a functional collagen molecule. Finally, we will discuss the regulation of collagen biosynthesis.

2. Structure of Procollagens

Procollagen, the precursor of collagen, is considered to represent the major intracellular form of collagen (for review, see Fessler and Fessler, 1978). It is also the molecular form that is discharged into the extracellular space by cells synthesizing collagen. Procollagen is larger than collagen because of peptide extensions, propeptides, at both the NH_2 and the COOH terminals of the collagen α chains (Fig. 6-1). Earlier work assigned propeptides to the NH_2 terminals only, but it is now abundantly clear that there are large propeptide domains at both ends of the proα chains. Most of the information about the structure of procollagen has come from studies on type I procollagen, but preliminary investigations of type II and type III procollagens indicate that these proteins have similar structures. The evidence for NH_2 and COOH propeptides in procollagen came from both chemical and morphological data. First, studies using vertebrate collagenases to cleave procollagen molecules into two fragments of unequal size (Chapter 8) indicated that both fragments contained extra peptides that were not present when collagen molecules were cleaved with the same type of enzyme (Tanzer et *al*., 1974; Byers et *al*., 1975; Fessler et *al*., 1975; Olsen et *al*., 1976). Second, immunological data showed that procollagen molecules contain two independent sets of antigenic determinants not present in collagen (Nist et *al*., 1975). Third, electron microscopic examination of SLS crystallites (see Chapter 1) made from procollagen showed extra pieces not found in collagen SLS at both ends of the aggregates (Tanzer et *al*., 1974; Hoffman et *al*., 1976). (Fig. 6-2).

Chemical characterization of the NH_2 propeptide of calf and sheep proα1(I) chains has indicated that the peptide contains three structural (and functional?) domains (Becker et *al*., 1976; Hörlein et *al*., 1979). The NH_2-terminal portion of the dermatosparactic calf and sheep peptides is about 100 amino acid residues long and is resistant to bacterial collagenase. The central portion is about 45

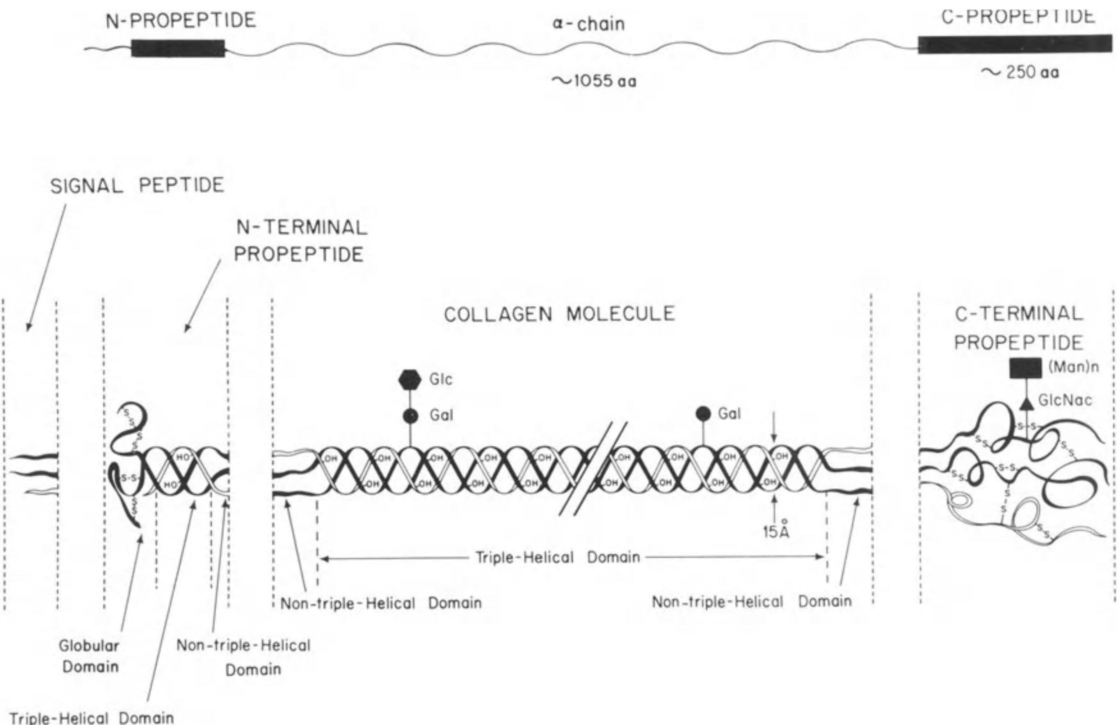
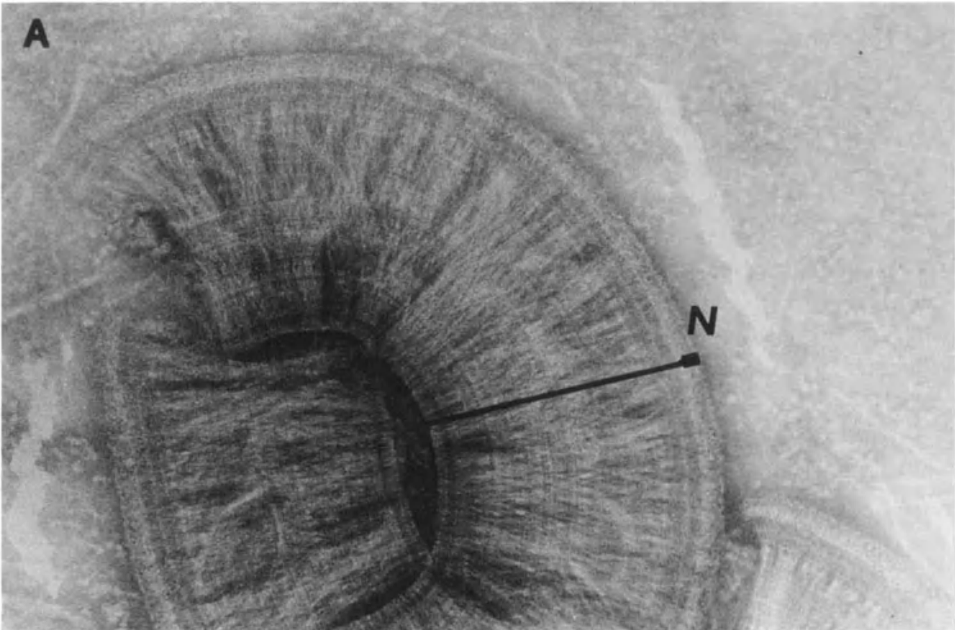


Figure 6-1. The domains of preproα chains and procollagen type I. During the synthesis of procollagen polypeptide chains on membrane-bound ribosomes, the chains are synthesized as preproα chains (top part of figure) with a hydrophobic signal peptide at their NH_2 terminal. The signal peptides are rapidly cleaved off the preproα chains as they enter the rough endoplasmic reticulum. (Modified from Prockop and Guzman, 1977.)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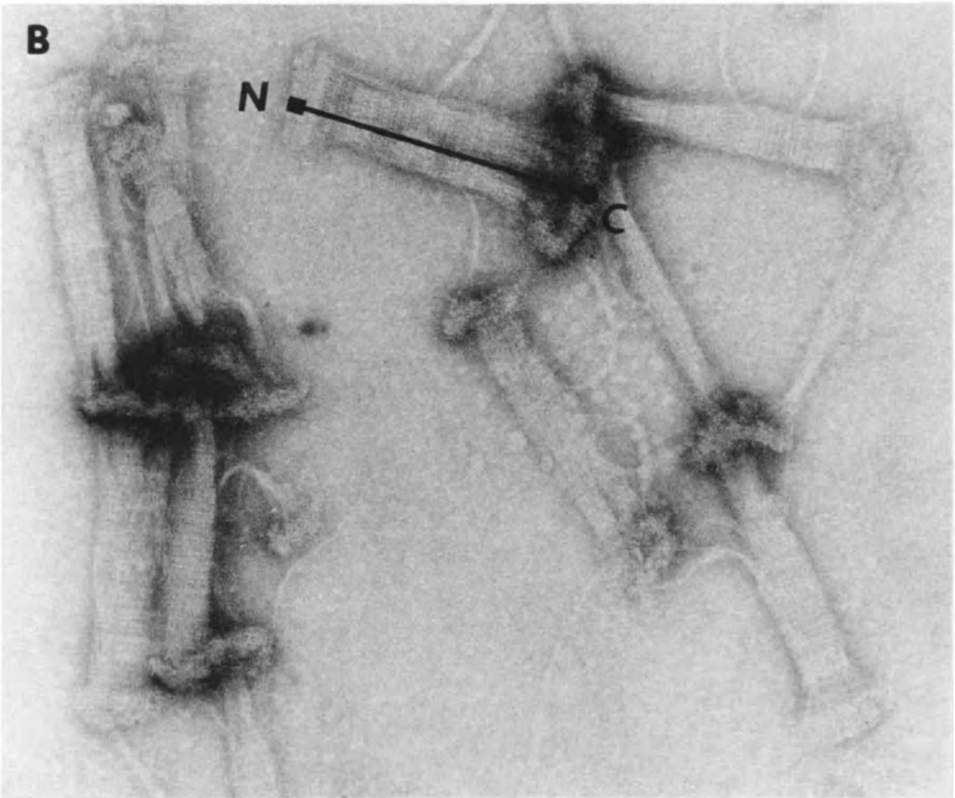
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residues long; it contains a collagenlike sequence with hydroxyproline and 30% glycine and is thus sensitive to bacterial collagenase. This "minicollagen" sequence is linked to the collagen α1(I) chain by a short connecting peptide (Fig. 6-1).

The complete amino acid sequence of the calf proα1(I) NH_2 propeptide* has been determined (Hörlein *et al.*, 1979). Also, the NH_2-terminal, collagenase-resistant domain of the sheep proα1(I) propeptide* has been sequenced (Rohde *et al.*, 1979). The sequences are almost identical in the two species and are similar, although not identical, to a partial sequence determined for the NH_2 propeptide of chick proα1(I) chains (Pesciotta *et al.*, 1980). A common feature among the chick, calf, and sheep proα1(I) NH_2 propeptides is the relatively large number of acidic amino acid residues at the NH_2 terminals of the peptides. In the central part of the NH_2-terminal globular domain, there are several cysteine residues and a high concentration of hydrophobic amino acid residues. The cysteine residues are all involved in intrachain disulfide bonds. Although sequence information for the type II procollagen NH_2 propeptide is not yet available, there are preliminary data available that suggest that this type of propeptide also contains only intrachain disulfide bonds similar to the type I peptides (Olsen *et al.*, 1976). In contrast, the type III procollagen NH_2 propeptide contains both intrachain and interchain disulfide bonds (Nowack *et al.*, 1976).

The collagenlike domain within the NH_2 propeptide contains glycine residues at every third position along the peptide chain. It also contains an unusually high content of proline and hydroxyproline. Consistent with this is the finding that the NH_2 propeptide of proα1(I) chains can form collagenlike triple-helical structures in solution (Engel *et al.*, 1977).

The NH_2 propeptide of proα2 chains appears to have the same overall structure as the proα1(I) peptide except that the NH_2-terminal, collagenase-resistant domain is much smaller. Although the complete amino acid sequence of this type of propeptide is not yet determined, the collagenlike central domain is probably similar to that of the proα1(I) peptide. Therefore, in a procollagen molecule in its native conformation, the central domains of proα1(I) and proα2 NH_2 propeptides together probably form a triple helix of high thermal stability.

* Chemical characterization of the NH_2 propeptides of calf and sheep type I procollagen has been possible through the discovery of a genetic defect, dermatosparaxis, in these animals. As will be discussed later in this chapter, in dermatosparaxis there is a defect in the normal conversion of type I procollagen to collagen in skin so that precursor molecules with an NH_2-terminal peptide extension (pN-collagen) accumulate in the skin. The proα1(I) NH_2 propeptides that have been sequenced were in fact derived from calf and sheep pN-collagen. In contrast, the chick proα1(I) NH_2 propeptide that has been characterized was derived from cells in culture and it was, therefore, a product of normal biosynthesis.

← **Figure 6-2.** SLS aggregates of type I pN-collagen (A) and procollagen (B) isolated from the medium of embryonic chick tendon fibroblasts incubated in suspension culture. The pN-collagen (see footnote above) reveals an NH_2-terminal segment (NH_2 propeptide) that is not present in control collagen SLS. The intact procollagen (B) reveals both NH_2- and COOH-terminal (COOH propeptide) segments. The aggregates were negatively stained with 1% potassium phosphotungstate. × 20,000.

During the conversion of procollagen in the extracellular matrix, the procollagen N-protease cleaves a peptide bond in the connecting peptide portion of the NH_2 propeptide leaving a short, non-triple-helical extension (telopeptide) at the NH_2 end of each collagen α chain (see Chapter 1). In the proα1(I) NH_2 propeptide, the enzyme cleaves a Pro-Gln bond (Hörlein *et al.*, 1979). The enzyme has an approximately 1000-fold higher affinity for native procollagen than for isolated proα chains (Tuderman *et al.*, 1978). It is believed, therefore, that the region containing the N-protease cleavage site has a distinct three-dimensional structure that is recognized by the enzyme.

The COOH propeptide of type I procollagen consists of three peptide chains that are linked by interchain disulfide bonds. The molecular weight of this propeptide trimer is 100,000 as determined by sedimentation equilibrium centrifugation (Olsen *et al.*, 1977). The amino acid composition is very different from that of the central collagen domain of procollagen and shows a relatively high content of acidic amino acid residues, cysteine, tyrosine, and phenylalanine. The COOH propeptides also contain mannose and *N*-acetylglucosamine, sugar residues that are not found within the collagen triple helix (Clark, 1979; Olsen *et al.*, 1977).

Recent studies that include a combination of cDNA nucleotide sequencing and classical amino acids sequencing have made it possible to formulate a detailed structural model for the type I procollagen COOH propeptide (Showalter *et al.*, 1980; Dickson *et al.*, 1981). The exact locations of the interchain disulfide bonds are not yet known, but the attachment site for a mannosyl oligosaccharide side chain (Fig. 6-3) and the locations of two (per peptide chain) of the intrachain disulfide bonds are known (Pesciotta, 1981).

In both the proα1(I) and the proα2 COOH propeptides, the oligosaccharide side chains contain two residues of *N*-acetylglucosamine attached to asparagine. There is somewhat more mannose in the proα2 chain than in the proα1 chain, 9 and 13 residues per chain, respectively (Olsen *et al.*, 1977). The significance of this is not known.

During the conversion of procollagen to collagen in the extracellular matrix, a procollagen C-protease cleaves a peptide bond at the NH_2 end of the COOH propeptide so that a short non-triple-helical extension (telopeptide) is left at the COOH end of each collagen α chain. In type I procollagen the C-protease cleaves an Ala-Asp bond in both the proα1(I) and the proα2 chains (Fuller and Boedtker, 1981; Dickson *et al.*, 1981).

Several functional roles have been proposed for the propeptides of procollagen, but none of these have been definitively proven. These proposed roles include: (1) chain association and triple-helix formation during molecular assembly: (2) intracellular transport and packaging of procollagen in secretory granules: (3) fibrillogenesis in the extracellular matrix: and (4) regulation of collagen synthesis. Definitive experimental support for any of these rules is still lacking, but preliminary data suggest that the COOH propeptide contains information required for polypeptide chain association (Rosenbloom *et al.*, 1976) and the NH_2 propeptide serves the role of a negative feedback inhibitor of collagen biosynthesis (Wiestner *et al.*, 1979; Paglia *et al.*, 1979).

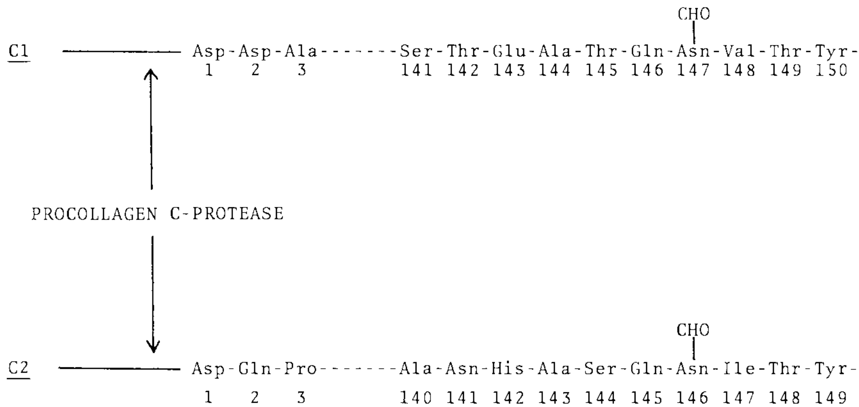


Figure 6-3. Partial amino acid sequences of the proα1(I) (C1) and proα2 (C2) COOH propeptides of type I procollagen. The cleavage sites of the procollagen C-protease and the attachment sites for the mannose-rich carbohydrate side chains (CHO) in the COOH propeptides are indicated. The amino acid residues are numbered starting with the NH_2-terminal aspartate residues of the two COOH propeptides.

3. Collagen Gene Structure and Regulation of mRNA Levels

Control of protein synthesis can be exerted through control of mRNA levels by (perhaps) regulation of gene transcription. In the case of collagen there is a good correlation between the rate of procollagen synthesis and the level of collagen mRNA in the cell (Adams *et al.*, 1977; Rowe *et al.*, 1978). For example, when chick cells are transformed with Rous sarcoma virus, the proportion of procollagen synthesized by the cells drops about 10-fold. At the same time, there is a 4- to 10-fold decrease in the amount of mRNA coding for collagen (Rowe *et al.*, 1978). As regulation of mRNA levels is likely to involve changes in transcription of collagen genes and/or changes in posttranscriptional processing of mRNA precursor molecules, it is clearly important to isolate and characterize collagen genes and study their transcription in well-defined systems.

3.1. Synthesis and Cloning of Collagen cDNA Probes

To obtain probes for assaying mRNA levels in cells and for isolation of collagen genes, several groups have attempted to synthesize and purify specific DNA copies of collagen mRNA molecules. This has been accomplished for both types of chick type I collagen chains [α1(I) and α2] (Lehrach *et al.*, 1978, 1979; Sobel *et al.*, 1978; Yamamoto *et al.*, 1980) and for human proα1(I) chains (Myers *et al.*, 1981). The cDNAs were prepared by using RNA isolated from either chick calvaria or human fibroblasts and enriched for high-molecular-weight polyadenylated RNA by a combination of chromatography on oligo-dT-cellulose

and sucrose gradient centrifugation (Fig. 6-4). As the mRNA molecules contain a poly(A) sequence at their 3′ ends, an oligomer of dT residues could be hybridized to the poly(A) sequence and was able to serve as a primer for the enzyme reverse transcriptase. The single-stranded DNA copies synthesized by the reverse transcriptase were made double-stranded with *E. coli* DNA polymerase I and amplified by molecular cloning in *E. coli* using a bacterial plasmid as a vector (Fig. 6-5).

Several bacterial clones containing recombinant plasmids with collagen cDNA inserts have now been isolated and characterized. Nucleotide sequence analysis of the inserts has provided useful information about the amino acid sequences of procollagen. Fuller and Boedtker (1981) have sequenced cDNA molecules that cover a large portion of the COOH end of α1(I) and α2 collagen chains and their complete COOH propeptides. Showalter *et al.* (1980) and Pesciotta *et al.* (1981) have used a combination of cDNA and peptide sequencing to identify the carbohydrate attachment sites in the type I COOH propeptides and to identify some of the intrachain disulfide bonds within the propeptide.

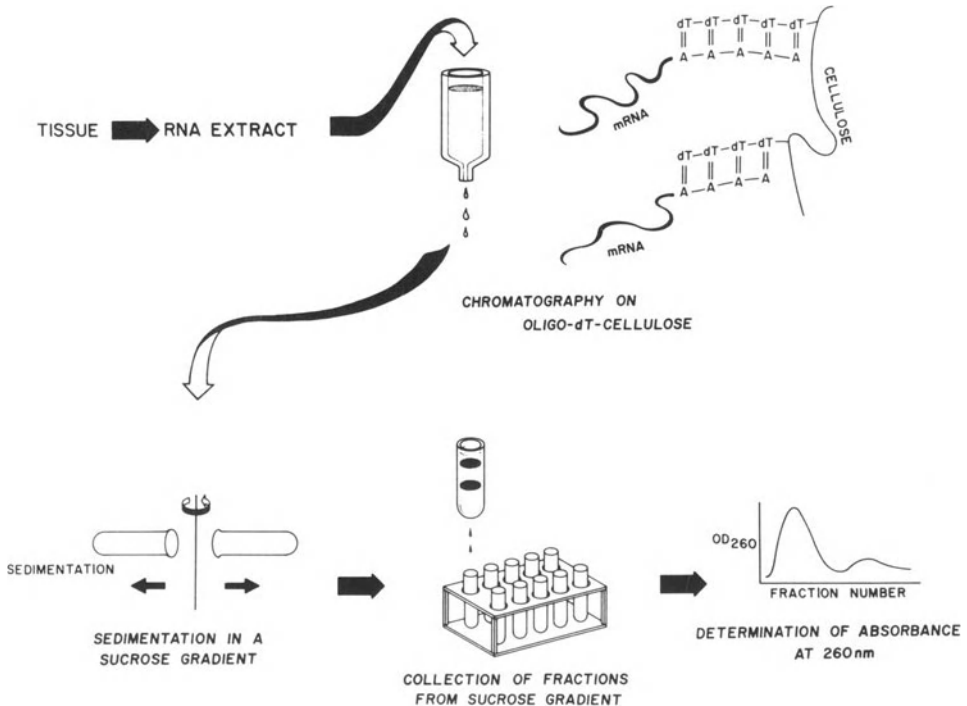


Figure 6-4. Procedure for the preparation of mRNA coding for collagen polypeptides. Tissues that are actively synthesizing collagen such as chick embryo calvaria or sterna are used for extraction of total RNA. The RNA is first fractionated by chromatography on an oligo-dT-cellulose column. The poly(A)-containing RNA molecules are bound to this column while ribosomal RNA is not. The RNA that can be eluted from the oligo-dT-cellulose column is then fractionated according to size by sucrose gradient centrifugation as indicated.

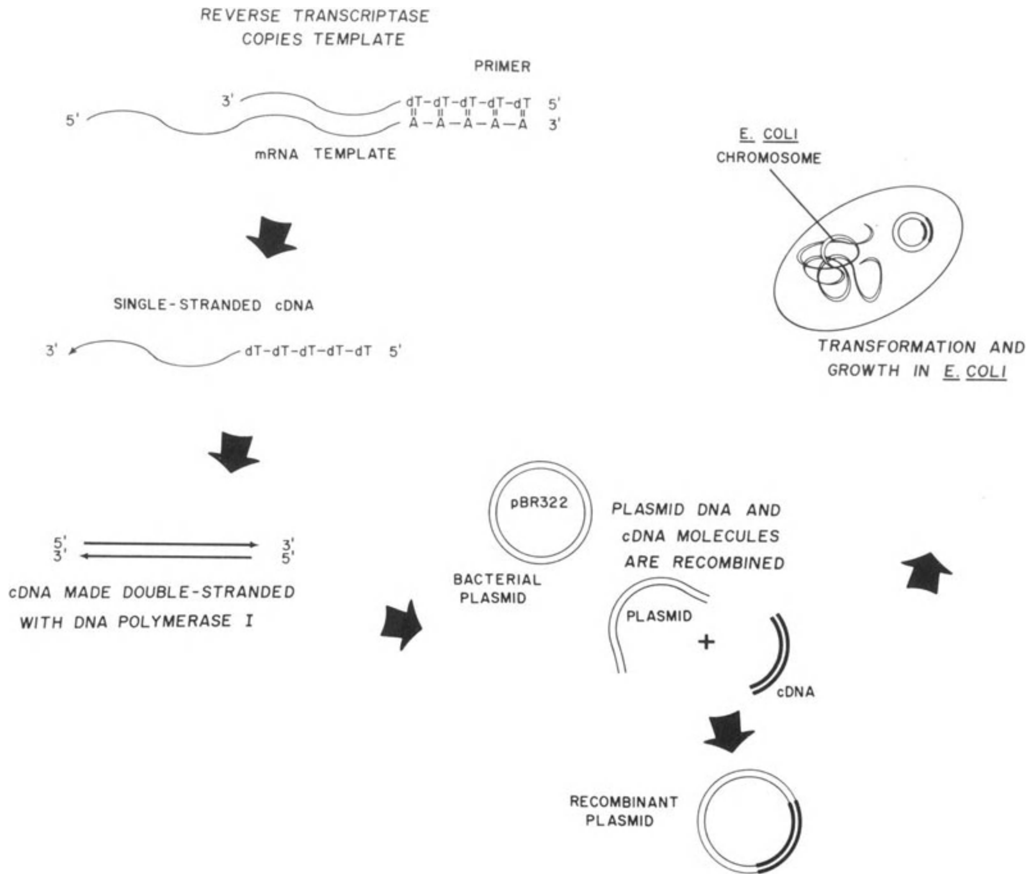


Figure 6.5. Procedure for the construction of recombinant plasmids containing sequences coding for collagen polypeptides. Collagen mRNA is first used as a template for the synthesis of single- and double-stranded cDNA using reverse transcriptase and DNA polymerase I. The double-stranded cDNA is then inserted into a restriction enzyme cleavage site in a plasmid vector using a "tailing" method or a "synthetic linker" method in a reaction that involves the enzyme polynucleotide ligase. The recombinant plasmid is grown in a suitable strain of *E. coli*, and appropriate clones containing the desired recombinants are isolated.

The elegance and power of recombinant DNA techniques are clearly illustrated by the rapid progress made in obtaining amino acid sequences of procollagen COOH propeptides through DNA sequencing. Of even greater importance, however, has been the use of cloned cDNA molecules as probes for isolation of collagen gene fragments from genomic libraries.

3.2. Isolation of Collagen Gene Fragments from Genomic Libraries

Genomic libraries from which collagen gene fragments have been successfully isolated include those of chicken, sheep, mouse, and human. They were

prepared by isolating 15- to 20-kb DNA fragments from partial restriction enzyme digests of total genomic DNA and ligating these fragments to the left and right arms of Charon 4A λ bacteriophage. The recombinant DNA was then packaged *in vitro* into infectious λ phage particles and amplified by growing the phage in *E. coli*.

Libraries have been screened for collagen genes using standard procedures. Briefly, the recombinant library phage was added to a lawn of *E. coli* grown on agar. After incubating the plates, DNA from phage plaques was transferred to nitrocellulose filters. The filters were dried and incubated with a ^{32}P-labeled cDNA probe. After washing and exposure to an X-ray film, "hot spots" on the developed film indicated the position of bacteriophage plaques that contained DNA sequences complementary to those of the probe (Fig. 6-6). Gene fragments with probe-specific sequences were then obtained by isolating and amplifying virions from such "host" plaques.

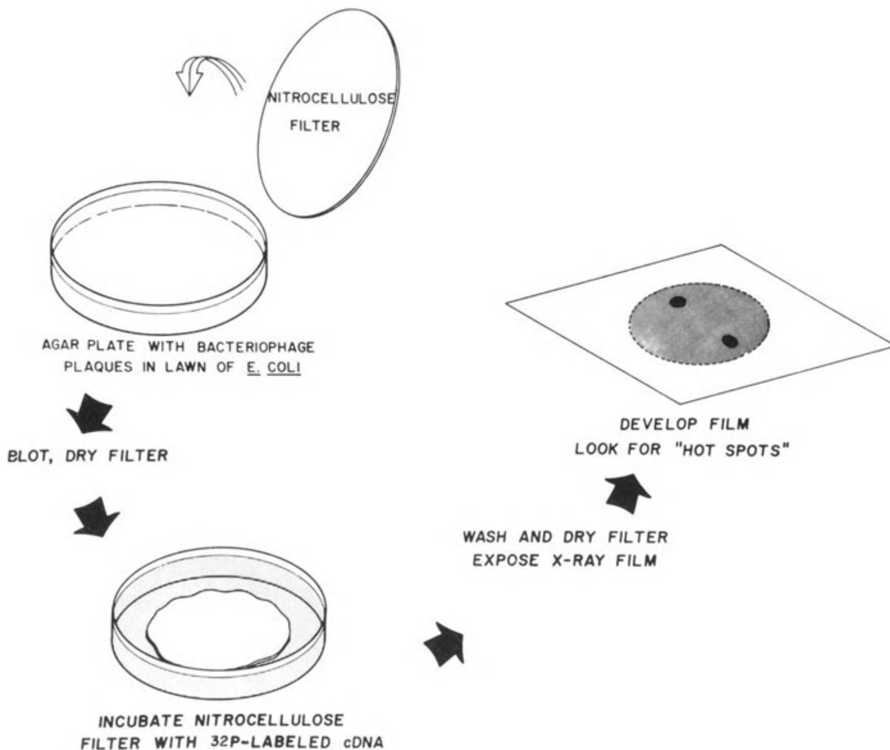


Figure 6-6. Procedure for screening a genomic library for specific genes. The recombinant phage containing restriction-enzyme-generated fragments covering the total genome is grown in a lawn of an appropriate strain of *E. coli*. The lysis plaques produced by the phage are screened for the presence of specific DNA sequences by transfer of a fraction of the DNA in the plaques to a filter of nitrocellulose using standard blotting techniques. The dried filters are soaked in a solution containing a ^{32}P-labeled cDNA probe under conditions that favor hybridization between the cDNA sequences and the genomic DNA sequences already bound to the filter paper. After washing off excess ^{32}P-labeled cDNA, the dried filter is placed on an X-ray film. Hot spots on the film indicate the position of phage plaques that contain genomic DNA sequences that hybridize with the ^{32}P-labeled probe. Virions in such positive plaques are picked from the agarplate and purified further by replating and rescreening.

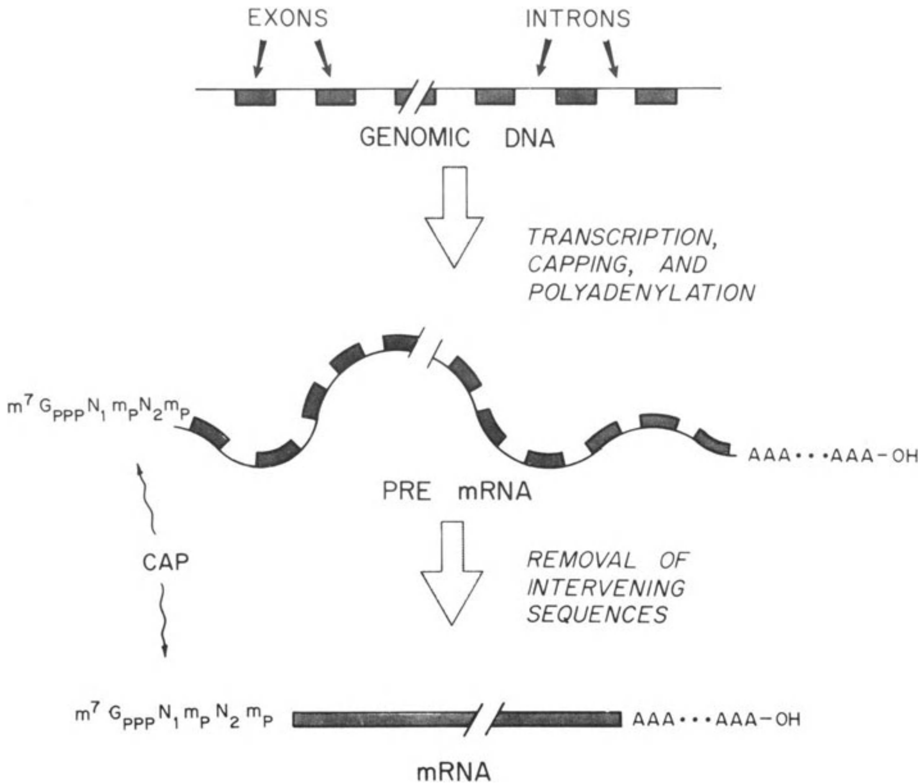
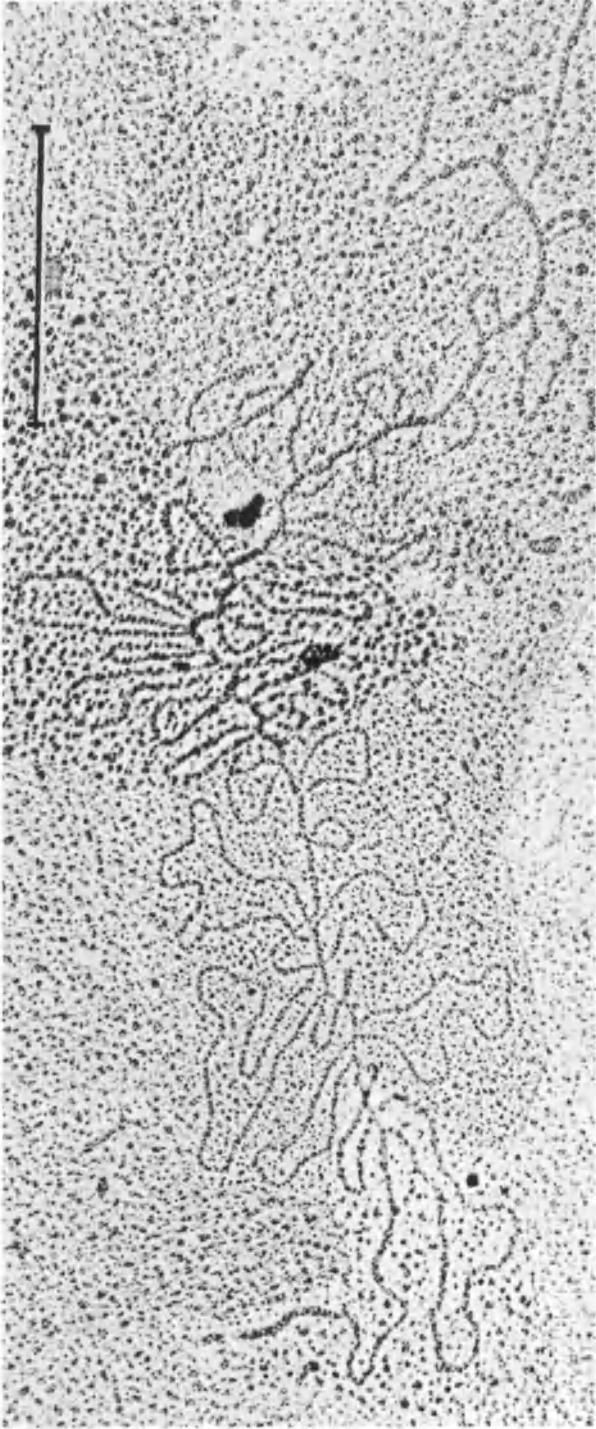


Figure 6-7. Most eukaryotic genes contain coding regions (exons) interrupted by noncoding intervening sequences (introns). The initial RNA transcripts of the genes are copies of both exons and introns. During maturation (processing) of the RNA, splicing enzymes remove the intron sequences, producing mature mRNA molecules.

3.3. Structure of a Collagen Gene

With such screening techniques, several chicken genomic clones spanning the entire proα2 collagen gene have been isolated (Ohkubo et *al.*, 1980; Wozney et *al.*, 1981). Although the complete gene structure has not yet been determined, the information already obtained shows that the gene is extremely large, about 40,000 base pairs. The organization of the proα2 gene is complex. Like many other eukaryotic genes (Fig. 6-7), it contains coding regions (exons) interrupted by noncoding intervening sequences (introns). Thus, the region of chromosomal DNA that contains the genetic information for a proα2 chain is considerably larger than the corresponding mRNA. The number of exons in the gene is probably around 50. Four of these exons, each 200–250 base pairs in length, are at the 3′ end of the gene and code for the COOH propeptide of proα2. The remaining exons are mostly 54 or 108 base pairs in length and code for the triple-helical region of the proα2 chain (Vogeli et *al.*, 1980) (Fig. 6-8). The gene structure may be different in other animal species. For example, a genomic DNA fragment, covering about half of the proα2 gene has been isolated from



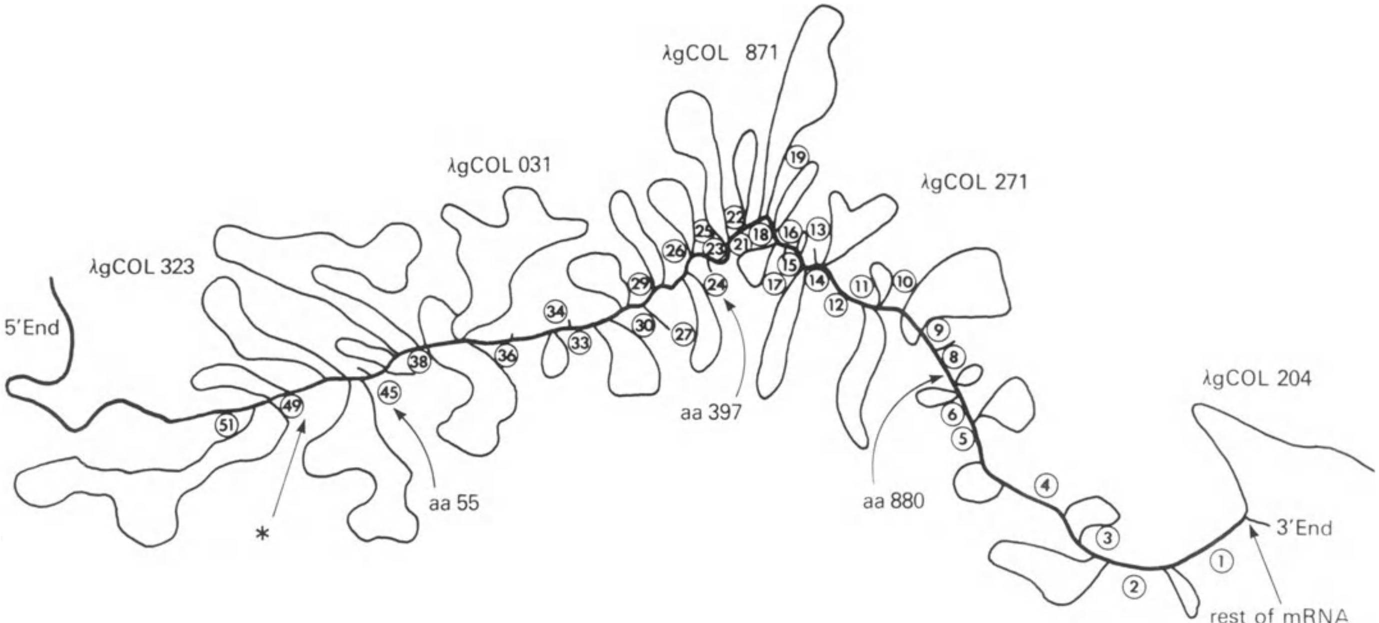


Figure 6-8. Electron micrograph and diagram showing the exon/intron structure of the chick proα2 collagen gene. The top part of the figure is a composite of several "R-loop" electron micrographs. The "R-loops" were prepared by incubating mRNA coding for type I collagen with one of several genomic clones coding for different portions of the proα2 chain under conditions that favor formation of stable DNA–RNA hybrids. As mRNA molecules contain sequences that are complementary to exon sequences but not to intron sequences, RNA and exons will form a double-stranded "core" in the "R-loop" structure with introns forming loops along this core. The bottom part of the figure is a tracing of the structure seen in the top part. The circled numbers indicate exons counted from the 3' end of the mRNA, and the amino acids 55, 397, and 880 in the α2 chain of collagen are indicated. The genomic clones λgCOL 204, λgCOL 271, λgCOL 871, λgCOL 031, and λgCOL 323 were used to obtain the "R-loop" structure. Bar =1300 base pairs. (From Vogeli *et al.*, 1981.)

sheep DNA (Boyd et *al*., 1980). Here, all the coding information for the carboxyl propeptide of proα2 appears to reside in one 850 base pair long exon at the 3′ end of the gene.

3.4. Regulation of Collagen mRNA Levels

Several types of data collectively indicate that the extent of procollagen synthesis by cells in culture is determined primarily by the levels of mRNA rather than by control of mRNA translation (Rowe et *al*., 1978). The question of how mRNA levels are regulated in cells that produce procollagen therefore becomes an important one. Changes in mRNA levels could be brought about by changes in the rate of synthesis and/or degradation of mRNA. At present, no data are available on this point.

4. Cotranslational and Posttranslational Modifications of Intracellular Procollagen

Numerous studies with subcellular fractions of cells show that when cells or tissues are incubated with radioactive proline, radioactive peptidyl hydroxyproline or collagenase-sensitive peptides can be recovered in microsomes and polysomes (for review, see Prockop et *al*., 1979). Such studies coupled with a demonstration of prolyl hydroxylase activity in microsomal fractions (Harwood et *al*., 1974) form the basis for the conclusion that collagen polypeptides are synthesized by membrane-bound polysomes and hydroxylated while still attached to the ribosomes.

There is good evidence that proα chains, like other secretory polypeptides, are synthesized with a hydrophobic signal sequence (see Blobel, 1980) at the NH_2 terminal of the nascent proα chains and that this signal sequence is rapidly processed in the RER (Palmiter et *al*., 1979; Graves et *al*., 1981). As a result, collagen polypeptides that contain this NH_2-terminal hydrophobic signal sequence can only be observed by translating collagen mRNA in a cell-free system without microsomal membranes.

4.1. Cell-Free Translation of Collagen mRNA

Because of the large size of collagen polypeptides, the corresponding mRNA molecules are among the largest mRNA molecules found in cells. They are, therefore, relatively easy to separate from other mRNA species. In most studies published to date, the RNA has been fractionated on oligo-dT-cellulose columns to separate ribosomal RNA from polyadenylated mRNA, followed by a sucrose gradient centrifugation to separate the high-molecular-weight RNA from low-molecular-weight species (Fig. 6-4).

When RNA is extracted from tissue culture cells or from a tissue that produces mostly type I collagen and the RNA is fractionated as outlined above, RNA directs the synthesis of polypeptide products of molecular weights about 180,000 (Boedtker et *al.*, 1976; Monson and Goodman, 1978; Rowe et *al.*, 1978) in cell-free systems (Fig. 6-9). It has been shown that in such systems the polypeptide products are longer than the proα chains secreted by cells. The designation preproα chains is therefore used for these cell-free products (Palmiter et *al.*, 1979). Both wheat germ and nuclease-treated reticulocyte extracts have been used to translate RNA from embryonic chick tissues and cultured chick fibroblasts into preproα1(I) and preproα2 chains (Boedtker et *al.*, 1976; Monson and Goodman, 1978; Graves et *al.*, 1981). Amino acid sequencing studies (Palmiter et *al.*, 1979; Graves et *al.*, 1981) suggest that the NH_2-terminal sequences of preproα chains are similar to signal sequences in other secretory proteins (for review, see Davis and Tai, 1980) in that they contain a preponderance of hydrophobic amino acid residues. The exact size of the signal sequences of preproα chains is not known, but in preliminary studies the signal peptide of preproα1(I) chains has been estimated to have a molecular weight of 5500–20,000. If these estimates prove correct, the signal peptides of preprocollagen chains would be much larger than prokaryotic and eukaryotic signal peptides characterized thus far (for review, see Davis and Tai, 1980). The possibility has been suggested that only the NH_2-terminal portion of the "pre" peptide of preproα chains represents the "true" signal sequence (about 15–25 amino acid residues), while the remainder could serve some other function (Olsen and Berg, 1979; Sandell and Veis, 1980); e.g., chain selection during molecular assembly.

The signal sequences would serve as vectors for ensuring that the nascent protein chains will cross the membrane of the RER (Blobel, 1980). As proα chains isolated from intracellular compartments have about the same molecular weights as proα chains of secreted procollagen molecules, the conversion of preproα chains to proα chains must be a rapid process, occurring either during translation on ribosomes or shortly after completion of polypeptide chain synthesis in the RER.

4.2. Intracellular Location of Posttranslational Modifications

The formation of a collagen triple helix that is stable at 37°C requires the presence of about 100 hydroxyproline residues per α chain (for review, see Prockop et *al.*, 1979). Synthesis of hydroxyproline is required, therefore, for the formation of a functional collagen molecule. Hydroxyproline is synthesized from prolyl residues after their incorporation into nascent peptides.

The synthesis of hydroxyproline occurs within the RER. In this compartment the newly synthesized proα chains are also disulfide linked into triple-stranded molecules. In addition, some of the lysyl residues are hydroxylated and glycosylated and the COOH propeptides are glycosylated. Whether all the posttranslational modifications are completed in this compartment is not clear.

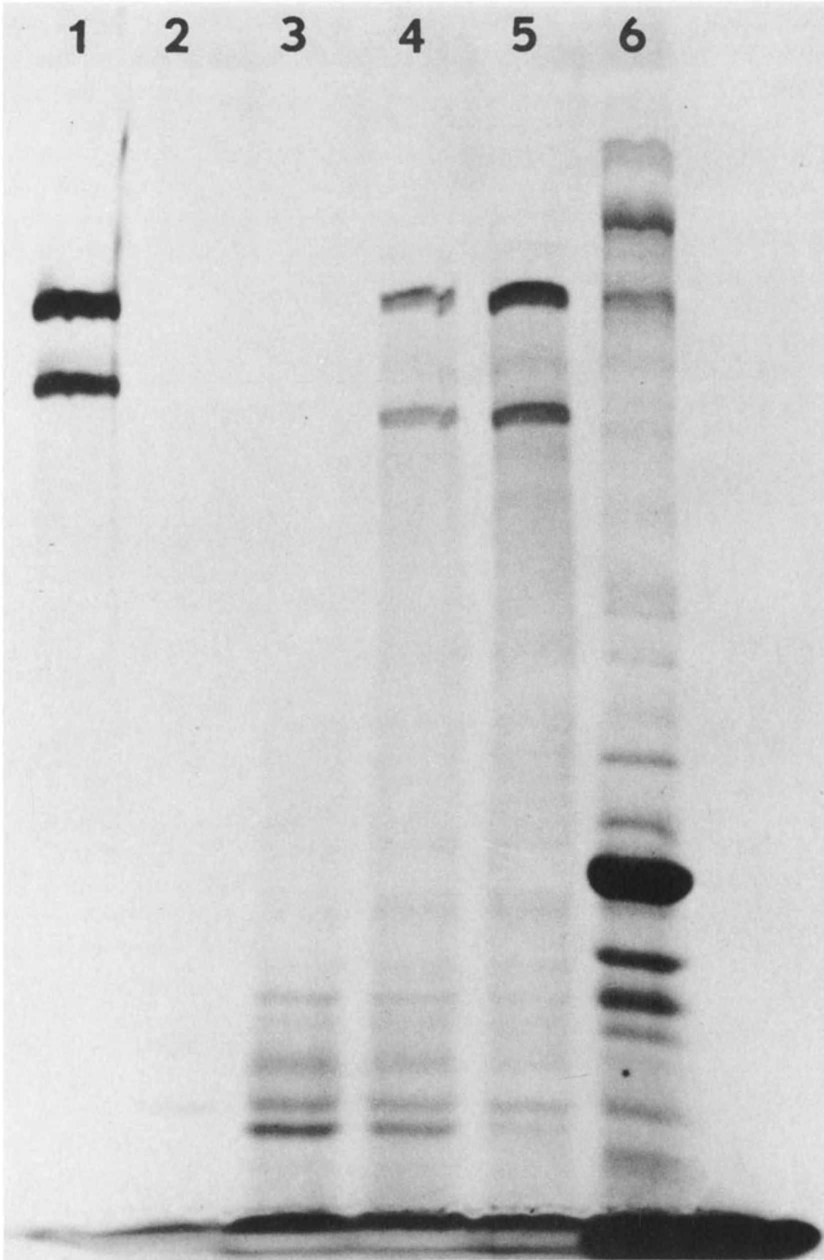
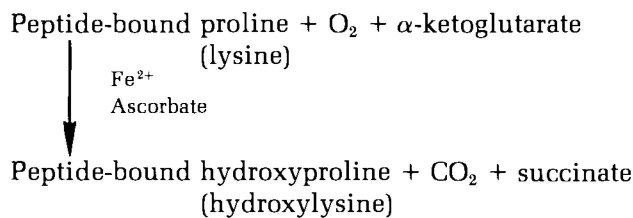


Figure 6-9. Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate of products obtained by translating chick embryo calvarial RNA in a reticulocyte lysate. Lane 1: proα1 and proα2 chains of type I procollagens secreted by chick embryo fibroblasts; lane 2: micrococcal nuclease-treated lysate, no exogenous RNA added; lane 3: micrococcal nuclease-treated lysate, calvarial 18 S RNA added; lane 4: micrococcal nuclease-treated lysate, total calvarial RNA added; lane 5: micrococcal nuclease-treated lysate, calvarial 28 S RNA added; lane 6: reticulocyte lysate not treated with micrococcal nuclease, no exogenous RNA added. (From Graves et *al.*, 1981.)

Subcellular fractionation studies suggest that posttranslational modification enzymes are distributed in several subcellular compartments (for reviews, see Fessler and Fessler, 1978; Prockop et *al*., 1979; Bornstein and Sage, 1980), but these studies are difficult to interpret because clean fractions, especially of the Golgi complex, were not obtained. The site of triple-helix formation is also unknown. However, hydroxylation of prolyl and lysyl residues and glycosylation of hydroxylysyl residues occur only on unfolded polypeptide chains. Therefore, if hydroxylation and glycosylation events do continue after procollagen has reached the Golgi complex, the triple helix cannot form until after the molecules have reached this compartment.

4.3. Hydroxylation of Prolyl and Lysyl Residues

Hydroxylation of prolyl and lysyl residues by three hydroxylases located in the RER are the best characterized modifications of procollagen (for reviews, see Kivirikko and Myllylä, 1981; Adams and Frank, 1980). Two of the hydroxylases convert some prolyl residues to 4-hydroxyproline or 3-hydroxyproline, and the third enzyme converts some lysyl residues to hydroxylysine. The three enzymes are mixed-function oxygenases. Their reactions involve the cofactors ferrous iron and ascorbate, and the cosubstrates oxygen and α-ketoglutarate. During the hydroxylation reaction, α-ketoglutarate is decarboxylated to succinate as the peptide substrate is hydroxylated.



The requirement for iron as a cofactor makes it possible to inhibit the hydroxylases in cells by introducing an iron chelator such as α,α'-dipyridyl (for review, see Prockop et *al*., 1979).

The three hydroxylases require a peptide substrate in a non-triple-helical conformation, and the susceptible prolyl and lysyl residues must be within specific amino acid sequences (for review, see Kivirikko and Myllylä, 1981). Prolyl 4-hydroxylase will only hydroxylate prolyl residues in the Y position of peptides containing X-Y-Gly sequences. Prolyl 3-hydroxylase will only hydroxylate prolyl residues in the X position of peptides containing X-Hyp-Gly sequences. Within the collagen domain of proα chains, lysyl hydroxylase appears to have the same position specificity as prolyl 4-hydroxylase in that it only hydroxylates lysyl residues in the Y position of peptides containing X-Y-gly sequences. However, within the telopeptide regions of proα chains, the lysine before glycine rule does not hold (Chapter 1). The hydroxylysines syn-

thesized within those regions are of special importance because they are converted to the corresponding aldehydes by lysyl oxidase and participate in cross-link formation in the extracellular matrix.

Lysyl hydroxylase has been extensively purified (for review, see Kivirikko and Myllylä, 1981) and apparently is a dimer of two subunits each of about 90,000 molecular weight (Turpeenniemi *et al.*, 1977). It is a microsomal enzyme and its solubility properties suggest that it is tightly associated with the membrane of the RER. It reacts more efficiently with increasing lengths of peptide substrate. Prolyl 3-hydroxylase has only been partially characterized (Risteli *et al.*, 1977), but prolyl 4-hydroxylase has been isolated as a pure protein from several sources (Berg and Prockop, 1973; Kivirikko and Myllylä, 1981).

The active prolyl 4-hydroxylase enzyme is a tetramer of molecular weight 240,000 with the subunit structure $\alpha_2\beta_2$, where α and β are two types of subunits of molecular weights about 60,000. The enzyme is a glycoprotein (Berg *et al.*, 1979), in that α subunits isolated from chick embryos have been shown to contain *N*-acetylglucosamine, galactose, and several residues of mannose. The β subunit also contains carbohydrate but less than the α subunit (Berg *et al.*, 1979). It is possible that the dissimilarity between the α and the β subunits may reflect the presence of two catalytic subunits (α) and two subunits (β) that are recognized by and bound to an RER membrane receptor thereby preventing the enzyme from being secreted along with procollagen.

Prolyl 4-hydroxylase is located within the RER, as shown by electron microscopy using ferritin-labeled anti-prolyl hydroxylase antibodies (Olsen *et al.*, 1975) (Fig. 6-10) and by its presence in microsomal subcellular fractions (Diegelmann *et al.*, 1973; Harwood *et al.*, 1974; Peterkofsky and Assad, 1976). This conclusion is further supported by the observation that prolyl hydroxylase activity can be released from isolated microsomes by detergent treatment (Diegelmann *et al.*, 1973). Also, when microsomes are isolated from cells treated with α,α'-dipyridyl so that prolyl hydroxylase is inhibited (see below), the microsomal fraction contains unhydroxylated collagen polypeptides. When such microsomes are incubated with cofactors for prolyl hydroxylase, the unhydroxylated collagen polypeptides are hydroxylated (Cutroneo *et al.*, 1974). The enzyme does not appear to be an integral membrane protein or to be tightly associated with the RER membrane, for it is easily soluble after mild detergent treatment of microsomes to disrupt the microsomal membrane.

Of special interest is the finding that although the active prolyl 4-hydroxylase is a tetramer of molecular weight 240,000, about 30–99% of the enzyme protein in cells is in an inactive form of molecular weight about 60,000 (for review, see Prockop *et al.*, 1979). This inactive form appears to consist entirely of the β subunit or a form related to this subunit that may be associated with cell membranes (Chen-Kiang *et al.*, 1977). Comparisons of various cells and tissues indicate that the ratio of active tetramers to the inactive form is roughly proportional to the rate of collagen synthesis. It has been suggested, therefore, that conversion of prolyl 4-hydroxylase from an inactive to an active form may be important in regulating collagen synthesis during development or during fibrosis. Recent data on the assembly of active, tetrameric prolyl 4-hydroxylase

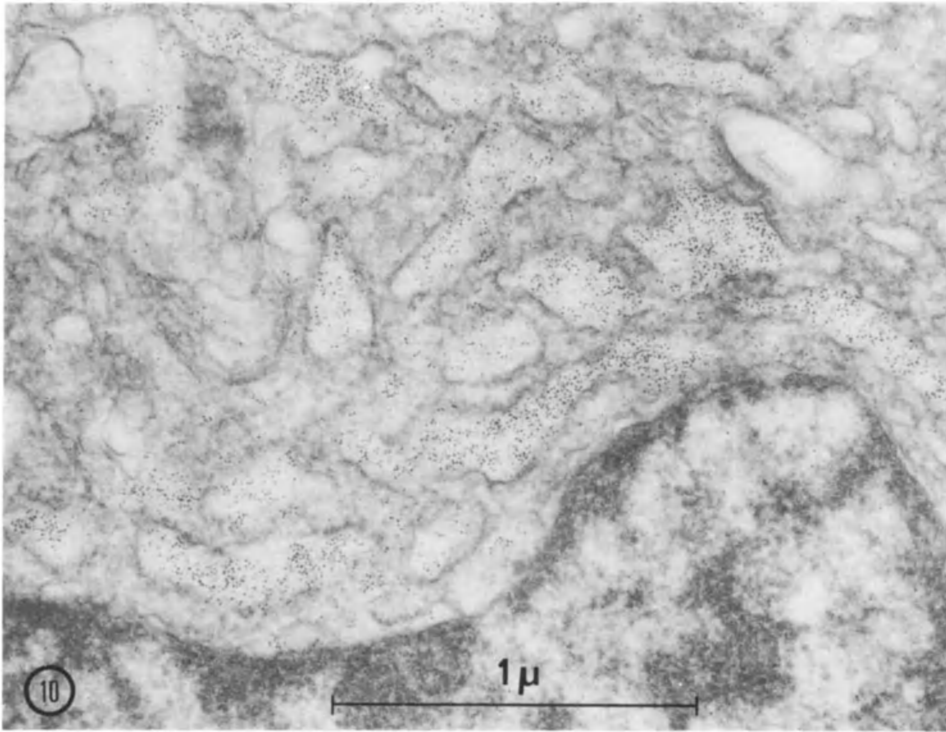


Figure 6-10. Electron micrograph showing part of a tendon fibroblast incubated with ferritin-labeled anti-prolyl hydroxylase antibody. The cells were fixed with 1% formaldehyde for 3 hr at 4°C, fragmented by homogenization, and incubated with the ferritin conjugate (1 mg ferritin and 0.2 mg IgG per ml) for 36 hr at 4°C. Note the heavy labeling of the cisternae of the RER and the absence of significant labeling of the cytoplasm except in places showing localized damage to the membranes of the endoplasmic reticulum. Bar = 1 μm. (From Olsen et *al.*, 1975.)

in embryonic chick tendon fibroblasts indicate that the inactive form serves as a precursor of β subunits in the active form of the enzyme (Berg *et al.*, 1980). This observation provides a basis for the attractive hypothesis that changes in the amount of active prolyl 4-hydroxylase can be brought about by changes in the rate of synthesis of α subunits. According to this view, assembly of active enzyme would involve the formation of tetramers by newly synthesized α subunits and preformed β subunits (Berg *et al.*, 1980).

4.4 Disulfide-Bond Formation and Triple-Helix Formation

The formation of disulfide bonds between proα chains contributes to the rate of formation and stability of the triple-helical conformation of procollagen. The NH_2 propeptides were initially envisioned as registration peptides (Speakman, 1971), but at least in type I and type II procollagens these peptides

contain only intrachain disulfide bonds (for review, see Prockop *et al.*, 1979). Therefore, the NH_2 propeptides are probably not sites for interchain recognition and initial chain association. As there are interchain disulfide bonds between the COOH propeptides, however, these peptides are now considered to contain the information that is required for proper chain association (Rosenbloom *et al.*, 1976). According to this view, the folding of the COOH-propeptide region of procollagen, stabilized by disulfide bonds, would precede the formation of the collagen triple helix. In support of this model is the observation that chain association and interchain disulfide-bond formation are processes that can proceed in the absence of prolyl hydroxylation and triple-helix formation (see Prockop *et al.*, 1979).

The formation of interchain disulfide bonds in procollagen occurs in the RER after polypeptide chain synthesis (Lukens, 1976). It has been suggested that a microsomal enzyme, disulfide isomerase, catalyzes the exchange of disulfide bonds and may correct disulfide bonds in procollagen (Anfinsen, 1973; Harwood and Freedman, 1978; Harwood, 1979).

The exact time or location for triple-helix formation is not yet known. Although some reports suggest that helix formation can occur while the polypeptide chains are stilll attached to ribosomes (Veis and Brownell, 1977), most available evidence favors a later stage of intracellular assembly (for discussion, see Prockop *et al.*, 1979). What is established, however, is the fact that hydroxyproline plays a critical role in stabilizing the triple helix under physiological conditions. Without any hydroxyproline residues, triple-helical collagen will melt around 25°C, whereas with about 100 hydroxyproline residues per α chain, the melting temperature is about 40°C (Berg and Prockop, 1973; Rosenbloom *et al.*, 1973). It is, therefore, clear that stable procollagen triple helices cannot form in cells at 37°C unless about half the proline residues have been hydroxylated by prolyl 4-hydroxylase. Several types of experiments in which the formation of the triple helix is prevented indicate that nonhelical procollagen is secreted by cells more slowly than helical procollagen. In fact, nonhelical procollagen is secreted with a rate constant that is only one-tenth of the rate constant for the secretion of helical molecules (Kao *et al.*, 1977, 1979). This finding probably explains the observed correlation between the rate of secretion of procollagen from cells and the level of hydroxylation of prolyl residues.

4.5. Glycosylation of Hydroxylysyl Residues

O-Glycosylation of hydroxylysine in the collagen domain of procollagen occurs by sequential action of galactosylhydroxylysyltransferase and glucosylgalactosylhydroxylysyltransferase (for review, see Kivirikko and Myllylä, 1979). The enzymes have been highly purified and shown to require hydroxylysine in polypeptides with a non-triple-helical conformation as substrate. Galactosylhydroxylysyltransferase transfers galactose from UDP-galactose to peptidyl hydroxylysine, forming peptidyl *O*-α-D-galactopyranosylhydroxyly-

sine. Glucosylgalactosylhydroxylysyltransferase transfers glucose from UDP-glucose to galactosylhydroxylysine, forming 2-*O*-α-D-glucosyl-O-β-D-galactopyranosylhydroxylysine (Fig. 6-11).

The extent of glycosylation of lysyl residues is variable for the different genetic types of collagen and is also variable in the same type of collagen from different tissues in the same organism. As the glucosylgalactosylhydroxylysyltransferase does not act on triple-helical substrates, the degree of glycosylation of lysyl residues will depend on the rate of triple-helix formation in cells. The function of O-glycosylation in collagen is not known. It is clear, however, that it is not essential for secretion of the molecule from cells, as collagen secretion does not appear to be impaired in patients with type VI Ehlers–Danlos syndrome characterized by lack of hydroxylysine and glycosylated hydroxylysine residues (Quinn and Krane, 1976).

4.6. Glycosylation of Asparagine Residues in Propeptides

The COOH propeptides of type I and type II procollagens are glycosylated by transfer of a mannose-rich oligosaccharide side chain from a dolichol phosphate intermediate to an asparagine residue in the polypeptide chain. Sequencing studies have shown that the acceptor site for the oligosaccharide side chain contains the sequence Asn-X-Thr, in agreement with studies on the structural requirements for *N*-glycosyltransferases (Pesciotta *et al.*, 1981). The synthesis of the dolichol phosphate intermediate is sensitive to tunicamycin. This drug, therefore, inhibits *N*-glycosylation of procollagen. Whether tunicamycin also inhibits the secretion of procollagen is not clear (Duksin and Bornstein, 1977; Tanzer *et al.*, 1977; Hously *et al.*, 1980).

The functional role of the mannose-rich oligosaccharide side chain in procollagen is not clear, but it is possible that the carbohydrate helps to protect procollagen from haphazard degradation after secretion.

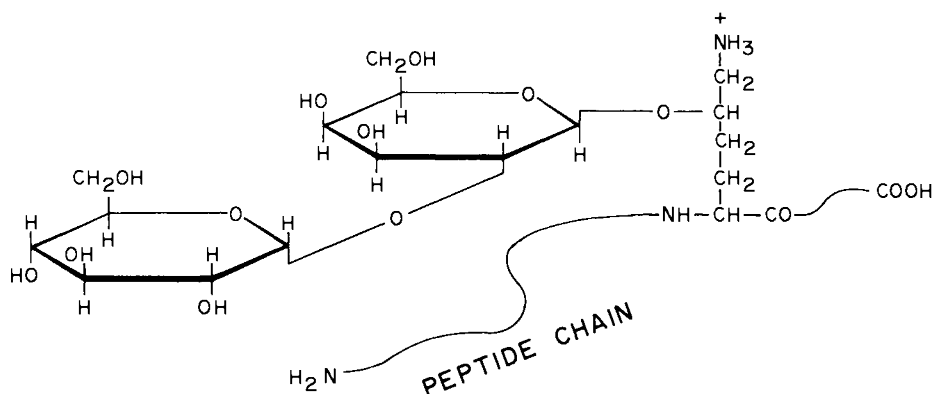


Figure 6-11. Structure of the disaccharide units attached to hydroxylysine in collagen.

5. Intracellular Transport of Procollagen

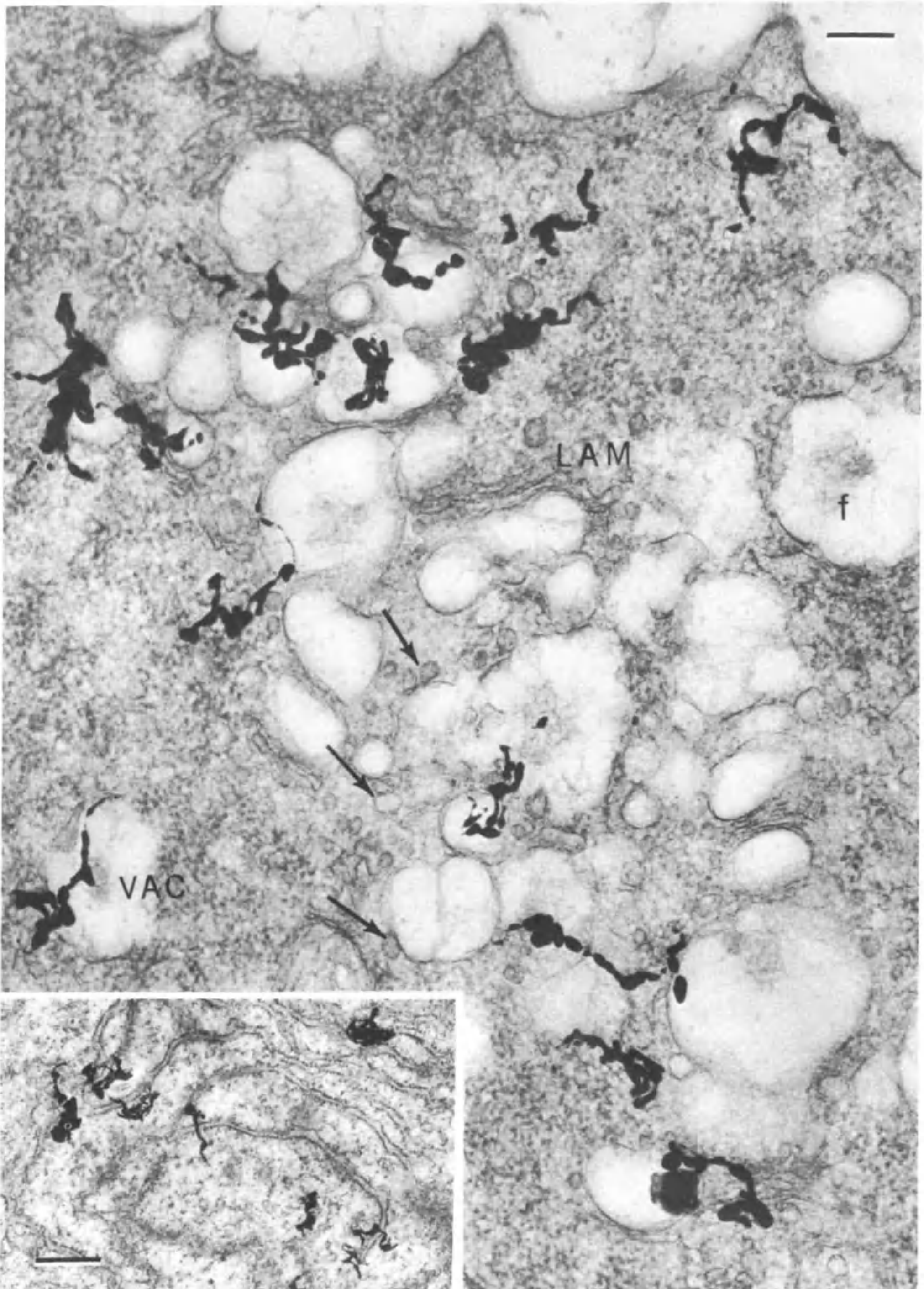
Secretion of procollagen involves transport from the RER to the Golgi complex, packaging in the Golgi region, and translocation of secretory vesicles. Some of these transport processes can be perturbed experimentally. For example, the secretion of procollagen is sensitive to local anesthetics, colchicine, vinblastine, cytochalasin B, uncouplers of oxidative phosphorylation, and the Na^+ ionophore monensin. As these agents have been shown to arrest the secretion of proteins in several other cellular systems, it is likely that procollagen is transported by ion- and energy-dependent mechanisms common to the export of other secretory proteins (see Palade, 1975).

5.1. The Role of the Golgi Complex in Procollagen Processing and Packaging

Early autoradiographic studies of collagen biosynthesis led to conflicting interpretations of the role of the Golgi complex in collagen secretion (for review, see Ross, 1975). Autoradiographic studies by Revel and Hay (1963) and Weinstock and Leblond (1974) suggested that all the proteins synthesized and exported by chondroblasts and odontoblasts, including collagen, were routed through the Golgi complex and packaged there into secretory granules prior to secretion (Fig. 6-12). In contrast, Ross and Benditt (1965) and Salpeter (1968) interpreted their autoradiographic data to indicate that fibroblasts and chondroblasts might use two different pathways for secretion of exportable proteins. One pathway would involve the RER–Golgi route; the other pathway, which might include collagen, could be a direct one from the RER to the extracellular space, circumventing the Golgi complex.

In all these autoradiographic studies, [^{3}H]proline was used as a precursor to label collagen polypeptides in fibroblasts, chondroblasts, and odontoblasts. An obvious problem in quantitating the data is the degree to which a particular cell type is incorporating proline only into products for excretion; proline incorporated into cytoplasmic structures (including membranes) would spend a longer time in the cell and could lead to the interpretation that not all of the secreted protein passed through the Golgi complex. On the other hand, a cell

Figure 6-12. Autoradiographs of sections of *Ambystoma* chondrocytes viewed with the electron ⟶ microscope. The tissue received [^{3}H]proline 15 min (inset) and 30 min (main micrograph) before fixation. At the earlier time interval, the isotope has been incorporated into a proline-rich product in the endoplasmic reticulum (inset), but has not yet reached the Golgi complex. At 30 min, much of the proline-rich product (presumably collagen in large part) has moved into Golgi vacuoles (VAC); it was probably transported to the Golgi vacuoles in small vesicles (arrows). Golgi vacuoles contain a finely filamentous material (f). The golgi lamellae (LAM) and vesicles show little label, but might contain secretory products in too dilute a concentration to expose grains in the emulsion. The curled black structures are silver grains in the autoradiographic emulsion. Inset, × 22,000, bar = 450 nm; main micrograph, × 45,000, bar = 220 nm. (From Revel and Hay, 1963.)


LAM
f
VAC

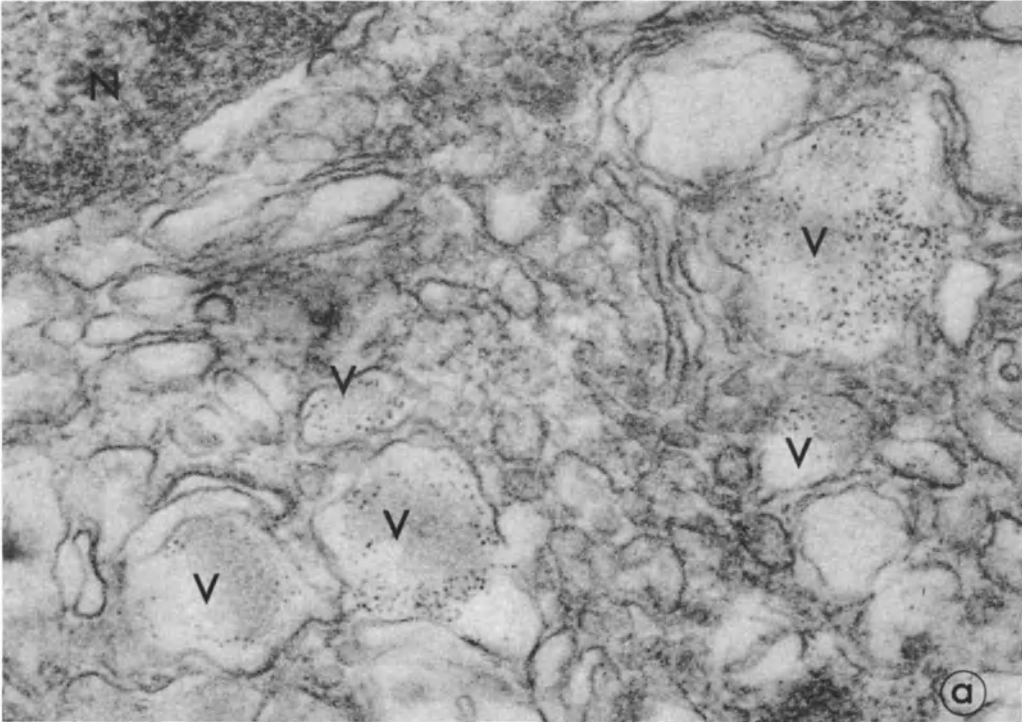
that is incorporating proline mainly into protein for secretion would yield data of a different type. Of interest here is the fact that the biosynthetic, intracellular collagen chains are proα chains, not collagen α chains. The propeptides of the proαchains represent almost 30% of the total chains, and their proline content is like that of most other globular proteins. Therefore, the relative proline content of proα chains is lower than that of collagen α chains. Unless a cell were producing mainly collagen, the course of procollagen through the cell might not be clear-cut in autoradiographic studies utilizing [^{3}H]proline.

The possibility that, in some cells, collagen is routed (e.g., in small vesicles) directly from the RER to the extracellular space cannot be ruled out (Nist *et al.*, 1975). It is now clear, however, that Golgi vacuoles do contain collagen and are involved in the processing and secretion of collagen. It has been demonstrated that collagenase-sensitive ^{14}C-labeled polypeptides can be chased from a microsomal fraction through a Golgi-enriched fraction to the extracellular medium of embryonic chick tendon fibroblasts (Harwood *et al.*, 1976). Also, in a Golgi-enriched subcellular fraction obtained from embryonic chick tendon fibroblasts, Harwood *et al.* (1976) could demonstrate the presence of disulfide-linked procollagen polypeptides. In addition, the presence of procollagen in Golgi vacuoles and cisternae of the endoplasmic reticulum has been demonstrated in chick tendon and cornea fibroblasts (Olsen and Prockop, 1974; Nist *et al.*, 1975) with ferritin-labeled antibodies against procollagen (Fig. 6-13). Finally, immunoperoxidase techniques have been successfully used to demonstrate procollagen in Golgi vacuoles of odontoblasts (Fig. 6-14).

5.2. The Packaging of Procollagen Aggregates in Secretory Granules

There is now a considerable amount of morphological evidence for the conclusion that procollagen is packaged in the form of cylindrical aggregates in Golgi-derived secretory granules, at least in some cell types. The data are especially convincing in the case of rat odontoblasts, for peroxidase-labeled anti-procollagen antibodies have been used to specifically label the aggregates in these cells (Karim *et al.*, 1979) (Fig. 6-15). It has been suggested that such aggregates could play the role of structural intermediates in fiber formation in the extracellular space (Bruns *et al.*, 1979), but the data available do not allow a definitive conclusion at this point (see Chapter 8 for further discussion).

Figure 6-13. Immunohistochemical localization of procollagen in tendon fibroblasts. The primary antibody (anti-proα1) was labeled with ferritin using a second antibody against it. The secretory organelles were permeabilized to antibody by partially homogenizing isolated fibroblasts, thus breaking the bounding membranes. (a) The contents of the Golgi vacuoles (V) are decorated with ferritin particles (small dots) indicating the presence of procollagen. N, nucleus. (b) The contents of the cisternae of the endoplasmic reticulum (ER) are also well decorated by the ferritin-labeled antibody, but the cell surface (CS) is not. Both × 120,000, bar = 80 nm. (From Nist *et al.*, 1975.) →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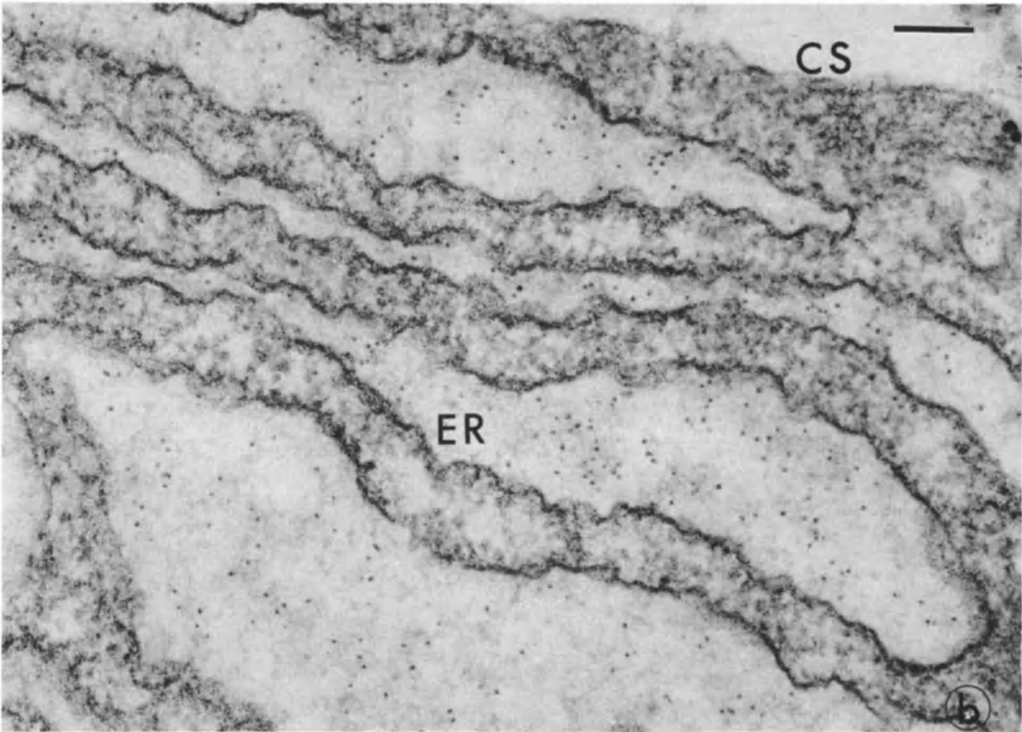
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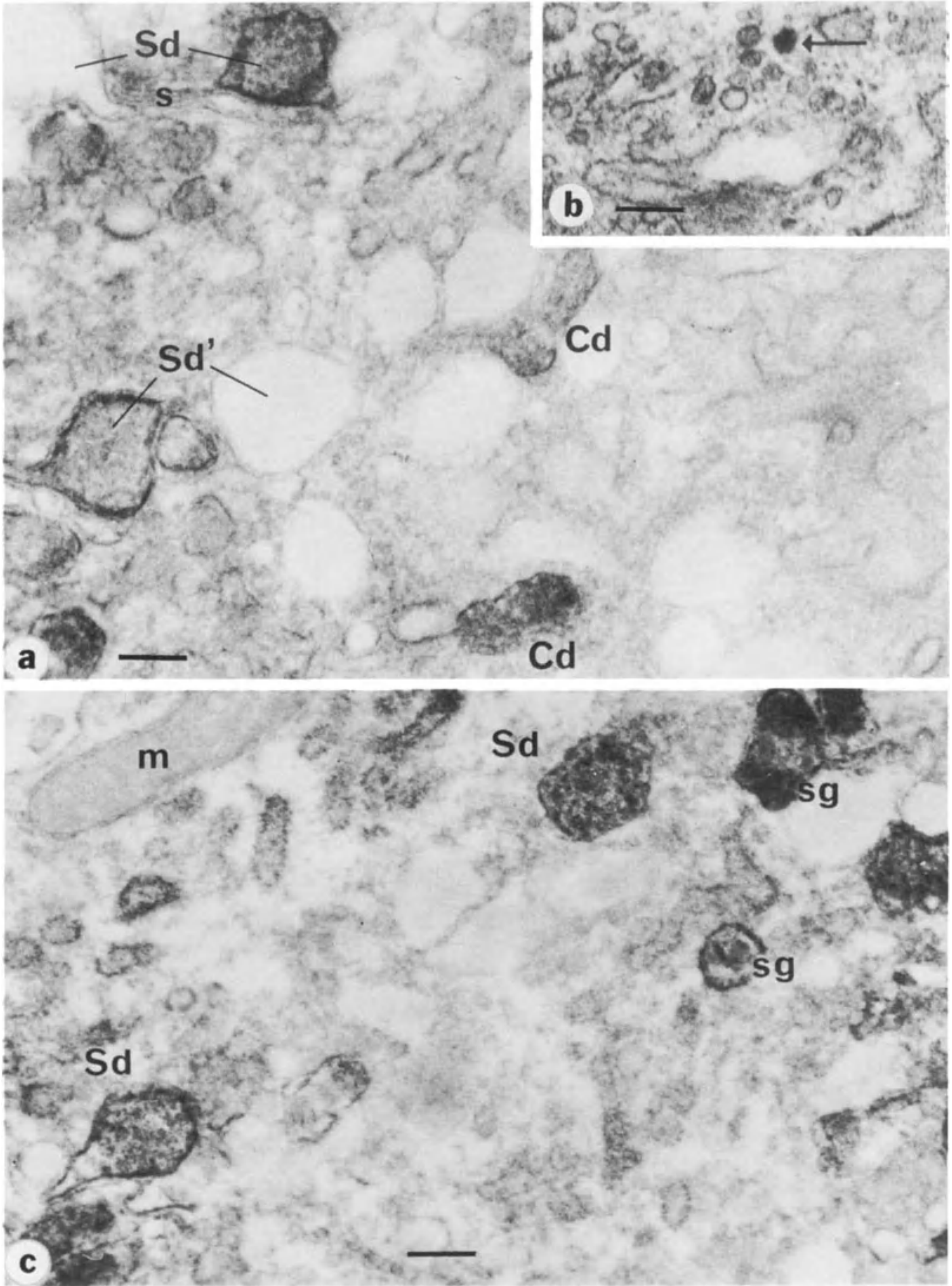
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6. Extracellular Processing of Procollagen

The propeptides of procollagen must be removed before the molecule can form stable collagen fibrils with normal structure. There is good evidence that two classes of proteolytic enzymes are needed for this processing of procollagen. One class of enzyme, the procollagen N-protease, is responsible for removal of the NH_2 propeptides. The second class of enzyme, the procollagen C-protease, removes the COOH propeptide from procollagen. It is conceivable that each class contains several enzymes that are specific for different genetic types of procollagens. In embryonic chick calvaria synthesizing type I procollagen *in vitro*, the removal of the NH_2 propeptide probably occurs at the time of secretion of procollagen from the osteoblasts, and it precedes the removal of the COOH propeptide (Morris *et al.*, 1975). The COOH propeptide appears in this system to be removed in a stepwise fashion, some time after secretion (Davidson *et al.*, 1977).

This sequence of events, however, is not a general one for all tissues and all procollagen types. In smooth muscle cell cultures, the COOH propeptide may be removed before the NH_2 propeptide (Burke et *al.*, 1977), and in type III procollagen, the NH_2 propeptide may be cleaved only to a limited extent (Fessler and Fessler, 1979). Type IV procollagen may not be processed at all (Heathcote et *al.*, 1978; Minor *et al.*, 1976; Dehm and Kefalides, 1978) or only to a very limited extent (Tryggvason et *al.*, 1980).

6.1. Procollagen Proteases

An enzyme that has a neutral pH optimum and removes the NH_2 propeptide from type I procollagen in an endoproteolytic cleavage has been partially purified from calf tendon (Kohn *et al.*, 1974) and embryonic chick tendon (Tuderman et *al.*, 1978). The chick enzyme appears to be a glycoprotein, as it binds to a column of concanavalin A-agarose and can be eluted from such a column with α-methyl-D-mannoside.

An interesting property of the N-protease is that it appears to cleave native procollagen at a much faster rate than denatured proα chains. It appears, therefore, that the proper recognition of the substrate by the enzyme requires a native conformation of the cleavage site.

← **Figure 6-14.** Odontoblasts stained with peroxidase-labeled anti-procollagen antibodies. (a) Golgi complex showing spherical distensions (Sd) and flattened portions of saccules (s). Some distensions are not stained; others are filled with reaction products. Two cylindrical distensions (Cd) are weakly stained. × 42,000, bar = 240 nm. (b) Here, a spherical distension is associated with transfer vesicles, one of which is strongly reactive (arrow). × 36,000, bar = 280 nm. (c) Another Golgi region showing two stained spherical distensions (Sd). The right part of the figure includes secretory granules (sg). Mitochondria (m) are unstained. × 36,000, bar = 280 nm. (From Karim *et al.*, 1979.)

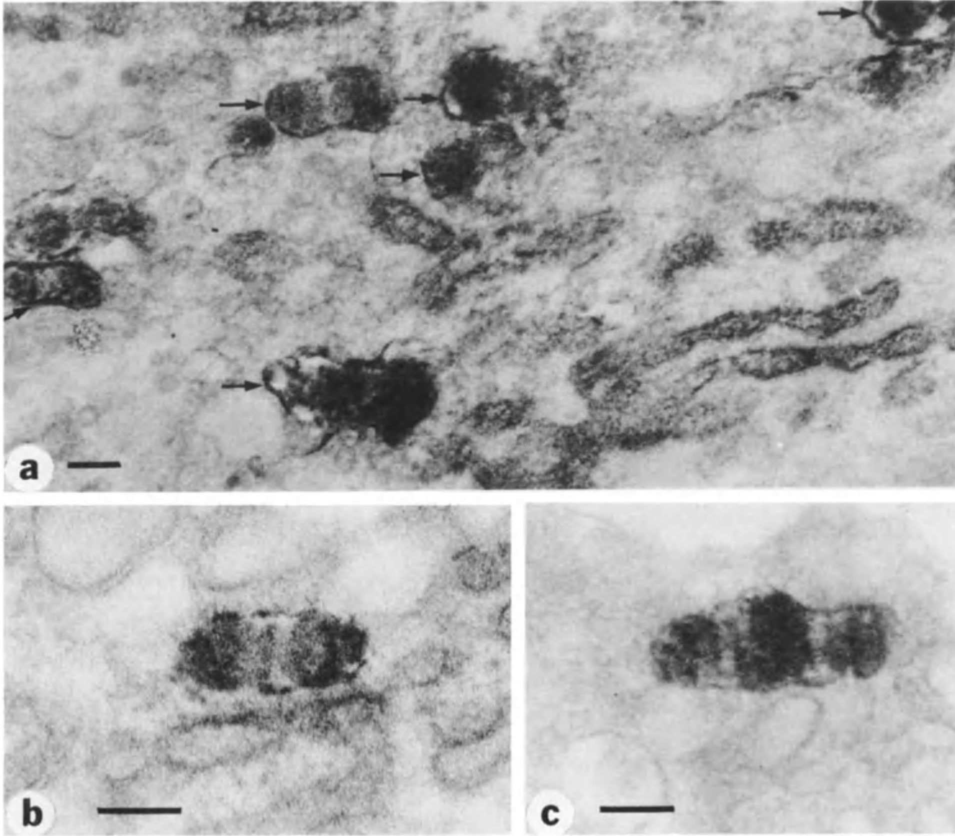

Figure 6-15. Apical cytoplasm of odontoblasts stained with peroxidase-labeled anti-procollagen antibodies. (a) In the upper right of this micrograph a typical secretory granule is cut longitudinally. Other granules are also reactive (arrows). Note the concentration of reaction product at the two extremities of the granules, giving them a bipolar appearance. Bar = 200 nm. (b) Higher magnification of such a granule. Bar = 200 nm. (c). This granule appears to be a double granule with a dilated dense area in the middle of the whole structure. Bar = 200 nm. (From Karim *et al.*, 1979.)

The enzyme activity that removes the COOH propeptide from type I procollagen is clearly different from that of the N-protease, but the enzyme has not yet been purified and characterized. There is preliminary and indirect evidence that the C-protease is also a glycoprotein (Duksin *et al.*, 1978). While the N-protease requires the substrate to be in a native conformation, the C-protease appears to be able to correctly cleave both native and denatured substrate polypeptides (Morris *et al.*, 1979).

6.2. Dermatosparaxis—A Procollagen Processing Defect

Dermatosparaxis, a genetic defect characterized by extreme fragility of skin, was first described in cattle (O'Hara et *al.*, 1970; Lenaers et *al.*, 1971) and later in sheep (Helle and Ness, 1972; Fjølstad and Helle, 1974). The disease is transmitted as an autosomal recessive character. The striking clinical manifestations are cutaneous. Large wounds are generated by relatively minor traumas (Fig. 6-16). In cattle, death ultimately occurs due to wound infection and septicemia. In sheep, the animals are more seriously affected and lambs usually succumb within a few days following birth. Electron microscopy of tissues of affected animals shows strikingly abnormal collagen fibrils in skin (Fig. 6-17). The fibrils are multibranched, twisted ribbons, irregularly dispersed within an excessive amount of amorphous material (O'Hara et *al.*, 1970; Fjølstad and Helle, 1974). Other tissues that contain type I procollagen, such as tendon and bone, appear normal or only slightly affected. That tendon and skin are affected to a different extent has also been demonstrated by X-ray diffraction studies (Cassidy et *al.*, 1980). Analysis of the molecular packing in collagen fibrils by low-angle X-ray diffraction shows that in dermatosparactic tendons most fibrils have a normal arrangement of collagen molecules, whereas in dermatosparactic skin few, if any, normal fibrils are present.

A molecular explanation for the abnormal collagen fibrils in dermatosparactic skin is offered by the observation that skin extracts from calves with the

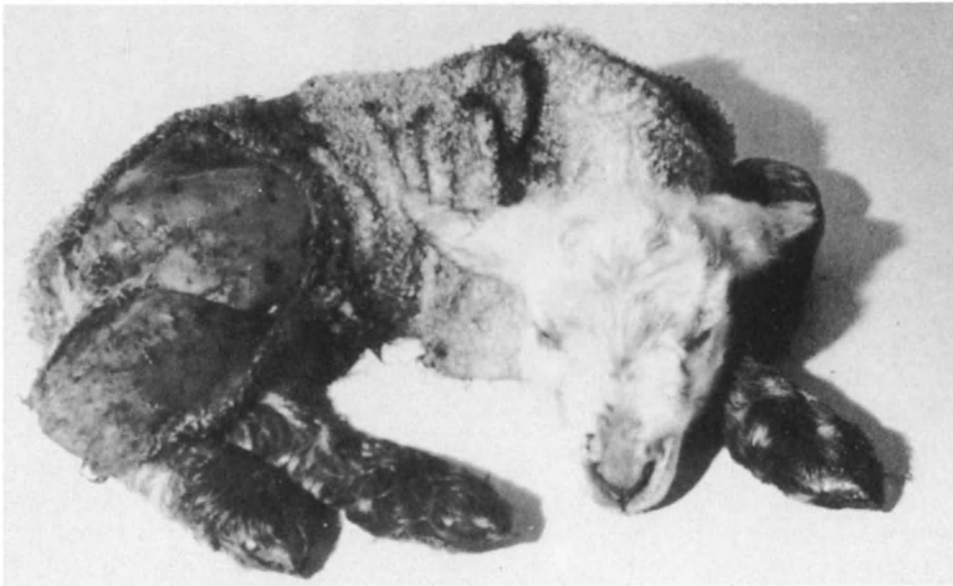

Figure 6-16. Dermatosparactic lamb 10 hr after birth showing lacerations of the skin. (From Fjølstad and Helle, 1974; courtesy of Dr. O. Helle.)

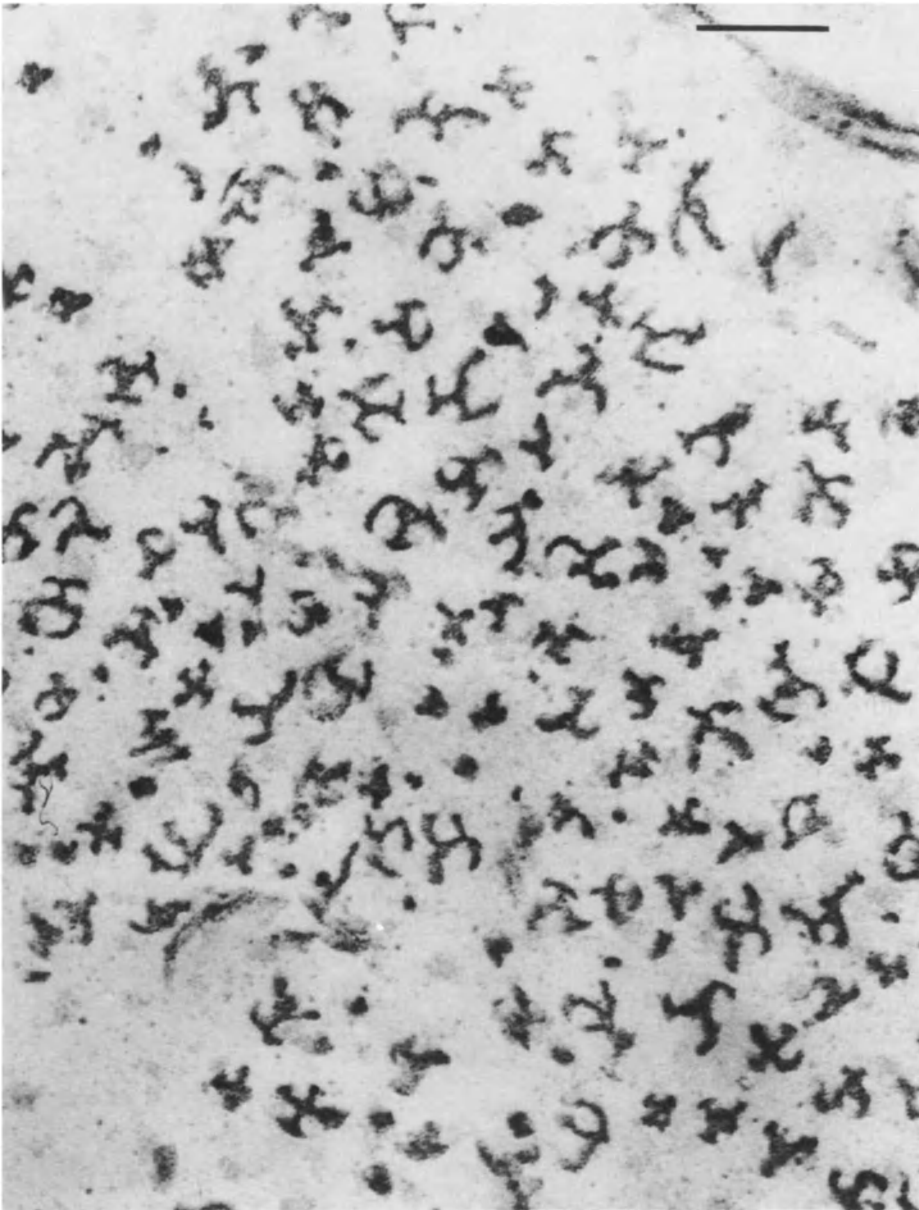

Figure 6-17. Cross section of collagen fibrils in the corium of dermatosparactic lamb showing the characteristic "hieroglyphic" appearance. × 90,000, bar = 200 μm. (From Fjølstad and Helle, 1974; courtesy of Dr. O. Helle.)

genetic defect are deficient in procollagen N-protease activity (Kohn et *al*., 1974). The lack of N-protease activity in the skin leads to the accumulation of molecules that contain the NH_2 propeptide. The abnormally twisted fibrils observed in dermatosparactic skin consist of molecules with the unprocessed NH_2 propeptides, as has been shown by direct immunostaining of the fibrils with ferritin-labeled antibodies directed against the NH_2 propeptide (Wick et *al*., 1978) (Fig. 6-18). Apparently, the presence of the NH_2 propeptide prevents the normal packing of the collagen molecules into cylindrical fibrils.

7. Regulation of Collagen Synthesis

The synthesis of collagen is subject to control on two levels. First, there are mechanisms operating in cells to modulate the types of collagen synthesized. Second, the relative amount of collagen produced by cells can be modulated by several environmental factors.

Substantial evidence indicates that the expression of genes for different collagen types is influenced by a variety of conditions. For example, the synthesis of type II collagen by chondroblasts is sensitive to cell density, Ca^{2+}, pyrophosphate, cAMP, and calcitonin (Deshmukh and Kline, 1976; Deshmukh et *al*., 1976, 1977; Deshmukh and Sawyer, 1977, 1978; Von der Mark et *al*., 1977). When chondroblasts are grown in monolayer cultures plated at low density, cells in the center of colonies produce type II collagen while cells in the periphery synthesize type I collagen (von der Mark et *al*., 1977). When Ca^{2+} is added to chondrocytes in suspension culture, a switch from type II to type I synthesis has been observed in some studies (Deshmukh and Sawyer, 1977).

Another possible example of "gene switching" is provided by studies of cloned lines of mouse embryonic carcinoma cells. These cells produce mainly type IV collagen. However, when retinoic acid is added to the cultures or the cells are plated at low density, the cells produce mainly type I collagen (Adamson et *al*., 1979). Although no data are available as yet, it is likely that "gene switching" involves a controlled differential transcription of collagen genes.

It is possible that the genes have to be in an "open" configuration (proved by accessibility to DNase I) in order to be transcribed, as has been suggested for globin genes (Stalder et *al*., 1980). Changes in the rate of transcription might control the level of collagen-specific mRNA molecules in cells and thereby the synthesis of each type of proα chain. The decrease in the rate of collagen synthesis observed when cells reach the stationary phase in cell culture, or when they are exposed to cortisol, cAMP, or transforming virus, may be due to decreased levels of mRNA. It is also conceivable that processing of initial RNA transcripts to mature mRNA molecules serves as a regulatory mechanism.

The production of collagen by cells is, however, also subject to control at the posttranslational level. This is illustrated well by the alterations in collagen production observed in scurvy. Ascorbate is a cofactor for prolyl hydroxylase

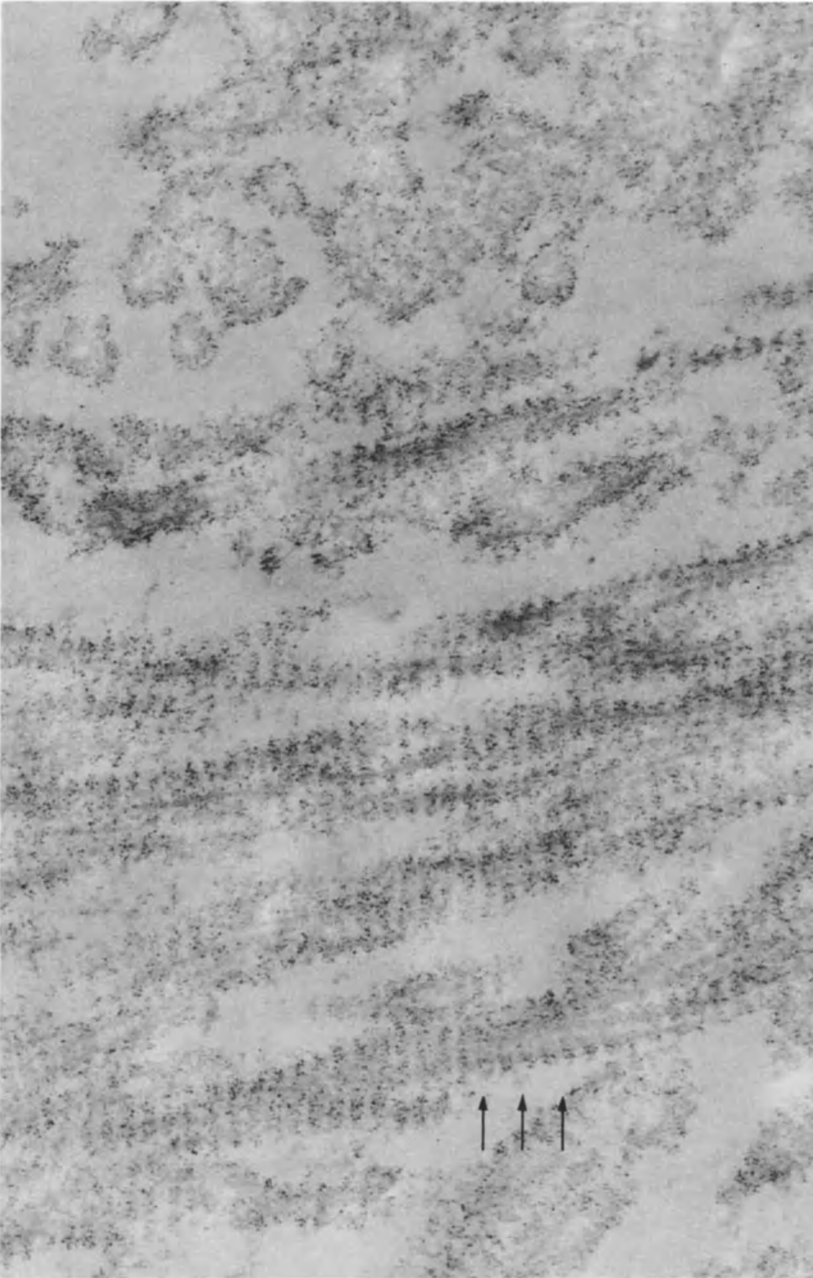

Figure 6-18. Dermatosparactic sheep skin stained with ferritin-labeled anti-pN-collagen type I. Note the localization of ferritin at regular intervals along the fibrils (arrows). × 60,000. (From Wick *et al.*, 1978, with permission.)

and is, therefore, needed for synthesis of hydroxyproline in collagen. In ascorbate deficiency, the proα chains are underhydroxylated and do not form stable triple helices at 37°C. As discussed above, such unfolded proα chains are secreted from cells at a low rate and do not form functional collagen fibrils.

The decreased rate of collagen production in ascorbate-deficient tissues is a pathological event. Prolyl 4-hydroxylase may also play an important role in the normal modulation of collagen production. The level of active enzyme generally follows the level of collagen production in different tissues and organs. It is possible, therefore, that the enzyme plays a rate-controlling role in collagen synthesis. As discussed above, changes in the amount of active enzyme can be brought about by changes in the synthesis of α subunits of the enzyme, as most cells contain a large reservoir of β or β-like subunits. How the synthesis of α subunits is controlled is not yet known, but it is likely that changes in mRNA levels are involved. Should this prove to be the case, the collagen system might provide an interesting model system for studies on the mechanism by which expression of a structural gene is coupled to the expression of the gene for an enzyme that modifies the structural gene product in a posttranslational event.

8. Concluding Remarks

The collagen gene family comprises a large number of genes whose products constitute the major protein components of the extracellular matrix of many animal tissues. We do not yet know the reasons why different tissues contain different types of collagens (see Chapter 1). However, it is likely that the different genetic types play important roles in embryonic development and morphogenesis and that the synthesis of each type is determined by a tissue-specific genetic program.

During the last decade a large amount of information has been accumulated about the posttranslational events involved in collagen biosynthesis. We have perhaps more detailed knowledge about the posttranslational modifications of collagen than of any other secretory protein, with the possible exception of proteoglycans (Chapter 5). The functional role of some of these modifications is known: 4-hydroxyproline allows the formation of stable triple helices at body temperature, hydroxylysine participates in the formation of stable cross-links. The functional importance of other modifications is obscure; the significance of O-glycosylation of hydroxylysine residues within the collagen domain and N-glycosylation of asparagine residues within the propeptide domain of procollagen is not understood.

Our understanding of the molecular basis for the modulation of collagen types during development and morphogenesis and the regulation of collagen production during connective tissue repair is still quite primitive. With the rapid progress in recombinant DNA technology, however, we can expect the situation to improve in the near future. Several considerations in fact would

seem to make the collagen system attractive as a model system for obtaining answers to general questions about gene regulation. First, a large amount of detailed chemical information about the protein products of different collagen genes and the posttranslational modifications of these proteins is available. Second, the different collagen genes are expressed according to a developmentally regulated program. Finally, collagen biosynthesis appears to be pathologically altered in several conditions, including both genetic defects and acquired diseases (see Prockop *et al.*, 1979; Bornstein and Byers, 1980).

It is likely that further work on the biosynthesis of collagen will lead to a better understanding of principles and mechanisms that extend beyond the realm of collagen cell and molecular biology. It is also likely that the most definitive and far-reaching conclusions will be generated by studies aimed at answering very precise and narrow questions, such as: (1) How does the src gene product modulate collagen synthesis in Rous sarcoma-infected cells? (2) How does switching among type I and type II collagen genes occur in chondroblasts? (3) Is a modulated expression of the α-subunit gene responsible for the regulation of prolyl hydroxylase activity in cells? (4) Is the expression of genes for prolyl hydroxylase and other posttranslational enzymes linked to the expression of collagen structural genes?

Obviously, the list can be extended to include a large number of interesting problems. With the appropriate experimental tools now becoming available, we can expect answers to many of these questions in the near future.

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Chapter 7

Matrix Assembly

ROBERT L. TRELSTAD and FREDERICK H. SILVER

1. Introduction

Matrix assembly in morphogenesis, growth, and wound repair is a sequential stepwise process that occurs in overlapping stages and usually involves more than one kind of matrix macromolecule. Combined morphological, biochemical, and physicochemical approaches are generating new information pertaining to the manner in which connective tissue macromolecules assemble. Some features of the assembly process can be studied *in vitro* under cell-free conditions using the purified components that are increasingly available as more matrix constituents are isolated and chemically characterized. Such studies have indicated that much of the assembly process is driven by physicochemical forces sufficient to generate specific aggregate forms that are very similar if not identical to those seen *in vivo*. The final structure of a matrix is therefore determined, in part, by forces of "self-assembly." Detailed evaluation of matrix assembly *in vivo*, however, indicates that many features of the process are regulated by cells, including aspects such as timing, site of assembly, and spatial orientation. Matrix assembly should therefore be considered a multistep process in which a successive series of physicochemical interactions of the matrix components occur within a context of morphogenetic patterning imposed by cells.

In this chapter we will describe structural and physicochemical properties of some matrix macromolecules and integrate this information into a consideration of matrix assembly deduced from morphological and experimental studies. The physicochemical properties of macromolecules are useful in defining the "boundary conditions" within which assembly processes must operate and thus provide considerable guidance for the analysis of the actual *in vivo* assembly sequences. For a more detailed discussion of physical biochemistry and macromolecular chemistry, the reader should consult several of the texts cited in the references, some of which have been written especially for students of biology (Flory, 1953; Tanford, 1961; Van Holde, 1971; Williams and Williams, 1973; Freifelder, 1976; Cantor and Schimmel, 1980). Other chapters in this

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volume have addressed the biosynthetic and biochemical characteristics of the various matrix constituents, as well as some of the ultrastructural features of matrix synthesis, processing, and secretion. Much of this information is basic to the present considerations, but will not be repeated here.

2. Chemical Sequences Dictate Matrix Structure

The chemical properties of each matrix constituent are dictated by the unique sequences of the subunits that comprise the polymer. Each of these subunits, whether amino acid or sugar, has only a few attributes that account for its properties, the most important being its potential for covalent-bond formation, charge, hydrophobicity, size, and allowed conformations. The manner in which these attributes are aligned along the axis or branches of linear polymers determines many of their structural properties. The isolation of various matrix macromolecules coupled with the subsequent biochemical determination of the sequential order of their subunits has been one of the major accomplishments of the past two decades in studies of matrix structure. We are now in a position to study matrix assembly with relatively complete chemical information for each reactant being available. This chemical sequence data coupled with physicochemical measurements of molecules in solution make it possible to draw conclusions about mechanisms of assembly at the molecular level, including the driving forces responsible and the rates possible. Of the matrix constituents that have been isolated to date, collagen is the only molecule for which such analyses have been conducted in detail. Although our treatment of assembly will focus on collagen, many of the principles involved should apply to the other constituents as well.

Having available the complete sequence of the $\alpha1$(I) chains from several species and that of the $\alpha1$(III) and $\alpha2$(I) chains has made it possible to engage in analyses of the collagen assembly process at the molecular and chemical levels. The profile of charged residues along the axis of the collagen molecule derived from amino acid sequence data for type I collagen is shown in Fig. 7-1. The distribution pattern of charged residues generated in this fashion is nearly identical to that obtained by densitometric tracings of electron micrographs of type I collagen segment-long-spacing (SLS) crystallites stained with metals. The SLS crystallite is a collagen aggregate that forms readily at low pH in the presence of ATP; the molecules lie in lateral register and amino acids of like charge are in apposition (see also Fig. 7-7 and Chapter 1). Because the crystallites are formed at low pH, most of the carboxylic acid side chains of aspartic and glutamic acids are protonated and uncharged. The cationic charges of the basic amino acids are probably complexed with the polyanionic material, usually ATP, that is used to induce the aggregates. Hydrophobic interactions between the molecules are maximized when the molecules are in register, and thus the molecules form SLS crystallites. At neutral pH, charge repulsions between collagen molecules prevent SLS formation even in the presence of ATP. When the crystallites are pipetted onto collodion-coated grids for electron

microscopy, ionized heavy metals such as uranyl ions are able to interact with the negatively charged amino acids at the slightly higher pH of the staining solution. The pattern of electron densities of the uranyl ions along the axis of the SLS is an accurate representation of the pattern of charge distribution along the molecule and is unique for each collagen type (Fig. 7-1).

When five type I collagen molecules are staggered by 234 amino acids to generate a 67-nm or 1-D overlap pattern (see Fig. 7-7 and Chapter 1) and the charge density along the axis of each molecule within the five overlapping 1-D segments is summed, the generated pattern of cross striations (Fig. 7-2) closely resembles that obtained from actual scans of native fibrils (Meek *et al.*, 1979). In order to understand how the charged and hydrophobic residues along the molecular axis are involved in fibril assembly, an analysis of some features of the mechanisms of aggregation is necessary; this will be followed in succeeding sections by discussion of the thermodynamics and kinetic aspects of the process.

3. Mechanism of Collagen Assembly

Early studies of collagen fibril formation *in vitro* focused on temperature-induced turbidity changes (optical density increases) that occur when neutral collagen solutions are heated. A typical schematic turbidity–time curve is shown in Fig. 7-3, illustrating that the mechanism of collagen heat gelation can be broken into distinct phases. During the initial period of heating or lag phase, no change in turbidity is observed. Wood and Keech (1960) proposed that fibril precursors or nuclei are formed during this period and that, when stable, they rapidly assemble, producing the large turbidity increase observed during the growth phase. Although a careful attempt to study physicochemical changes that occur during the lag phase by viscometry and sedimentation equilibrium has not revealed the presence of aggregates (Comper and Veis, 1977a), laser light scattering studies (Silver *et al.*, 1979) have demonstrated that during this period the translational diffusion coefficient decreases from that of a single collagen molecule, 0.78×10^{-7} cm^2/sec (Obrink, 1972; Fletcher, 1976; Silver *et al.*, 1979; Gelman and Piez, 1980), to that expected of small aggregates, 0.35×10^{-7} cm^2/sec. Studies by Gelman and Piez (1980) confirm that the diffusion coefficient decreases during the lag phase, and recent studies (Silver, 1981) suggest that the weight average molecular weight at the end of this period is about 930,000.

The size and shape of the aggregates formed during the lag phase have been predicted on the basis of calculations (Silver *et al.*, 1979) to be 4-D staggered dimers and trimers (Fig. 7-4) with translational diffusion coefficients of 0.5×10^{-7} and 0.35×10^{-7} cm^2/sec, respectively, which agrees with subsequent molecular weight measurements (Silver, 1981). When fully formed fibrils in tissues are disassembled, as is readily accomplished by placing rat tail tendons in 0.1 M acetic acid, a series of aggregates are obtained that consist of 4-D staggered dimers, trimers, and tetramers (Silver and Trelstad, 1980), suggesting

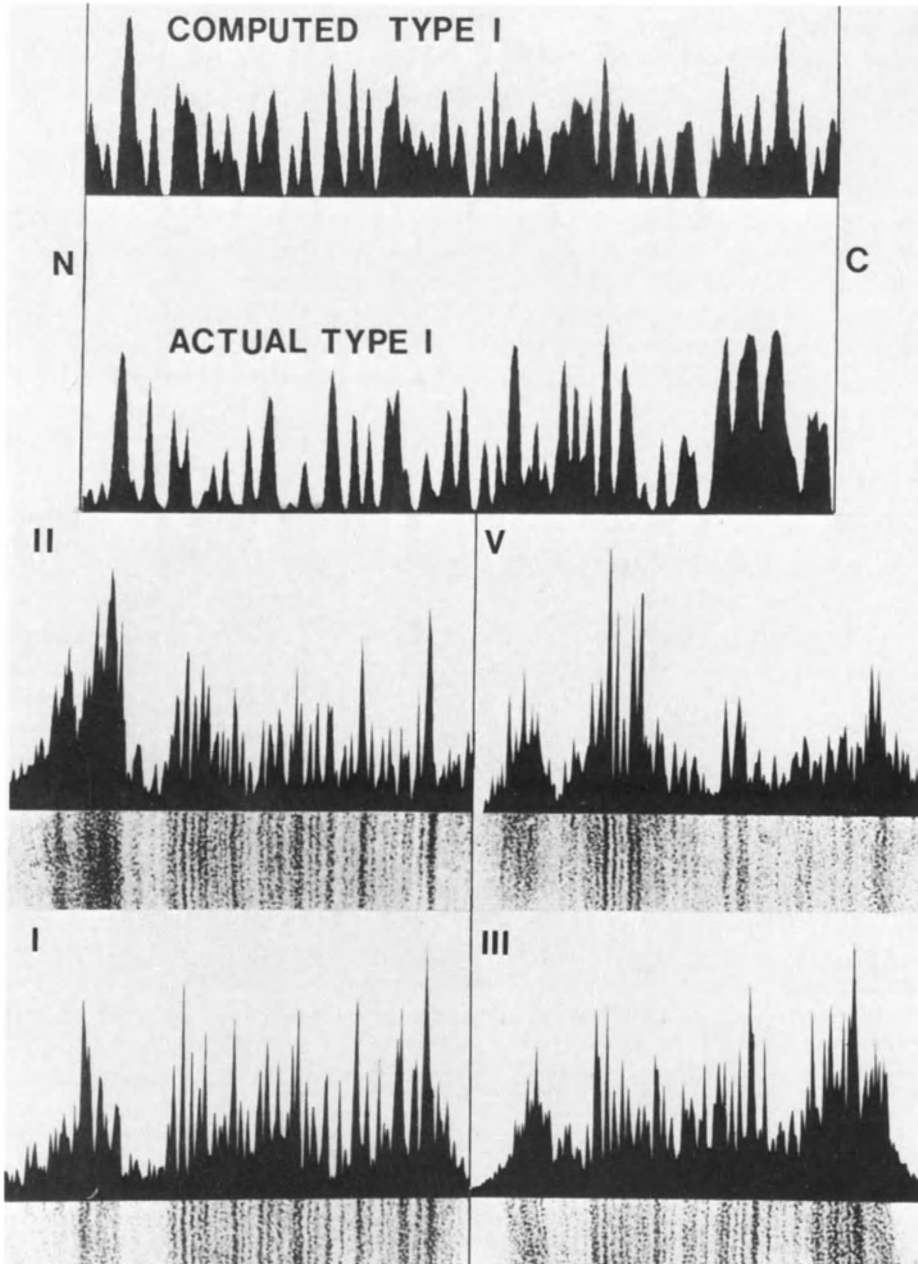


Figure 7-1. (Upper) Axial charge profiles of type I collagen computed (top) from the positions of charged amino acids as determined by amino acid sequence analyses of α1(I) and α2(I) chains and from an actual (bottom) axial densitometric scan of a type I collagen SLS crystallite stained with uranyl acetate and phosphotungstic acid (provided by Dr. R. Bruns). The NH_2 terminal (N) is at the left, the COOH terminal (C) at the right. (Lower) Electron micrographs of four SLS crystallites from chick collagen types I, II, III, and V and their actual densitometric scans. The NH_2 terminal is at the left for each; staining was with uranyl acetate only.

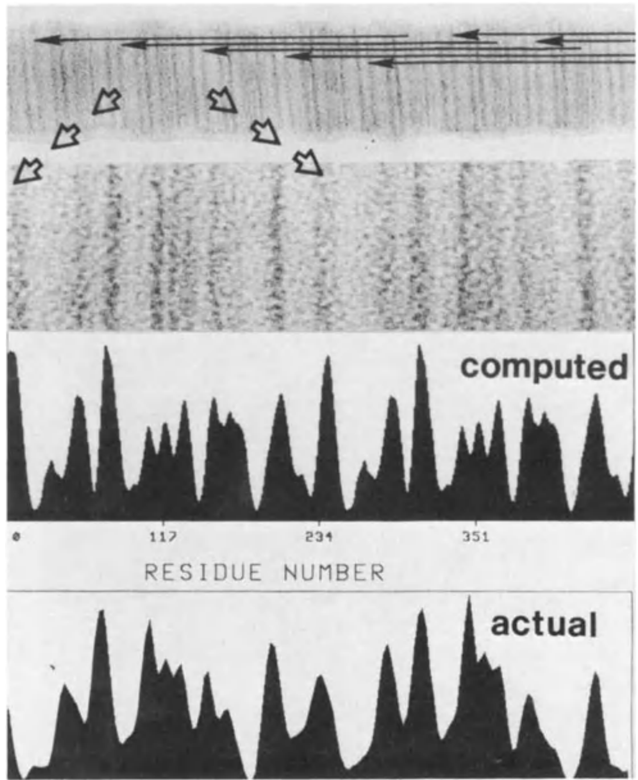


Figure 7-2. The upper electron micrograph is a type I collagen fibril teased from a rat tail tendon, stained with uranyl acetate, and examined without sectioning. The positions and polarities (arrowheads indicate NH_2 terminals) of 1-D staggered collagen molecules are indicated by the thin arrows. The distance between arrowheads is 67 nm. The lower micrograph shows a portion of a different fibril at higher magnification. The open arrows indicate the comparable segment of the fibril in the upper micrograph. The computed axial charge profile of a native type I collagen fibril was generated by summing the positions of charged amino acids in five collagen molecules staggered by 234 amino acid residues. The residue number refers to the position of the amino acids in the molecule whose NH_2 terminal lies at the beginning of the 1-D staggered set. An actual axial densitometric scan of a type I collagen fibril stained with phosphotungstic acid is shown at the bottom. Upper micrograph, × 216,000; lower micrograph, × 821,000.

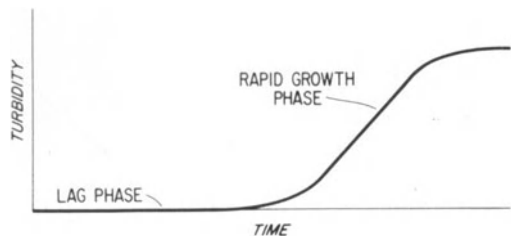
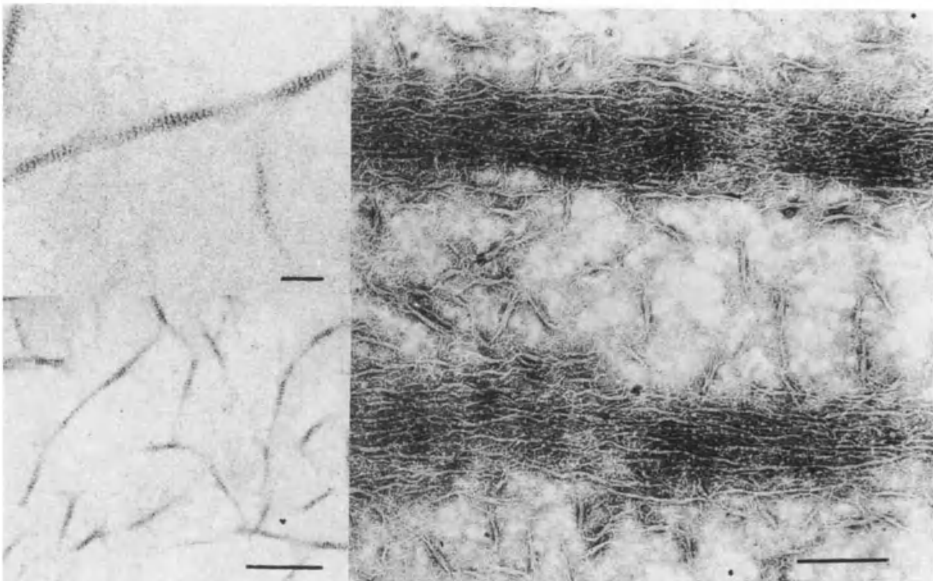


Figure 7-3. Schematic turbidity–time curve for type I collagen illustrating the lag phase, during which long linear aggregates form; and the rapid growth phase, during which the long linear lag-phase structures aggregate laterally into wider forms. (For a theoretical discussion, see Silver and Trelstad, 1979.)



that the process of self-assembly under certain conditions is reversible and that 4-D staggered structures constitute some kind of intermediate in fibril organization. Further evidence for 4-D staggered intermediates during the initial stage of *in vitro* fibrillogenesis comes from measurements of solution behavior of molecules (Ananthanarayanan and Veis, 1972; Bernengo *et al.*, 1978), from modeling (Doyle *et al.*, 1975), from cross-link analyses (Kang, 1972; Eyre and Glimcher, 1972), and from the ultrastructure of fibril fragments (Zimmermann *et al.*, 1970; Silver and Trelstad, 1980). The overlapping ends of the molecules in the 4-D staggered configuration are thus of particular interest in regard to their role in fibril assembly. At the same time, these overlapping ends are important because they are the major site of covalent cross-linking between the molecules (Eyre and Glimcher, 1972). The nonhelical ends of the molecule have been shown to have substantial influence on the rate and extent of fibril formation *in vitro* (Hayashi and Nagai, 1973; Comper and Veis, 1977a,b; Gelman *et al.*, 1979), and recently Helseth *et al.* (1979) have suggested a more complex conformational change at these ends. Although lysine-derived cross-links do not appear to form during the lag phase, chemical reduction of the aldehyde precursors of these cross-links prolongs this step (Gelman *et al.*, 1979; Brennan and Davison, 1980).

The lack of turbidity change during the lag phase is a characteristic of linear self-assembly systems (Silver and Trelstad, 1979). It is of interest to note that the lack of turbidity change during the lag phase was initially incorrectly interpreted as indicating that no quantitatively significant aggregation was occurring beyond the formation of a few "nuclei." Subsequent analysis showed (Silver and Trelstad, 1979) that the doublings in molecular weight (from monomer to 4-D dimer), which should have doubled the turbidity (scattering), were nearly offset by a doubling of the interference generated by the aggregate itself (Fig. 7-5). Although all linearly assembling macromolecules demonstrate these phenomena, the differences in angle dependence of scattering and inter-

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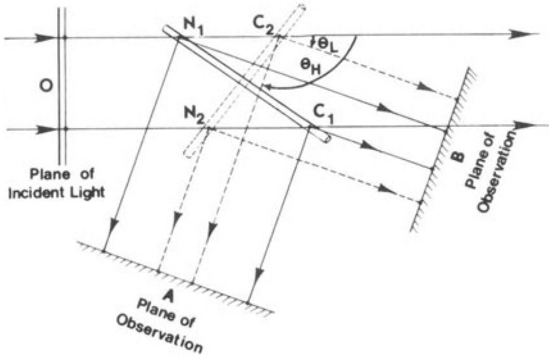
Figure 7-4. A schematic series of assembly stages of type I collagen derived from studies using light scattering and electron microscopy to identify intermediate forms. The individual monomers interact in a 4-D stagger to form dimers and trimers (top). In the 4-D trimer at the upper right and elsewhere, the numbers refer to segments of length D along the 4.4-D-long molecular axis. Number 1 is at the NH_2 terminal, 2 is at the interface of the first D segment with the second, and so on. A 4-D staggered configuration is the same as an overlap of 0.4 D. The 4-D trimers laterally associate to form an aggregate consisting of approximately five trimers or 15 molecules. If the trimers overlap in a 1-D pattern, a relatively compact structure is formed (middle right), which can add to other like structures in a linear and/or lateral fashion to build a longer subfibril as illustrated by the larger central structure. The electron micrographs are from a collagen solution sampled at the end of the lag phase of the assembly sequence and applied to a collodion-coated grid and negative stained with phosphotungstic acid (right) and positive stained with uranyl acetate (left top and left bottom). The collagen molecules have aggregated into tactoidal structures that contain a 1-D stagger order in that a faint periodicity of 67 nm is apparent (left). In the top micrograph, three aggregates, each with a 1-D periodic staining pattern, are aligned end to end. These tactoidal aggregates appear to add to each other to form long narrow subfibrils. The narrow subfibrils laterally entwine at a later stage to form wider fibrils (for details see Trelstad *et al.*, 1976). Right micrograph, × 40,000, bar = 300 nm; left bottom micrograph, × 34,000, bar = 300 nm; left top micrograph, × 81,000, bar = 67 nm.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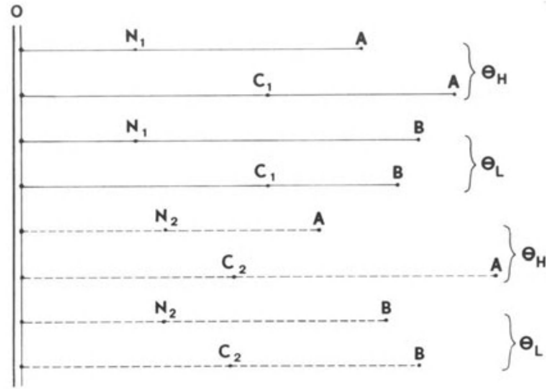
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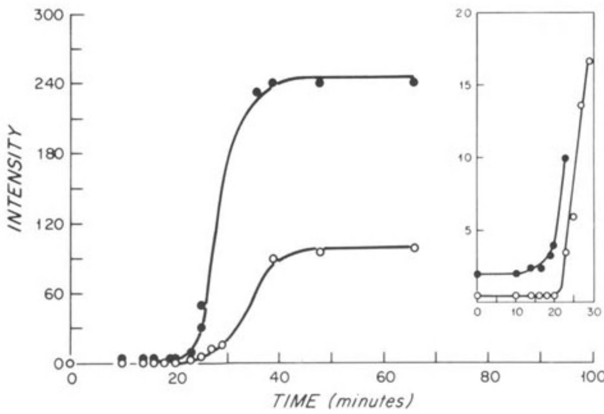
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ference make it possible to detect the early aggregates that are formed in the lag phase. As shown in Fig. 7-5, although the intensity of light scattered at 90° remains constant, that extrapolated to 0° changes during the lag phase (Silver, 1981). From a mechanistic point of view, the fact that the rate of change of molecular weight with time increases significantly after the molecular weight reaches 930,000 (Silver, 1981) suggests that further growth probably occurs by the addition of trimers and not by the addition of monomers.

Lateral growth of linear trimers occurs during the time when the turbidity increases rapidly until the molecular weight reaches about 4×10^6 (i.e., 15 molecules) (Silver, 1981). At this time, another increase in the rate of change of molecular weight with time occurs, suggesting that a subunit with about five linear trimers is an important kinetic unit from which fibrils grow (Fig. 7-4). Further growth occurs by end-to-end and side-to-side addition of these units. Conceivably, the rate of end versus lateral growth determines the width to length ratio of the final fibril.

4. Thermodynamic Analysis of Assembly

4.1 General Considerations

Although the apparent initial step in *in vitro* collagen assembly is the formation of 4-D dimers, these analyses do not reveal the driving force for the process. From thermodynamics, however, we know that in order for a process to occur spontaneously, without any energy from the environment being added, the free energy of the products must be lower than that of the reactants. The free energy change, ΔG, of a system is related to changes in the enthalpy (the sum

Figure 7-5. The scattering of light by a long rodlike molecule is dependent on the angle between the incident light and the observer. The diagram (derived from Tanford, 1961) illustrates the differences in path length for light scattered from different regions (C and N) of a rod-shaped molecule such as collagen. The collagen molecule is shown at two different orientations with respect to the incident light. The paths of the light scattered at a low angle (θ_L) from positions C and N from each molecule to a detector at position B are indicated by the broken lines; the paths of the light scattered at a high angle (θ_H) from these same positions to a detector at position A are indicated by the solid lines. The differences in path lengths from the plane of the incident light (O) to the plane of the observers (A or B) are shown. The path length difference is always greater for the greater scattering angle. Because interference is directly related to path length differences, scattering data obtained at 90° are less sensitive than scattering data extrapolated to 0°. Turbidity measurements made in a standard spectrophotometer are proportional to the light scattered at 90°. The most accurate light scattering measurements are thus those that are extrapolated to 0°. The differences between type I collagen in the lag phase at 26°C at 0 and 90° are illustrated in the graph. At 90° (open circles), the intensity of scattering and time of apparent increase (detailed in the box on the right) are less apparent and occur later than that seen at 0° (closed circles) (Silver, 1981). The 0° values were obtained between angles of 30 and 80° and extrapolated to 0°. Careful examination in this manner of the lag phase when apparently "nothing" was happening has revealed the presence of aggregate forms consistent with 4-D staggered dimers and trimers.

of kinetic and potential energy factors), ΔH, and changes in the entropy (the randomness of the system), ΔS, as follows:

$$\Delta G = \Delta H - T\,\Delta S \quad (1)$$

Kinetic energy is composed of a translational component (Brownian motion), a vibrational component, and a rotational component and is important in matrix assembly because collisions between macromolecules (translational energy) in the correct conformational state(s) (rotational energy) must occur before two macromolecules can interact. Once two macromolecules are in the correct rotational states and are near each other in space, potential energy or bonding dictates whether they will stick or collide elastically. The two types of bonding that lead to lower potential energies, the favored state of a system, are covalent bonds and secondary or van der Waals bonds. Formation of a covalent bond lowers the potential energy by about 100 kcal/mole, whereas formation of a secondary bond lowers the potential energy by at most 5 kcal/mole. Most matrix assembly reactions occur without the formation of covalent bonds. Accordingly, the formation of the weaker secondary bonds, which include dispersive bonds, electrostatic bonds, and hydrogen bonds, is important in assembly. Dispersive bonds, which arise from the attraction of two nonpolar atoms, lower the potential energy from 0.05 to 0.20 kcal/mole. Electrostatic bonds can be either attractive or repulsive and can lower or raise the potential energy as much as 5.0 kcal/mole. Hydrogen bonds can be considered electrostatic bonds due to the partial charge on the atoms involved. Hydrophobic bonds are weak dispersive bonds promoted by the presence of water.

In summary, the enthalpy of a state is defined as consisting of kinetic and potential energy components as indicated in equation (2). The potential energy is either covalent (P_c), electrostatic (P_e), or dispersive (P_d), while the kinetic energy is primarily translational (E_t) and rotational (E_r). By definition, the enthalpy change during assembly is related to the change in both the total number of bonds as well as the kinetic energy of collagen and water molecules, which can be calculated using equation (2).

$$H = P_c + P_e + P_d + E_t + E_r \quad (2)$$

The entropy term, S, is proportional to the number of conformations (N) or rotational states that are possible for a macromolecule and to the number of positions in space, N, that can be occupied by a small molecule such as water:

$$S = k \ln(N) \quad (3)$$

where k is Boltzmann's constant. If the number of rotational states or the volume occupied by a molecule is increased, then the entropy increases according to equation (3) and the net free energy decreases according to equation (1). For this reason, increasing the molecular motions of a large macromolecule or increasing the volume occupied by a small mobile molecule (which occurs by

the addition of heat) results in a lower free energy. An assembly process that is enthalpy driven has a net negative enthalpy change, while an entropy-driven process has associated with it a large positive entropy increase.

4.2. Thermodynamics of Collagen Assembly

Self-assembly of collagen molecules into fibrils occurs spontaneously at neutral pH. Therefore, there is a net negative change in free energy associated with this process. Based on solubility measurements (Cooper, 1970) and fibril growth studies (Haworth and Chapman, 1977), the free energy change is between −12 and −23 kcal/mole. It is important to ask whether this free energy change is a result of formation of new bonds, that is, of lowering the enthalpy, or is a result of increased disorder or randomness of the system, that is, of increased entropy.

At room temperature, the enthalpy change associated with collagen gelation is about 25 kcal/mole (Cooper, 1970), which is similar to that found for activation of self-assembly (Haworth and Chapman, 1977), and indicates that heat must be added to the system in order for this process to occur. This amount of heat is too large to be explained only in terms of an increase in kinetic energy resulting from increased translational and rotational motions, as these terms are roughly proportional to the product of the gas constant and absolute temperature, which amounts to less than 1 kcal/mole at room temperature. Therefore, the required heat must be due to an increase in potential energy that would arise if bonds were broken. The bonds that are broken are in all likelihood secondary bonds, as self-assembly can occur in the absence of covalent intermolecular cross-links and under such circumstances is reversible.

In trying to understand which bonds are broken, it is helpful to think about the secondary bonds that hold the collagen molecule together. The triple-helical structure of the collagen molecule is believed to be stabilized by two hydrogen bonds per triplet: one bond is formed between the carbonyl oxygen and the amide hydrogen of amino acid residues on different α chains, and the second is thought to be a water-mediated hydrogen bond between another set of residues in the same triplet (Ramachandran and Ramakrishnan, 1976). Hydrogen-bonded water on the outside of the triple helix (Privalov *et al.*, 1979) is present and may play a role in preventing collagen–collagen interactions, as it is well known that the collagen helix–coil transition temperature is increased by removal of water. Thus, water molecules must hydrogen bond to the outside of the molecule and form a hydration layer covering the amino acid side chains that would otherwise be exposed. This layer of hydration amounts to between 0.24 (Chapman *et al.*, 1971) and 0.6 g/g collagen (Dehl, 1970; McClain and Wiley, 1972; Fung *et al.*, 1974), and corresponds to between 4 and 10 hydrogen bonds per triplet. Thus, the 20 kcal/mole that is required for fibril formation and that accounts for the enthalpy change is probably derived from the breaking of about four water-mediated hydrogen bonds associated with the exterior of each triplet.

Because the net enthalpy of self-association is positive, the process cannot be energy driven and thus requires a net positive change in the entropy to explain its spontaneity. In fact, measurements of the changes in entropy during fibril assembly indicate a net positive increase, and thus entropy drives the process (Cooper, 1970; Haworth and Chapman, 1977). The large entropy increase has been attributed to the disruption of the ordered water shell that surrounds the collagen molecules. The water shell itself is stabilized by hydrogen bonds between water molecules, especially adjacent to hydrophobic amino acid side chains, and also by hydrogen bonds between the water and other more polar amino acids. Loss of this ordered water shell leads to greater randomness of the water molecules and thus a net increase in the entropy of the system. The macromolecules at the same time become more ordered by charge–charge and hydrophobic interactions, but this negative change is quantitatively much less than the positive increase for the surrounding solvent molecules.

As indicated by equation (3), a net increase in entropy is also associated with an increase in the kinetic energy of both collagen and water as heat is added to the system. Local unfolding at the triplet level has been shown by hydrogen-exchange studied (Privalov *et al.*, 1979) and electron spin resonance (Hearn *et al.*, 1979). The latter studies also indicate that an increase in the mobility of the collagen molecule occurs discontinuously near room temperature. Other studies (Jelinski and Torchia, 1979) suggest that the molecules are not totally rigid or motionless even in the fully formed fibril. Whether the loss of the water shell is a result of an increased mobility of the molecule or whether the two factors act synergistically is unclear, but both are extremely important in driving collagen self-assembly.

Presumably, the water shell is initially removed at those regions that are devoid of imino acids, i.e., the regions 0.4 D from each end of the molecule. These ends of the molecules, which do not have a triple-helical structure, can more readily undergo changes in conformation (Helseth *et al.*, 1979) and engage in charge–charge and hydrophobic interactions (Hulmes *et al.*, 1973) and thereby enter into the formation of 4-D staggered linear aggregates and 1-D staggered lateral aggregates.

All proposed models of the collagen fibril point to some ordering phenomenon (enthalpy related) that results in a 1-D or multiple-D stagger of neighboring molecules. Thermodynamically, these models imply that energy must play a role in fibril stability. Otherwise, we are at a loss to explain the order of collagen fibril structure. We must conclude, therefore, that although the process of fibril formation *in vitro* is entropy driven, this applies only to the overall process of removal of an ordered water shell. Specific interactions between charged and hydrophobic amino acid residues that occur after the water shell is removed may result in a lower enthalpy, but this decrease in enthalpy is much smaller than the increased enthalpy required to remove hydrogen-bonded water. Further thermodynamic studies on collagen fibrils and collagen in solution are required to determine the exact nature of the interactions between collagen molecules that result in the 1-D stagger.

Elastin assembly or coacervation is also believed to be entropy driven because this process is similar to collagen self-assembly in that it requires thermal energy for initiation. Urry (1978) has proposed that elastin coacervation is driven by the formation of hydrophobic bonds and may also require the removal of a hydration layer.

5. Kinetic Analysis of Assembly

5.1. General Considerations

Although thermodynamics reveals whether or not a process can occur and defines the driving force of the reaction, it does not reveal how fast the process occurs. A transition-state model is helpful to understand the factors that affect the rate of matrix assembly. This model suggests that matrix assembly occurs through the formation of an activated or transition state that in some cases can have a higher free energy than the reactants. In this case, free energy in the form of heat or kinetic energy must be added to activate the reactants. The rate of formation (R_{AB}) of the product AB from the reactant molecules A and B is given by

$$\begin{aligned} R_{AB} &= K\,[A][B] \\ K &= K_0 \exp(-E_a/RT) \end{aligned} \tag{4}$$

where K is the rate constant and the brackets indicate molar concentrations. Temperature dependence of the rate constant is related to the activation energy (E_a) where K_0 is the rate constant at some standard condition of the temperature, T, and R is the gas constant. From the activation energy and the temperature dependence of the rate constant, we can analyze the kinetics of assembly.

5.2. Kinetics of Collagen Assembly

The activation energy for collagen assembly has been reported to be between 20 and 58 kcal/mole (Williams et *al*., 1978; Comper and Veis, 1977), which is very close to the net enthalpy increase of 25 kcal/mole (Cooper, 1970) that occurs during self-assembly (Section 4.2). This indicates that activation probably involves removal of hydrogen-bonded water and that no additional heat is required after activation. The activation step, which is the formation of linear dimers and trimers, is highly temperature dependent, showing a large increase in rate as the temperature is increased (Wood and Keech, 1960; Cassel et *al*., 1962; Comper and Veis, 1977; Gelman et *al*., 1979). By contrast, lateral growth does not show the same temperature dependence (Comper and Veis, 1977). Increased collagen concentration and decreased ionic strength (between

0.3 and 0.15) increase the rate of linear growth (Wood and Keech, 1960; Cassel *et al.*, 1962; Williams *et al.*, 1978), suggesting that electrostatic interactions are involved in early dimer and trimer formation. The increase in rate with temperature and concentration can be explained as an increase in the collision frequency between monomers, which results in an increase in the number of dimers and trimers formed. From a physical point of view, collagen molecules in solution are rotating (tumbling) and translating at a rate that is proportional to the absolute temperature and inversely proportional to the viscosity. As the temperature is increased, the number of collisions between molecules increases. As shown in Fig. 7-6, interactions between the ends of two tumbling rods are most likely to occur if the volume occupied by each molecule is close to that of the spherical domain through which it can rotate. Because the rotational diffusion coefficient of a rod is inversely proportional to the cube of the length, a length increase of a factor of 3 decreases the rate of tumbling by a factor of 27. As the tumbling rate decreases, lateral collisions become more probable.

As the number of trimers formed increases, so does the probability that these species will grow laterally. The increased rate of fibril growth (Hayashi and Nagai, 1973; Honya and Mizunuma, 1974) that occurs in the presence of surfactants, which lower the surface free energy of water, suggests that the water shell is disrupted by these agents.

6. Ultrastructure of Matrix Aggregates

An understanding of matrix assembly requires approaches quite different from those necessary for the characterization of the final form or morphology of the matrix. The final form reflects the last steps in the assembly sequence and so this form can only give clues to the manner of its assembly. It is essential, nonetheless, to have a fairly accurate description of the final form of an ag-

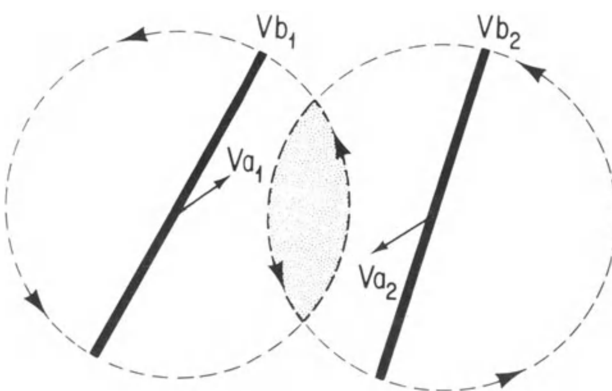


Figure 7-6. Schematic diagram of translation and rotation of two rods that occupy overlapping spherical domains. The diagram shows the instantaneous projections of two overlapping domains onto the plane of the paper. The motion of each molecule can be characterized by the translational velocity of its center of mass (Va_1 and Va_2), the direction of which is strongly dependent on the strength of intermolecular interactions. In addition, the velocity (Vb_1 and Vb_2) of a point at the end of each rod is characterized by the sum of its translational and rotational velocities.

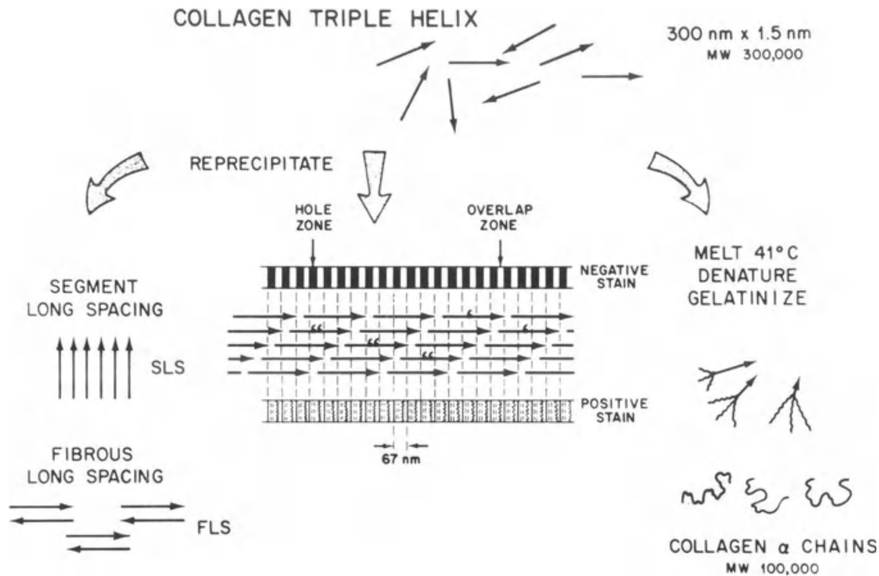


Figure 7-7. Traditional two-dimensional presentation of collagen packing in SLS (segment-long-spacing), FLS (fibrous-long-spacing), and native fibrillar forms. These models were derived from electron microscopic study and have now been refined both by kinetic studies of fibril assembly (see Fig. 7-4) and by X-ray diffraction studies of the final fibril form. For the beginning student, the 1-D stagger of adjacent molecules is most readily seen in this presentation. Note that an intermediate subassembly consisting of a 4-D staggered dimer (′) or trimer (″) is easily accommodated in this two-dimensional model. As discussed elsewhere (Chapter 1), collagen is denatured into component α chains when heated above 37°C.

gregate before a complete model of its manner of construction can be conceived. Most of the data that are available concerning the form of the final matrix structures, such as collagen fibrils, basement membranes (basal laminae), proteoglycan aggregates, elastin fiber and microfibrils, and the fibronectin network in the matrix on the cell surface, are derived from ultrastructural and X-ray diffraction studies. In the preceding discussion, we have focused attention on the assembly process in general and that of type I collagen in particular. In order to discuss the ultrastructural features of collagen fibril assembly, it will be helpful to briefly review features of the final fibril structure that have been defined by ultrastructural, X-ray diffraction, and biochemical studies.

Ultrastructural studies were the first to demonstrate that the length of individual type I collagen molecules, visualized directly and in unstaggered lateral aggregates or SLS crystallites, was approximately 4.4 times the length of the major repeating pattern of 67 nm, or 1 D unit, seen in the native fibril (Gross *et al.*, 1954; Hall, 1956; Olsen, 1963). A two-dimensional representation of collagen fibril structure was deduced from these studies (Fig. 7-7), in particular from a photographic synthesis in which the striated pattern of the SLS crystallite was staggered on itself to generate a pattern similar to that seen in native

fibrils (Hodge and Petruska, 1963). Subsequent studies using amino acid sequence data and computer assistance, as shown in Fig. 7-2, have confirmed these early observations (Meek *et al.*, 1979). Further studies of collagen types I, II, and III have shown that these collagens form typical as well as unusual or polymorphic fibrils *in vitro* and have confirmed the general model that the collagen fibril is comprised of molecules staggered and interwoven with each other into a ropelike structure (Kuhn *et al.*, 1964; Bard and Chapman, 1968; Bruns *et al.*, 1973; Doyle *et al.*, 1975; Bruns, 1976; Lapiere *et al.*, 1977).

The simple planar representation of the collagen packing pattern illustrated in Fig. 7-7 for type I collagen is a satisfactory first approximation of fibril organization, but it is not sufficient to explain the three-dimensional packing of staggered monomers. A number of models have been proposed to account for the three-dimensional order in the fibril, and involve formation of a microfibril of two, four, five, or eight molecules (Miller, 1976). The microfibril model that has been given the most consideration is the five-strand model of Smith (1968). This model is formed by distributing five molecules in 1-D stagger on the surface of a cylinder such that each molecule is staggered by 1 D unit from adjacent molecules. Adjacent microfibrils could then be staggered by an integral value of D and the summed packing of the entire set would be a D periodic fibril.

X-Ray diffraction studies of intact native type I collagen fibrils have supported the microfibril model in that an equatorial reflection indicated a 3.8-nm substructure, which is close to the 3.5-nm diameter predicted for the five-strand microfibril (Miller and Wray, 1971; Veis and Yuan, 1975). However, a number of the reflections present in the X-ray patterns have not been satisfactorily explained by the various models that have been proposed, and alternative explanations have been presented. Katz and Li (1973) suggested an hexagonal packing of collagen molecules in the fibril and more recently Hulmes and Miller (1979) have proposed that the collagen molecules in type I collagen fibrils are packed on a quasi-hexagonal lattice that is sheared both axially and laterally. This packing arrangement does not explicitly require discrete substructures of fixed dimensions within the final fibril structure and does not require or predict that a specific subunit(s) participates in the assembly sequence. Trus and Piez (1980) have modified the quasi-hexagonal model and proposed that a compressed microfibril structure fits the X-ray diffraction and ultrastructural data better. The location and lateral extent of the covalent cross-links that are present between adjacent molecules have independently led to a proposed fibril substructure that includes features of the compressed microfibril (Bailey *et al.*, 1980). While most attention has been focused on a microfibril model, arguments continue for an organization of molecules within the fibril in other patterns such as the quasi-hexagonal model (Hulmes and Miller, 1979) or a layered smectic liquid crystal (Woodhead-Galloway, 1980). Detailed arguments for these various models are provided in several recent reviews (Miller, 1976; Fraser *et al.*, 1979; Bornstein and Traub, 1979; Woodhead-Galloway, 1980).

Ultrastructural studies of connective tissues in fixed and thin-sectioned or freeze-fractured preparations have added important details of the organization of matrix aggregates *in vivo*. Individual collagen fibrils can be seen to be comprised of thinner subfibrils that, when slightly dissociated by various solvents, describe a helical or twisted ropelike course along the fibril axis (Bouteille and Pease, 1971; Lillie *et al.*, 1977; Hasty and Hay, 1977; Ruggeri *et al.*, 1979). In most fibrils there is near-perfect alignment of the banding pattern across the entire fibril, indicating that the subfibrils are normally packed in some kind of extended lateral register. It has not been possible to specifically identify a collagen type by its striated pattern or diameter, although fibrils of types II and III generally are narrow (Hay *et al.*, 1978). Quantitative analysis of the diameters of type I collagen fibrils in sectioned materials suggests that within the relatively wide distribution of fibril widths, varying between 16 and 500 nm, all fibrils are comprised of a subfibril that is 8 nm in diameter (Parry and Craig, 1979; Squire and Freundlich, 1980). Such an 8-nm subfibril would be consistent with four 3.5-nm microfibrils related to one another by a 4_3 screw axis and resulting in a microfibril of 8.5-nm diameter (Fraser *et al.*, 1979).

The macroperiod of the collagen fibril does not seem to be rigidly set at 67 nm, but rather differs depending on tissue source, tissue preparation, and apparent tensile stress (Gillard *et al.*, 1977; Stinson and Sweeny, 1980; Brodsky *et al.*, 1980). The periodicity of skin collagen fibrils (65.2 nm) is shorter than that found for tendon fibrils (67.0 nm) from the same species, and this difference does not seem to relate to their proportional content of collagen types I and III (Lapiere *et al.*, 1977; Brodsky *et al.*, 1980), but rather to possible noncollagenous constituents such as glycosaminoglycans within the fibril structure (Pease and Bouteille, 1971; Nakao and Bashey, 1972).

The length of individual collagen fibrils and their sites of origin and insertion are not known. Collagen fibrils in tendons interface with muscle cells in an interdigitating fashion that suggests that the convoluted surface of the muscle is the site of fibril termination (Nakao, 1975). The connection between the collagen and the muscle may be mediated by an elastic protein, connectin (Maruyama *et al.*, 1977). The insertion of collagen fibrils into the basement membrane of the epidermis of the fish *Fundulus heteroclitus* and shark has been described (Nadol *et al.*, 1969; Wainwright *et al.*, 1978). Recent evidence discussed below suggests that the site of collagen fibril formation by several kinds of fibroblasts is a deep recess in the cell surface.

7. Ultrastructure of Collagen Assembly Intermediates

The ultrastructure of the assembly sequence for type I collagen has been studied during the lag and growth phases of the typical heat gelation reaction (see Fig. 7-3). The earliest aggregates that can be observed by electron microscopy are narrow structures, approximately 5 nm in diameter, which then laterally aggregate to form wider aggregates that are generally compact in their

central region and frayed at their ends. A 67-nm or 1-D-stagger packing pattern is apparent in the compact central region of these early forms. These aggregates with their splayed ends then appear to adlineate and interdigitate in a tandem manner to produce a longer, linear subfibrillar aggregate (Fig. 7-4). These subfibrillar aggregates are found frequently entangled with each other in a ropelike manner, suggesting that lateral growth occurs by entwinement of the subfibrils. These studies indicate that the fibril assembly process is a multistep sequence in which subassemblies of potentially many discrete forms are central to the assembly sequence. They suggest that linear growth involves addition of subassemblies to the end of a growing subfibril, whereas lateral growth requires a separate process of lateral association of the subfibrils (Trelstad *et al.*, 1976).

If type I collagen labeled with ^{125}I is added to an *in vitro* polymerizing solution, an asymmetric pattern is observed in the addition of labeled molecules to the fibrils such that the axial rate of growth of the NH_2 terminal is 1.2 to 1.7 times greater than that found for the COOH terminal (Haworth and Chapman, 1977). In subsequent quantitative studies using dark-field electron microscopy to define changes in mass along the forming fibril axis, Holmes and Chapman (1979) found that the number of molecules per cross section of the fibril increased by 4–5 molecules per D period at the NH_2 terminal and by 8–10 at the COOH terminal. These studies indicate that linear fibril growth occurs by the addition of multimers to the end of a subfibril, possibly preferentially to the NH_2-terminal end.

Transmission electron microscopic studies of other matrix components including the chondroitin sulfate proteoglycan monomer and its aggregated forms with hyaluronate, elastin, and fibronectin have also yielded information concerning the organization of these monomers and aggregates. Details of these structures are presented in other chapters.

8. Cells Regulate the Multistep Assembly Process

It is evident from the preceding discussion of some of the physicochemical and ultrastructural features of collagen fibril assembly and from consideration (Chapter 2) of the formation of proteoglycan–hyaluronate–link protein aggregates that these assembly processes *in vitro* are not simple one-step monomer–polymer transitions, but rather are complex multistep events. Additional complexity is apparent from an examination of the matrix assembly process *in vivo*, where one adds onto the continuum the events of biosynthesis, posttranslational enzymatic processing and intracellular packaging, discharge into the extracellular space, and extracellular enzymatic processing.

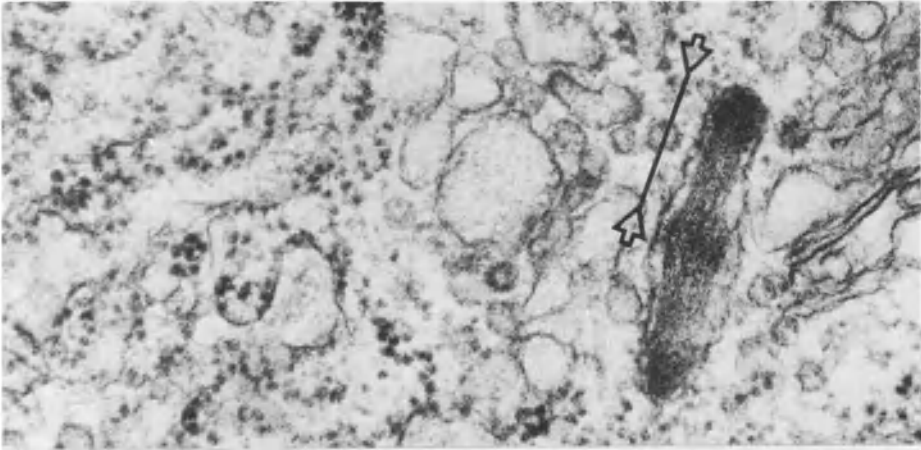
A number of the intracellular steps in the biosynthesis of collagen, elastin, fibronectin, and the proteoglycans have been discussed in other chapters. The synthesis and processing of matrix macromolecules require considerable spatial organization of the biosynthetic machinery during the sequential steps from the cisternae of the endoplasmic reticulum through the Golgi apparatus to

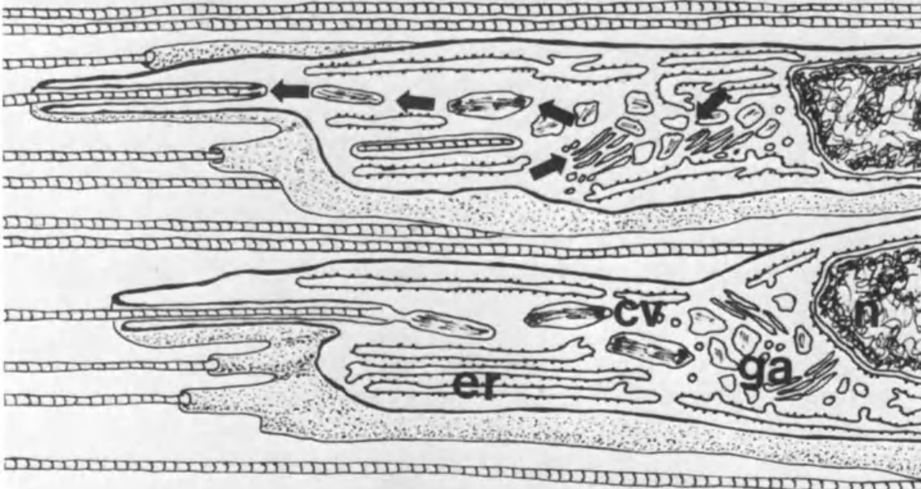
the final secretory vacuoles. During this intracellular processing, events are likely to occur that are of importance for the subsequent matrix assembly. For example, the compartmentalization of the secretory products within secretory vacuoles most likely involves mixing of different macromolecules together including important processing enzymes such as the procollagen proteases and lysyl oxidase (which might remain inactive until the ionic environment of the extracellular space is encountered).

The intracellular compartment is thus a site for secretory product condensation and perhaps mixing with processing enzymes, and it is also a site where the initial interactions between matrix macromolecules of possible significance to subsequent assembly sequences can occur. In many connective tissue cells, filamentous material presumed to be aggregated collagen molecules can be seen in the condensation vacuoles of the Golgi apparatus, and even the endoplasmic reticulum may contain a visible product that labels with anti-collagen antibodies (Nist *et al.*, 1975). Some of the Golgi vacuoles of chondrocytes reveal rodlike structures that presumably contain collagen, while others contain proteoglycan granules and filaments (Figs. 2-3, 5-6). Aggregates that resemble SLS and end-overlapped SLS have been observed in a number of different cell types (Figs. 7-8, 7-9) including odontoblasts, epithelial cells, and fibroblasts (Trelstad, 1971; Weinstock, 1972, 1977; Weinstock and Leblond, 1974; Trelstad and Hayashi, 1979; Bruns *et al.*, 1979). Weinstock (1977) has found similar structures in the predentin matrix, and Bruns *et al.* (1979) have found SLS in cell culture media, suggesting that such aggregates, under some conditions, can be discharged into the extracellular space.

It is useful to recall that SLS crystallites do not form *in vitro* at neutral pH in the absence of ATP because under these conditions there is maximal repulsion between like charges. Therefore, the procollagen aggregates seen intracellularly may be complexed with other charged macromolecules that allow their formation. Perhaps more important than the specific structure of these aggregated forms for the current discussion is the recognition that monomeric forms of collagen probably do not exist in the extracellular space *in vivo*, i.e, that initial interactions of importance to matrix assembly seem to occur within the cell under cellular regulation. The relationship between the aggregate collagen forms that occur in cells and those, such as the 4-D dimer, that have been defined by *in vitro* assembly studies remains to be determined. The intracellular aggregates in a number of cells are often longer than single procollagen molecules and, in the chick tendon and cornea, can appear as end-overlapped dimers and trimers (Trelstad and Hayashi, 1979; Bruns *et al.*, 1979).

The vectorial discharge of matrix constituents into the extracellular space is another way by which cells exercise influence over the matrix assembly process *in vivo*. The cytoskeletal and energy-dependent transport (Ehrlich and Bornstein, 1972; Kruse and Bornstein, 1975) of secretory vacuoles to the cell surface for discharge does not result in their random accretion around the entire perimeter of the cell, but usually results in their preferred localization to one region or pole of the cell. The vectorial or polarized discharge of secretory products is a well-recognized phenomenon in exocrine and some endocrine

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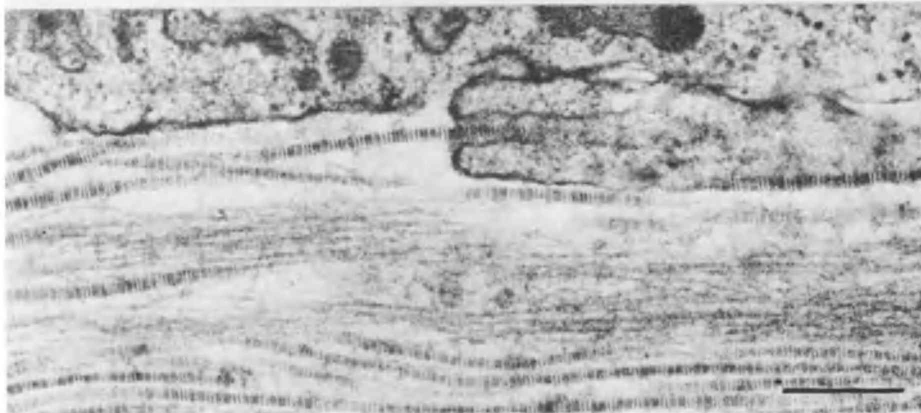


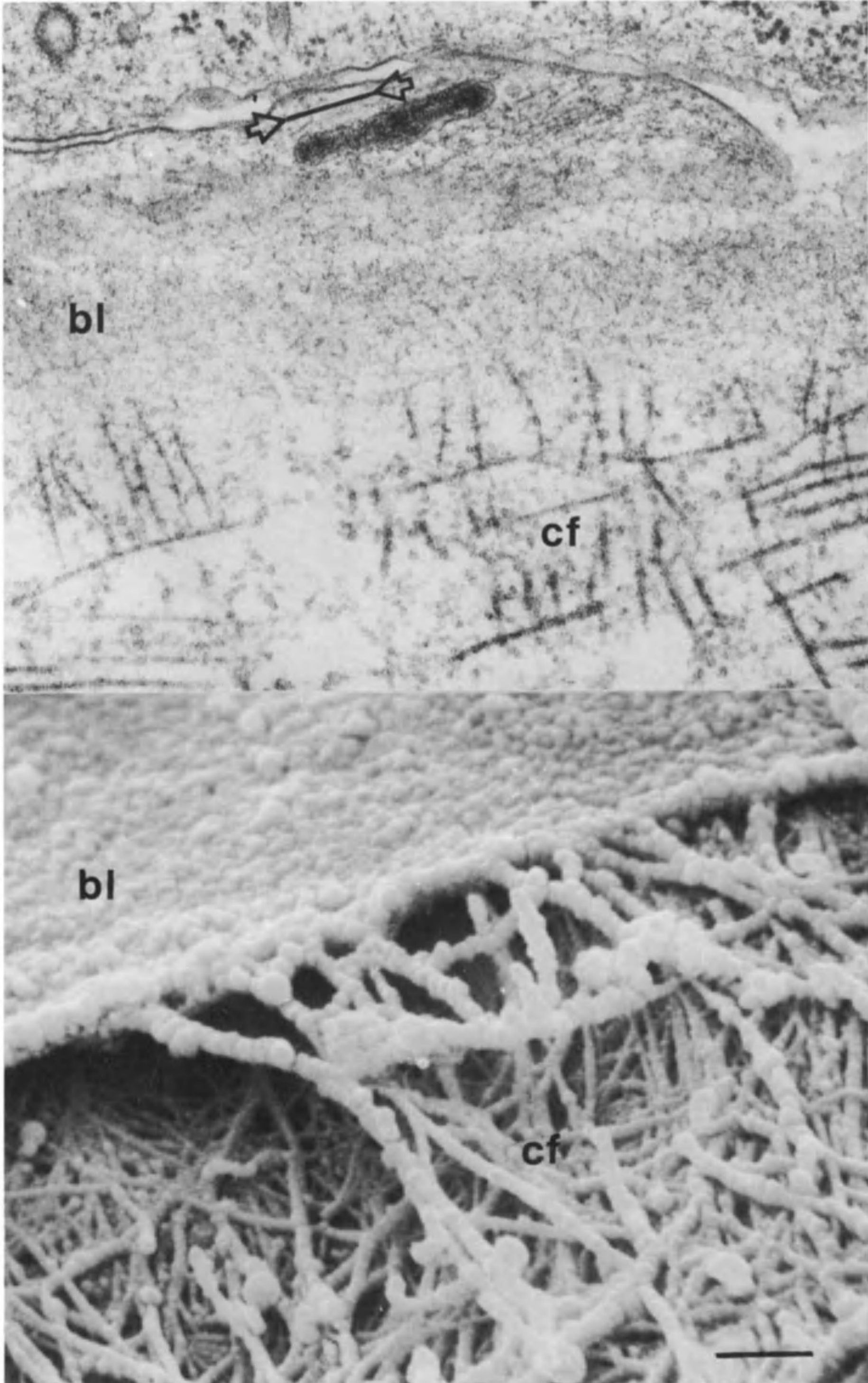
glands and is generally associated with the coordinate, polarized positioning of the Golgi apparatus in the secretory pole of each epithelial cell.

Similar coordinate, polarized patterns of intracellular organelles have been described in fibroblasts, osteoblasts, odontoblasts, chondroblasts, and matrix-producing epithelia (Trelstad, 1970, 1977; Weinstock and Leblond, 1974; Trelstad and Hayashi, 1979; Garant and Cho, 1979; Holmes and Trelstad, 1980). The polarized discharge of matrix components from odontoblasts, osteoblasts, and the corneal epithelium are perhaps the best examples of how cells might influence the organization of the architecture of the matrix by polarized discharge. In each of these cases, the matrix constituents are discharged from one face of a group of aligned cells and the matrix thus grows by accretion as a layer. In bone, a tissue that grows by apposition of new matrix on existing matrix, cells are progressively pushed away from the old matrix by the new products polymerizing on the surface of the cell that abuts the old matrix.

The site of assembly of matrix aggregates in the extracellular space is of obvious importance to the generation of matrix architecture. From autoradiographic studies of a number of connective tissues, the site of matrix deposition is near to or a short distance from the cellular perimeter (Revel and Hay, 1963; Trelstad and Coulombre, 1971; Weinstock and Leblond, 1974). Newly synthesized components do not diffuse distances much greater than the dimensions of the cells, i.e., less than 20 μm. These sites of matrix assembly must be unique in that they represent the site at which, for example, the collagen fibrils grow in length.

Type I collagen assembly in the developing chick embryo tendon occurs close to or in association with the surface of the tendon cell (Trelstad and Hayashi, 1979). The tendon cell surface is infolded at many sites, and such infoldings or recesses contain collagen fibrils (Fig. 7-8). The chick tendon cell discharges its collagenous secretory product, presumably as a procollagen, into these recesses, where the discharged material then apparently adds to the end of a growing fibril. For tendon formation, such an arrangement would provide means for the cell to control the length and orientation of the tendon bundles as they form.

← **Figure 7-8.** The upper electron micrograph illustrates a collagen condensation vacuole in an embryonic chick tendon cell. These vacuoles contain a number of procollagen molecules, packed side by side, that are about to be discharged into the extracellular space. The line between the arrows indicates the length of a fully processed collagen molecule (300 nm); there are thus at least two sets of molecules in some kind of end-overlapped configuration within the vacuole. The diagram illustrates the probable secretory route of collagen from fibroblasts in the chick tendon. Following synthesis in the endoplasmic reticulum (er), the procollagen is transported to the Golgi apparatus (ga), where the procollagen is packaged and where aggregation begins to occur. These aggregates are then moved to the cell surface in condensation vacuoles (cv) and are discharged into the extracellular space. The site of discharge is into deep recesses in the cell's surface, a configuration that allows the cell to control the growing end of the fibril and to add collagen aggregates in response to growth requirements. The lower electron micrograph illustrates a cell process containing a recess from which a collagen fibril extends. Upper micrograph, × 65,000, bar = 300 nm; lower micrograph, × 56,000, bar = 300 nm. (From Trelstad and Hayashi, 1979.)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An analogous, but uniquely different circumstance occurs in the embryonic chick cornea, where a matrix comprised of collagen types I and II, proteoglycans, and fibronectin is synthesized by the epithelial cells and deposited beneath the basal surface of the epithelium (Hay and Revel, 1969; Trelstad and Coulombre, 1971; Toole and Trelstad, 1971; Trelstad et *al.*, 1974; von der Mark et *al.*, 1977; Linsenmayer et *al.*, 1977; Kurkinen et *al.*, 1979). All of the "prepolymerized" matrix components produced by the epithelial cells must traverse the basement membrane. A similar situation exists in the basement lamella of fish and amphibians (Revel and Hay, 1963; Nadol et *al.*, 1969). The site at which collagen begins to assemble into fibrils in the cornea appears to be at or near the lamina densa of the basement membrane (Fig. 7-9). That is, secreted materials exit from the epithelial cells and do not diffuse away from their basal surface, but rather are transiently incorporated into the basement membrane. It is within the interstices of the basement membrane that fibril assembly apparently begins, and the ends of the collagen fibrils in the highly ordered subepithelial stroma are thus embedded in this structure.

In other connective tissues, the site of matrix assembly may not occur immediately at the cell surface, but rather some short distance from it. In these circumstances, the existent matrix that surrounds the cell may either hinder or facilitate the diffusion of the preassembled matrix components to the sites of assembly and may also serve as a template for matrix assembly. The template role of the matrix is poorly understood, but in considering the manner in which the final three-dimensional form of a tissue is established, it is reasonable to expect that it is accomplished in a stepwise manner in which the order at one stage contributes to the order that subsequently develops. Templates for matrix deposition have been described in a number of tissues for collagen, elastin, and proteoglycans (Ross and Bornstein, 1969; Myers et *al.*, 1971; Trelstad and Coulombre, 1971; Humphreys and Porter, 1976; Frederickson et *al.*, 1977). The restriction or hindered diffusion of large macromolecules through a preexistent matrix is intuitively apparent, but the unexpected finding of circumstances in which there is facilitation of diffusion is, perhaps, not (Preston et *al.*, 1980). In some conditions, facilitated diffusion *in vitro* is coupled with the transient

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← **Figure 7-9.** Electron micrographs of the basal surface of the embryonic chick corneal epithelium. (Upper) This micrograph is slightly oblique to the corneal surface and illustrates the basal lamina (bl) as a continuous, feltlike network that merges with the underlying matrix of orthogonal collagen fibrils (cf). The collagen synthesized by the corneal cells is packaged in vacuoles similar to those seen in the tendon (Fig. 7-8). The line between the arrows indicates the length of a fully processed collagen molecule (300 nm). In this case, the site of discharge of the collagen is across the epithelial basal surface, and the manner in which the discharged collagen assembles into fibrils appears to involve transient incorporation into the basal lamina. The growing ends of the fibrils are apparently anchored within the basal lamina, and this structure is the site at which addition of intermediate aggregates occurs. (Lower) Scanning electron micrograph of the basal lamina (bl) and underlying collagen fibrils (cf) of a chick cornea. The epithelial cells have been removed from the upper surface of the basal lamina. The basal lamina is a granular, ruglike structure from which fibrils emerge to enter into the underlying stroma of collagen fibrils and glycosaminoglycans. Upper micrograph, × 45,000, bar = 300 nm; lower micrograph, × 14,500, bar = 1 μm.

development of ordered structures whose probabilities of occurrence under equilibrium conditions are low and whose growth involves nonequilibrium phenomena (Nicolis and Prigogine, 1977; Prigogine, 1980).

The final three-dimensional morphology of each tissue within a species is monotonously reproduced in every individual of that species. It seems unlikely *a priori* that the polymerization process for any of the matrix constituents is simply "self-assembly" in the extracellular space. There is too much order to conclude that the assembly process is divorced from "production," and the biosynthetic element, the cell, is surely involved in an active way in the assembly of the matrix.

9. Matrix Macrostructure and Function

In the preceding discussions, we have focused on the microstructure of some of the matrix components as revealed by biochemical, physicochemical, and ultrastructural techniques. In this and the following section we consider a number of the macroscopic properties of the matrix that reflect tissue-specific differences in assembly processes.

The capacity to produce composite extracellular matrices that assemble into multiple forms reflects a successful stratagem of multicellular organisms to segregate cells into functional units of tissues and organs able to contend with the forces of gravity and work in unison to transmit the forces necessary for movement. The shape and size of higher organisms are defined by spaces, partitions, and unique forms of the matrix. In the embryo, the extracellular matrix is the scaffolding that helps determine tissue patterns, and in the adult, it serves to stabilize these same patterns. The mineralized matrices of the bones and teeth are stiff, hard structures, whereas the nonmineralized cartilages are flexible and compressible and serve as joint cushions during compression and translation of joints. The ropelike organization of tendons and ligaments provides them with the capacity to withstand large forces without stretching more than a few percent of their length, making movement possible. Elastic blood vessels transiently store pulse pressures generated by the heart and thus ensure a relatively continuous flow of blood. The stretchable, tough, and tight-fitting skin is a collagenous shield that serves to keep undesired materials out and desired materials in, whereas its counterpart covering the eye, the cornea, is a transparent lattice of collagen fibrils serving both barrier and optical functions. In the kidney, filtration of small waste molecules from larger blood proteins is accomplished by the glomerular basement membrane, a structure whose chemistry and organization are described in detail in Chapter 11.

Many connective tissues are birefringent, that is, they have two refractive indices when examined under polarizing optics, owing to the ordered arrangement of their constituent macromolecules. Highly oriented structures allow light to pass in one plane of polarization more rapidly than light in a perpendicular plane, and comparisons of the slow and fast waves reveals details of the organization of the object. Practically, the slow and fast waves are detected by

differences in light intensity and/or color and in black-and-white photomicrographs obviously only as levels of light intensity. The observed intensity of any birefringent object is dependent on the geometric relationship between the plane of the incident polarized light and the object itself. Accordingly, the birefringence pattern of an oriented material in a polarizing microscope changes with changes in the positioning of the specimen. In bone, for example, the collagen fibrils are organized into layers within which all fibrils course in the same direction. Adjacent layers of fibrils are ordered in different directions, thereby causing a change in the relative birefringence of the layers (Fig. 7-10). this alternating pattern of fibril orientation provides increased strength for bony tissues, and the orientation of the fibrils is related to the stresses to which the bone is subjected. Collagen fibrils of cartilage are less oriented than those of bone, whereas the collagen fibrils of ligaments are highly ordered (Fig. 7-10). Skin is also comprised of interlacing bundles of collagen fibrils, but in a less structured pattern than that of bone (Fig. 7-10).

The different patterns of type I collagen-rich tissues are apparent from a comparison of skin, intervertebral disc, and tendon (Fig.7-11). In skin the collagen fibrils form a wickerwork of interlacing bundles, whereas in the annulus fibers of the intervertebral disc they are present in broad sheets in which the orientations of the collagen fibrils in adjacent sheets are in different directions (Fig. 7-11). In tendons there is an unusual periodic change in the polarization pattern of the bundles of collagen, which has been explained by a planar crimp of the collagen fibrils along the tendon axis (Fig. 7-11). This pattern allows for extension of tendinous structures without actual extension of the collagen fibril, thereby serving to absorb impact tensile loads without generating large amounts of heat, an important biomechanical function of tendons.

The pattern of the collagenous matrix in the submammalian cornea is highly ordered, consisting of alternating layers of collagen that describe a right-handed spiral in a manner resembling a cholesteric liquid crystal (Fig. 7-12). Remarkably, this pattern is the same in both eyes and therefore bilaterally asymmetrical (Trelstad and Coulombre, 1971). For further discussions of the many interesting macroscopic and microscopic patterns described in vertebrate tissues, the books by Neville (1976) and Bonner's abridgment of Thompson (1969) are recommended. Stevens (1974) presents an excellent general treatment of patterns in natural structures.

10. Biomechanics

The macroscopic physical properties of matrices reflect the content and conformation(s) of the individual macromolecules as well as their three-dimensional or supramolecular structure. Matrix macromolecules that adopt a randomly coiled form, such as elastin (Hoeve and Flory, 1958, 1974; Dorrington and McCrum, 1977), are easily deformed and give flexiblity to the matrix. The flexibility of these macromolecules is due to the ability of the network chains to adopt many different conformations of similar free energy (Hoeve and Flory,

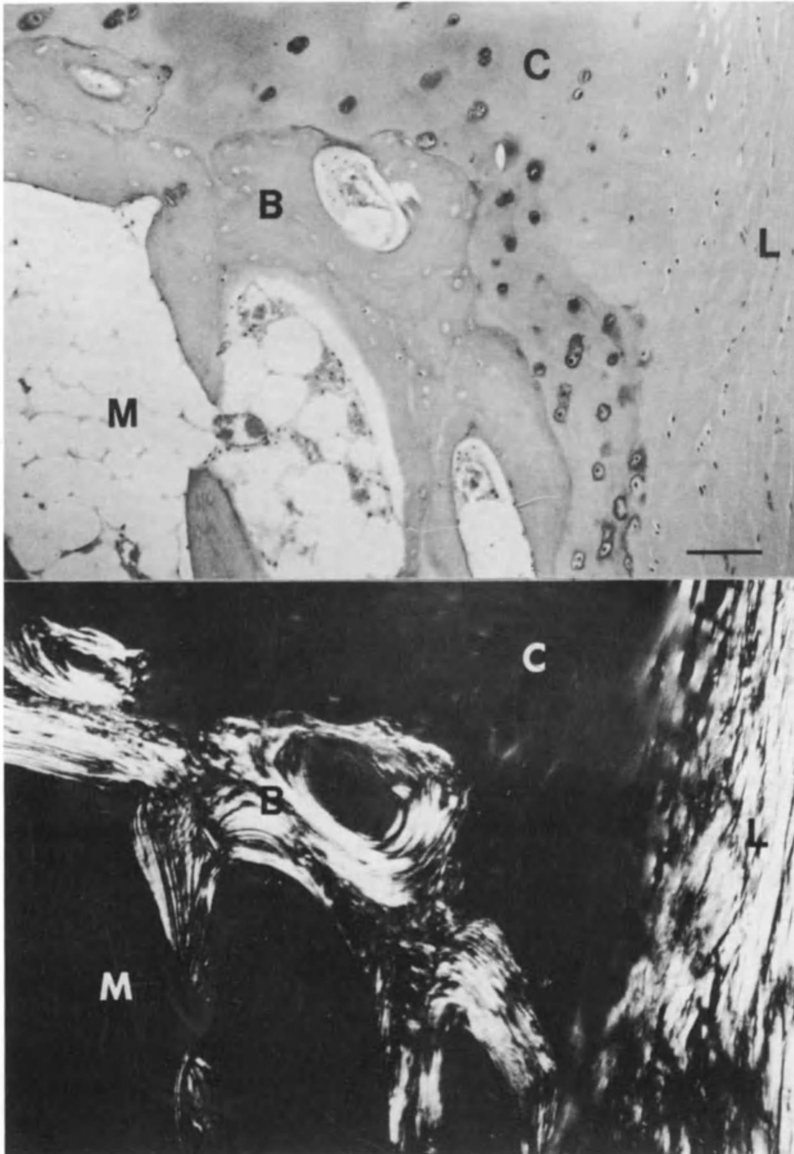

Figure 7-10. The upper light micrograph is from the edge of a finger joint and illustrates cartilage (C), bone (B), bone marrow (M), and ligament (L). The differences in the underlying organization of the matrices of these tissues are not readily apparent in standard microscopic preparations, but if the same specimen is viewed between crossed polarizers as illustrated in the lower micrograph, marked differences in the organization of the matrices in the cartilage, bone, and ligament are obvious. The ligament consists of bundles of collagen fibrils whose axes are generally in one direction, imparting to it strong birefringence. The cartilage, by contrast, contains few oriented fibrils and thus little birefringence; accordingly, this region of the section appears dark in the lower micrograph. The bone reveals a layered structure organized in sheets in both a flat and a circular configuration in which some regions appear very bright and others not. Simply turning the specimen in the microscope indicates that the dark areas in the bone are so only because of their angle with respect to the incident polarized light. On the other hand, the cartilage, being relatively nonbirefringent, is dark at all sample positions. Both × 112, bar = 100 μm.

Figure 7-11. Light micrographs from skin (top), annulus fibers from the intervertebral disc (middle), and tendon (bottom) viewed in a polarizing microscope at the same magnification. Each tissue illustrates a difference in the orientation of the component fibrils, their width, and their geometric distribution. The skin type I fibrils are organized into a wickerwork of cablelike structures. As illustrated later (Fig. 7-14), this woven structure gives the skin a pliable quality. The type I collagen fibrils in the intervertebral disc (middle) are organized as sheets within which the collagen fibrils all course in the same direction (arrows) and between which there is a shift in orientation. The type I collagen fibrils in the tendon (bottom) are oriented in a general sense along one axis, but the fibril contains a regular crimp such that the birefringence alternates. The crimp is best explained by the collagen fibrils being distributed in a standing planar wave and probably serves biomechanical functions (see Fig. 7-13). All × 380, bar = 25 μm.

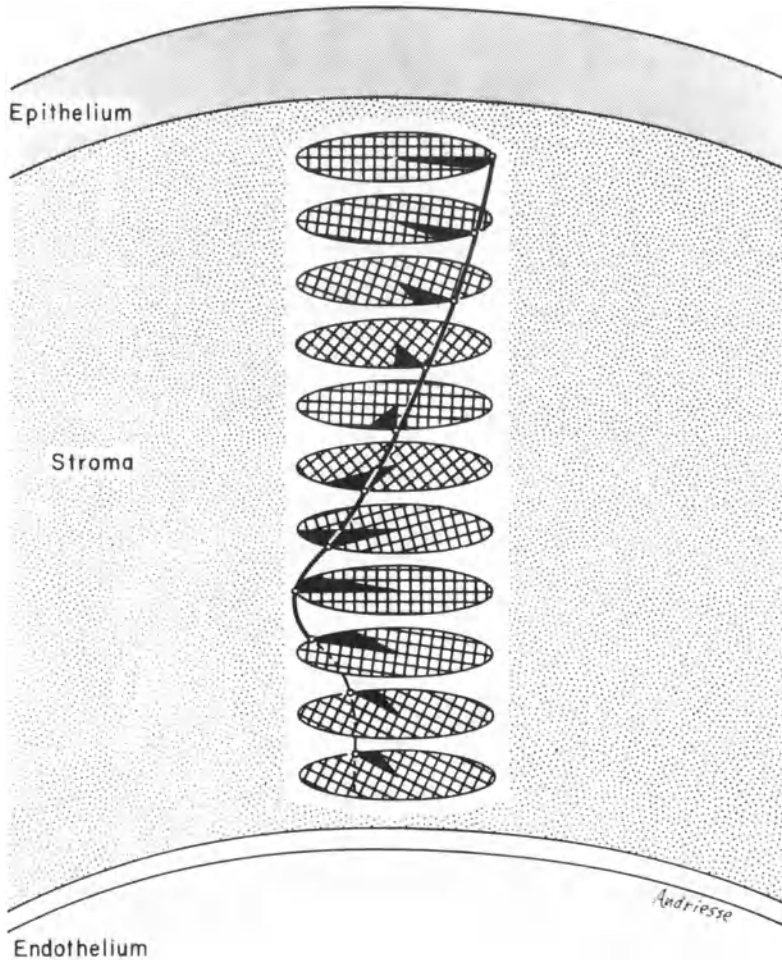

Figure 7-12. The chick corneal stroma is composed of layers of collagen fibrils in which the fibrils in each layer course in the same direction and those in adjacent layers in nearly a perpendicular direction. A pair of adjacent layers thus form an orthogonal set of fibrils as illustrated by the grid on the flat disc. The orientation of the orthogonal grid shifts in a clockwise direction proceeding from the outer epithelium to the inner endothelium. The net angular displacement from outermost to innermost layers in the chick is about 220° (Trelstad and Coulombre, 1971). Near the posterior portion of the cornea, the shift stops and all subsequent layers are in register. This remarkable pattern is identical for both eyes and thus does not show mirror symmetry about the animal midplane. All submammalian corneas are organized on this general theme. (Trelstad, unpublished.)

1974) without the formation or destruction of bonds. The ability of matrices that contain elastin to recover from large deformations is due to the large entropy gain that occurs when the stress applied to a randomly coiled macromolecule is released.

Helical or rigid macromolecules like collagen impart stiffness to matrices and prevent large deformations. In some matrices such as aorta and tendon, the collagen fibrils are crimped to allow smaller reversible deformations without significant energy loss. This is important in matrices that are in a continuous state of loading (force applied) and unloading (force removed), for any energy loss results in heat buildup that could damage the surrounding tissues.

In matrices that contain significant numbers of collagen fibrils, the stiffness is increased either by increasing the volume fraction of collagen or by orienting the fibrils and fibers along the direction in which the force is applied. For this reason, tendons are stronger when stretched along the fiber axis than other collagen-containing matrices that contain randomly oriented collagen fibers such as skin. For example, Fig. 7-13 is a stress–strain curve for a stress-bearing tendon. For small strains, i.e., a small change in length over the original length, a low-modulus region occurs. The modulus or stiffness is defined as the slope of the stress–strain curve. This region reflects the uncrimping of collagen fibrils and is totally reversed when the stress is removed. At higher strains, the curve becomes almost linear (like an ideal solid) before yield and fracture occur at a strain of about 10%. Tendon fails in tension by slippage of bundles of fibrils

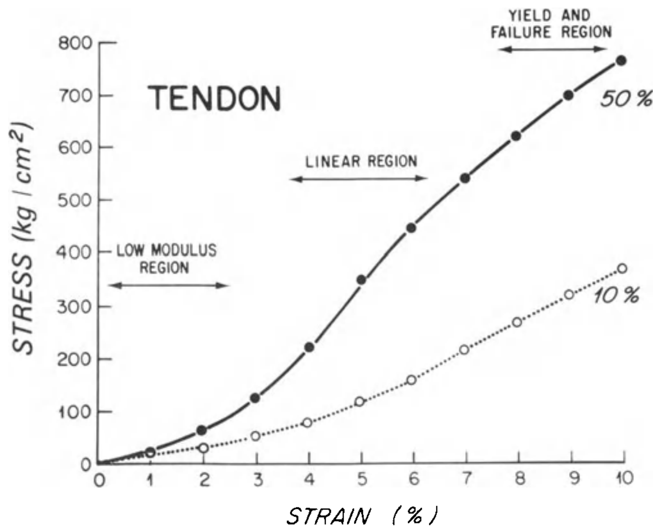

Figure 7-13. Stress–strain curves for wet rat tail tendon in tension at strain rates of 10 and 50% per minute. The dependence of the modulus (slope of the stress–strain curve) on the rate of extension illustrates viscoelasticity, which is prominent in connective tissues. The early part of each curve, or toe region, represents the straightening of the crimp pattern in the collagen fibrils (see also Fig. 7-11). The linear region represents the rapid increase in tension on the fully extended fibrils, and the yield and failure region represents slippage of bundles of fibrils past each other.

past each other, whereas skin fails by tearing or ripping. The stress–strain curve for skin in tension (Fig. 7-14) is quite different from that for tendon. Tendons fail at strains of about 10%, whereas skin fails at strains in excess of 100%. As tendon is almost entirely composed of type I collagen fibrils, the stress–strain behavior of tendon is actually the stress–strain relationship of individual collagen fibrils. The fact that skin can deform about 10 times that of tendon before it fails suggests that collagen fibrils in skin are either: (1) "kinked" like the filaments in a nylon stocking and therefore must be straightened out before the fibrils are deformed; or (2) in series with elastin, a more deformable connective tissue component.

Another important comparison to be made between the stress–strain curves of skin and tendon involves the stress at failure. Tendons deform very little because the collagen molecules in tendon fibrils are oriented almost parallel to the axis. The collagen fibrils in skin, however, are more randomly oriented (see Fig. 7-11) and orient along the tensile axis when skin is stretched. In skin, the fibrils cannot totally orient along the axis of stress because of their random orientation; accordingly, this organization of the matrix lowers the tensile strength of this tissue. In addition, if collagen fibers are in series with elastin fibers, failure will occur initially in the elastin because of its lower tensile strength. The elastic tendon in the domestic fowl behaves as a dual-network system in which one network, the elastin, accounts for the low-modulus proper-

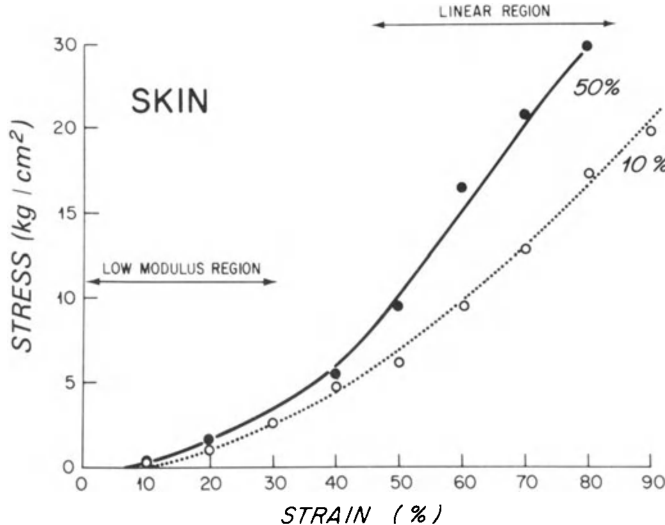

Figure 7-14. Stress–strain curves for wet rat back skin in tension at strain rates of 10 and 50% per minute. Note the lower stress and higher strain in comparison with the stress–strain curve in tendon (compare also the calibration of the ordinates and abscissas). Both of these tissues are primarily comprised of type I collagen fibrils. The wickerwork alignment of fibrils in the skin, however, produces a much different fabric from that of the tendon in which the fibrils are more uniformly aligned (Fig. 7-11). The three-dimensional architecture is a major determinant of the differences between skin and tendon in stress–strain properties.

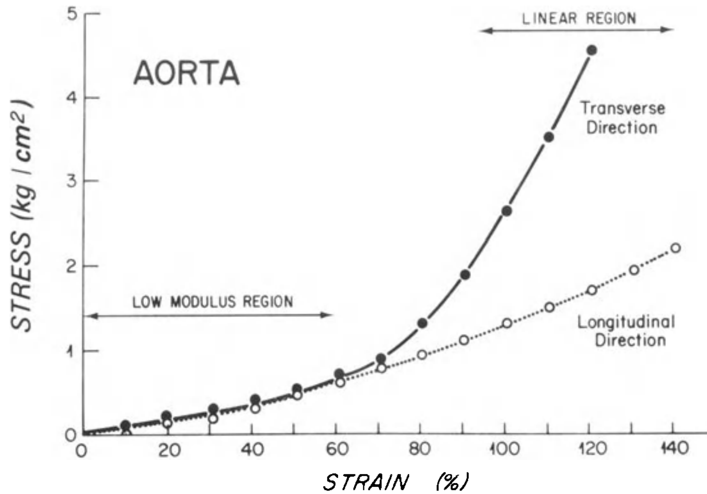

Figure 7-15. Stress–strain curve for the thoracic aorta of a 37-year-old male with Marfan's syndrome. The high extensibility in the longitudinal direction at very low stress levels as compared to control aortas suggests that permanent deformation (creep) may occur in this disorder and that the formation of structural defects in the vessel wall for biomechanical reasons may account for the tendency of these patients to form aneurysms (ballooning) and double-barreled channels that may rupture.

ties and the second network, the collagen, accounts for the high-modulus properties (Oakes and Bialkower, 1977).

The time dependence of the stress–strain curve is termed viscoelasticity. Stress–strain curves of skin and tendon are dependent on the rate at which the strain is applied, as shown in Figs. 7-13 and 7-14. At the molecular level, this time dependence reflects the time required for macromolecules to adopt new conformations. As stress is applied, each macromolecule is extended, resulting in a new conformation. At high strain rates, connective tissues are stiffer (stress–strain curve of larger slope) because the macromolecules do not have a chance to adopt new conformations. Intermolecular chain slippage also results in the time-dependent behavior of connective tissues.

An example of pathological changes that occur in connective tissue mechanics is seen in Marfan's syndrome. Normally, the stress–strain curve of an aorta in the longitudinal direction is very similar to that in the transverse direction (Yamada, 1970). However, as shown in Fig. 7-15, in Marfan's syndrome it is possible to observe large differences in the stress–strain behavior parallel and perpendicular to the axis of the aorta. In addition, very small stresses can cause dissections or separation to occur between the layers within the vessel wall. These biomechanical changes are manifestations of structural changes that occur in the aorta that probably result in the dilatation of the aortic valve, ballooning (aneurysm) of the aorta, and aortic dissections that are commonly seen in patients with this disorder (Pyeritz and McKusick, 1979).

Perhaps the most important aspect of the mechanical response of matrices is the type of bonding that connects the macromolecular components. It is well known that collagen and elastin form cross-linked networks that make these macromolecules insoluble under physiological conditions. Without cross-links, these networks would fall apart during continued force application. In addition, physical as well as covalent interactions between collagen, elastin, and other macromolecules modify the mechanical behavior of these networks.

The mechanical behavior of matrices as discussed above is dependent on several factors such as macromolecular structure, content, supramolecular organization and bonding that give matrices the potential for playing vastly different structural roles. An important aspect of the cell biology of the extracellular matrix in the future will be to further extend our understanding of the relationships that exist between macromolecular and supramolecular structure and macroscopic tissue function.

11. Concluding Remarks

Structural hierarchy in life forms extends from the atomic to the macroscopic level. With the information now available characterizing many of these levels and the ability to distinguish each, it is possible to cross their boundaries and examine their interfaces. At such boundaries are often found the interactions that lead to elucidation of the mechanisms involved. We know that major determinants of the development, maintenance, and restoration of three-dimensional form in animals are the assemblages of extracellular macromolecules. Of these, the best described are the collagens. Knowledge of the molecular architecture of the collagens, their physicochemical properties, assembly sequences, mechanical stabilization by cross-linking, and three-dimensional distributions can now be coupled with information about their genetic diversity and manner of biological regulation by cells. In this chapter we have tried to show the continuum that extends across the various levels of biological order as it pertains to matrix constituents, with the intention of describing the mechanisms of the genesis and stabilization of form in normal, deranged, and repair processes. We have emphasized a combination of both physical and biological approaches, for alone either approach tends to oversimplify the study of matrix organization.

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Chapter 8

An Essay on Biological Degradation of Collagen

JEROME GROSS

1. Introduction

The limitations of language very often tend to confuse concepts. We are intuitively led to believe that the cleavage of a molecule is a "degradative" or catabolic activity. However, enzymatic removal of portions of molecules serves the purpose of activating them if they are enzymes or altering their behavior so that they may now form a highly ordered superstructure, as in the case of the fibrinogen–fibrin conversion or procollagen–collagen transformation in fibrillogenesis. The inactive precursor molecules are "degraded" to shorter sizes that permit their participation in the process for which they were designed. In the case of morphogenesis at the tissue level, total destruction of the extensive collagenous lamellae of the tadpole tail during metamorphosis involves highly synchronized and selective removal of collagen fibrils, the function of this process being to prepare the animal for its transition from an aqueous to a terrestrial environment. On another level during embryonic development, highly localized and in some cases massive programmed death of cells is an important phase of morphogenesis (e.g., limbs and nervous system). From the viewpoint of the dismantled structural or cellular element, it is undergoing degradation, but viewed from the higher level of the tissue or the organism as a whole, the process is merely a phase of essential remodeling to facilitate overall function.

Turnover (perhaps more accurately described as physiological degradation and removal) of formed collagen fibrils, when measured by the rate of decay of radioactivity in structures such as skin or tendon of healthy adult animals, has been recorded as exceedingly slow compared with turnover of other proteins (Thompson and Ballou, 1956). Half lives were reported to be of the order of weeks or months when total collagen has been examined. In studies in which animals were pulse labeled with radioactive proline or glycine and newly synthesized neutral-salt-extractable collagen studied, half lives of the order of

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hours were measured (Harkness et *al*., 1954; Jackson and Bentley, 1960); however, these do not represent degradation but rather the rapid conversion into insoluble forms of the extractable fractions. It would appear that the turnover rate, or dissolution, of the collagen fibrils in the extracellular space is truly very slow compared with most "soluble" proteins. We do not at this time have enough data to discuss relative rates of degradation and removal for the various types of collagen under different circumstances.

When a tissue undergoes rapid remodeling, as it does in early embryonic or postnatal development, or when abrupt physiologic changes in tissue mass occur, as in the postpartum uterus, in repair, regeneration, and hormonally induced modeling of the skeletal system and other connective tissues, the rate of collagen removal is relatively rapid and its half-life may be measured in terms of days, perhaps hours. It is possible that localized regions, perhaps even on a microscale (as at the insertion points of ligament or tendon fibrils into bone or muscle), in which there is considerable and rapidly varying mechanical tension, undergo turnover at rates very different from those in the bulk of the tendon or bone. The documentary data for this conjecture are not yet available.

It is useful to consider the dismantling of collagen from the viewpoint of several different types of biological processes: (1) constructive morphogenesis, consisting of new or original structure formation, as in embryologic development and including remodeling of old structures for new or changing function; (2) repair or replacement of tissue parts that involves a debridement process in which old scaffolding must be grossly removed to clear the field for fresh replacement; (3) pathologic, indiscriminate tissue destruction, as for example in lytic tumor invasion or abscess formation. It is not unlikely that in each of these three categories, cellular behavior and function, and direct regulation of enzyme activity with respect to collagenolysis, will be markedly different.

1.1. Examples of Physiologic Removal of Collagen in Remodeling

Perhaps the best example of collagen removal in morphogenesis is the early development of a long bone. The earliest embryonic anlage is a condensation of mesenchymal cells that initially are enmeshed in type I collagen. A gradual transition into cartilage occurs with the appearance of type II, characteristic of the latter tissue (Linsenmayer and Toole, 1977). It is not at all clear whether the original type I collagen fibrils are removed and replaced or whether they are simply overwhelmed by the newly deposited cartilage type and remain in place. The cartilage then calcifies, a bony collar is deposited, and internal vascularization and ossification begin. Consequent removal of the inner layers of bone, accompanied by rapidly increasing amounts of periosteal bone, results in a large marrow cavity (see Bloom and Fawcett, 1975). This process requires remodeling and enlargement of the marrow cavity by removal of type I collagen of the recently deposited bone.

Clearly, this whole complex process involves many types of enzyme activities needed for removal of mineral, noncollagenous macromolecules, such as

proteoglycans and glycoproteins, and collagen itself, which probably requires more than just collagenase. Perhaps the most intriguing process is that of synchronization of the dismantling of structural elements and their replacement by new ones of the same or different type. Which cells are responsible for these different aspects and how do they coordinate their various activities? Unfortunately, there is essentially no knowledge of that last process and only fragmentary information concerning the enzyme systems involved in effecting these changes. We do not even know whether a specific collagenase is involved in the removal of bone collagen (type I), which may not be the same enzyme that degrades cartilage type II collagen.

Hormone-mediated metamorphosis of the anuran tadpole is another dramatic example of remodeling. Under the influence of thyroid hormone, there is a nearly complete change in shape and function of an entire animal, converting it from an obligatory aquatic life to an air-breathing, amphibious existence in a matter of days. During transition, swimming capability must be maintained even as the tail is being resorbed (Fig. 8-1). This requires progressive change in structure while rapid normal removal progresses in an orderly manner. The dense collagenous lamellae of the tail fin are rapidly dismantled, yet there is continuous new synthesis and replacement with new collagen fibrils of a very transient and continuously changing scaffold. *In vivo* labeling with [^{14}C]proline during the process indicates replacement of old fibrils with new, and a selective sparing or protection (at least temporarily) of the newly synthesized and deposited fibrils (Fig. 8-2).

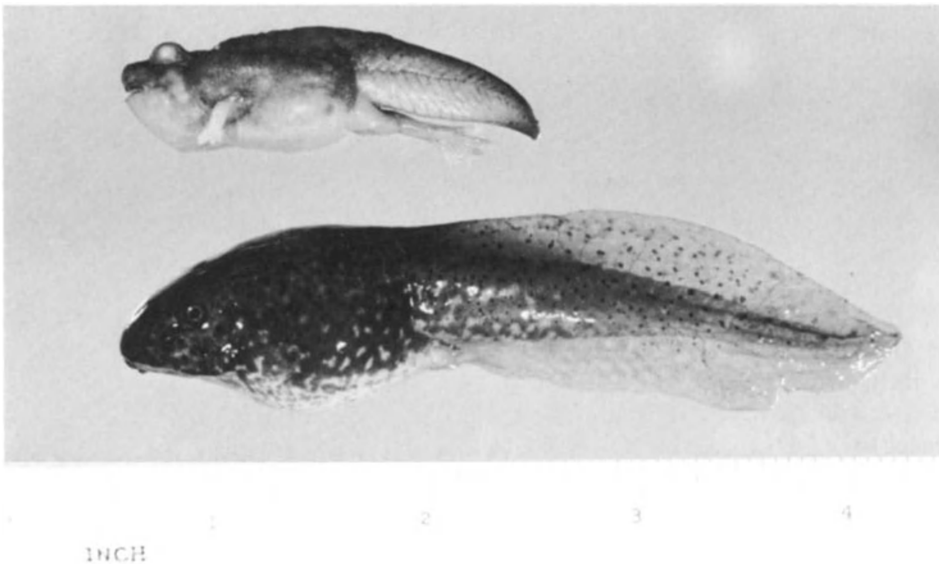


Figure 8-1. Resting tadpole, *Rana catesbeiana*; above, tadpole after treatment with thyroxin for 9 days. (From Lapiere and Gross, 1963.)

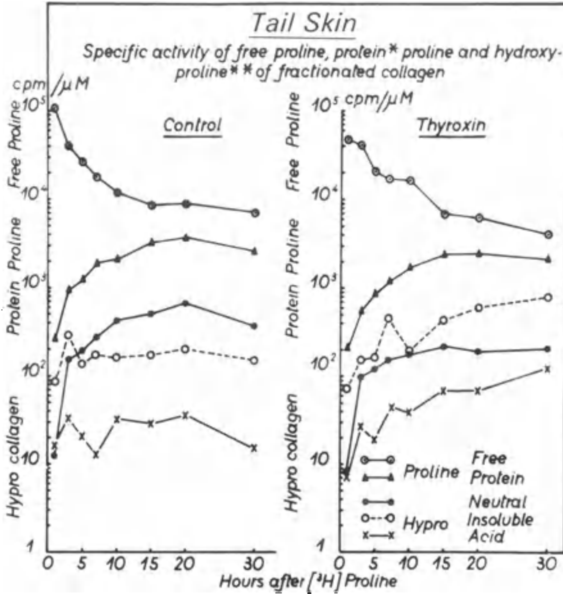


Figure 8-2. The specific activity of the imino acids (cmp/μmol) is plotted versus time (hours) after injection of the tracer (at time 0). Note the reversal in levels of specific activity of neutral-salt-extractable (newly synthesized) and insoluble (cold) collagens from the nonmetamorphic to the metamorphic state. *Proline from the protein not precipitated after distilled water dialysis of the neutral salt extract. **Collagen represented by hydroxyproline (From Lapiere *et al.*, 1964.)

Looking at a single problem raised by constructive, physiologic collagenolysis, as in bone development or tail fin removal in amphibian metamorphosis, one might ask: is the fibril or group of fibrils removed uniformly along its length, in units that can be measured in millimeters? This would require a relatively uniform concentration of enzyme molecules maintained long enough to dissolve the entire linearly localized structure. It seems unlikely that active collagenase would be present free in the tissue in a region as long and asymmetric as this, if it were secreted by a cell at a distance. Is it not possible that the cytoplasm of the enzyme-secreting cell might extend, in close proximity, all along the length of the structure to be removed? A morphologic observation of the relationships between cells and the orthogonally arrayed collagenous layers of the remodeling backskin in the metamorphosing tadpole strongly suggests that this indeed might be the case (Fig. 8-3). Using immunofluorescence studies of collagenase secreting rheumatoid synovial cells, cultured on collagen films, Woolley *et al.* (1979) have also demonstrated that the enzyme is localized to the substrate under the cell and its processes.

It is likely that little or no active collagenase is free in the tissue in physiologic remodeling, probably because it is either released in a latent form, to be activated by some as yet unidentified tissue protease, or has been blocked by inhibitors free in the tissue. Inhibitors are known to be synthesized and secreted, at least in culture, by fibroblasts (Table I; see Murphy and Sellers, 1980, for review). If the cytoplasm of a collagenase-synthesizing cell were closely applied to the fibrils slated for removal, it could maintain a high concentration of specific enzyme over considerable areas, activate the zymogen form, and overwhelm by simple concentration any inhibitor that might be present in the intervening region. The implication here is that inhibitor is not secreted by the

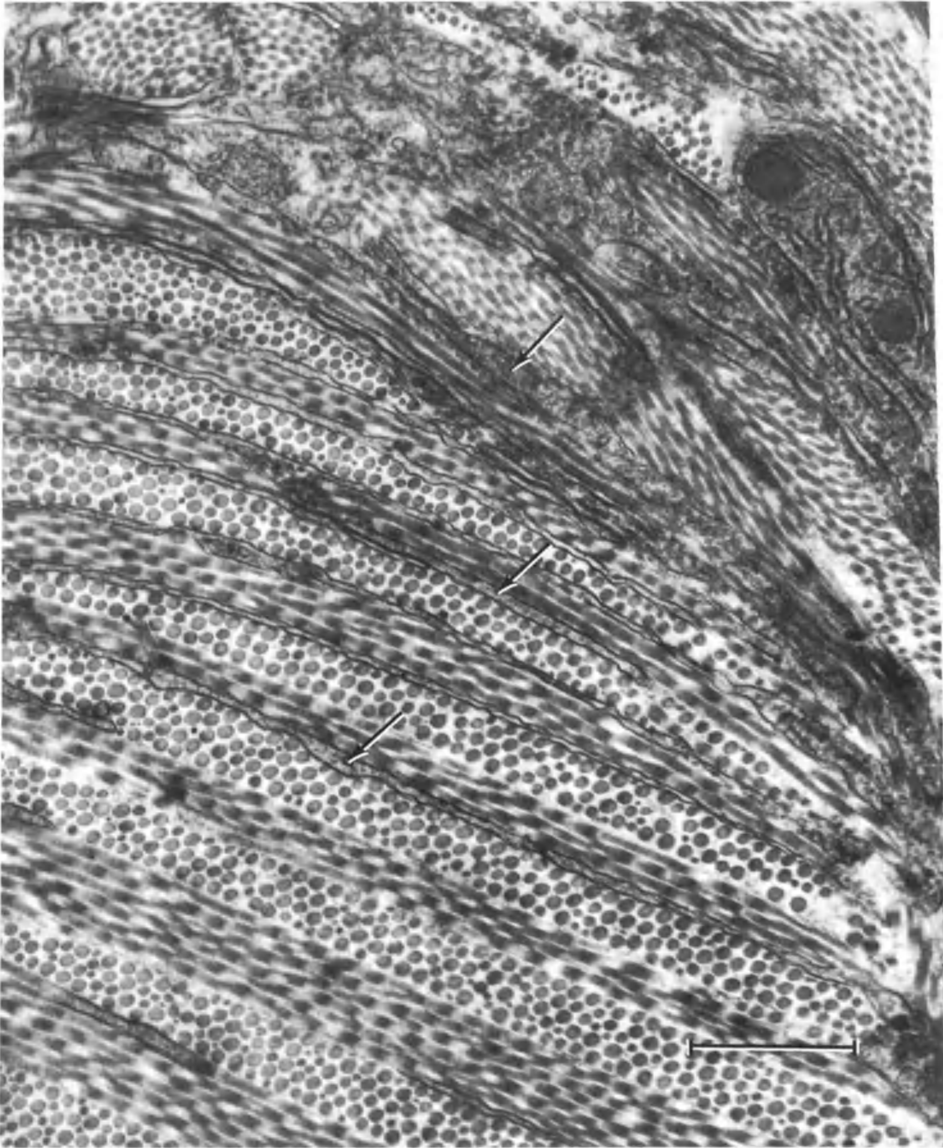


Figure 8-3. Electron micrograph of backskin of tadpole treated 9 days with thyroxin. Note cytoplasm (arrows) applied to collagenous laminae in the region of dermis closest to the surface where remodeling is most active. Also note the considerably fewer cellular elements between the more deeply located laminae. Bar = 400 nm.

same cell or at least at the same time as is collagenase. A close association such as that required here is observed not only in the remodeling tadpole backskin, but also in resorbing bone (Vaes, 1980) and in the attack of rheumatoid synovial cells (pannus) on cartilage (Woolley *et al.*, 1980). In those situations in which collagen fibrils appear to be within the cytoplasmic domains of fibroblasts in

Table I. Summary of Reported Inhibitors of Vertebrate Collagenases and Methods of Enzyme–Inhibitor Dissociation[a,b]

Collagenase inhibitor	Source	Collagenase–inhibitor reactivating agents	Key references[c]
α2-macroglobulin, mol. wt. 725,000	Human skin extracts	Sephadex G-150 chromatography	Eisen *et al.* (1971)
	Human serum	3 M NaSCN (5% recovery)	Abe and Nagai (1972)
	Synovial fluid	3 M NaSCN	Abe and Nagai (1973)
	Bovine serum	3 M NaSCN, trypsin (60% recovery)	Birkedal-Hansen *et al.* (1975b)
	Serum, tissue culture media	Trypsin, plasmin	Akroyd *et al.* (1979)
β_1-anti-collagenase, mol. wt. 40,000	Human, dog serum		Woolley *et al.* (1976, 1978)
	Serum, tissue culture media	Trypsin, plasmin	Akroyd *et al.* (1979)
Polycations, e.g., lysozyme, mol. wt. 14,300	Egg white (cartilage)	ND[d]	Sakamoto *et al.* (1974)
Cationic protein			
mol. wt. 11,000	Bovine cartilage, aorta extract	ND	Kuettner *et al.* (1976)
mol. wt. about 22,000	Bovine cartilage extract	ND	Roughley *et al.* (1978)
mol. wt. 40,000	Pig leukocyte cytosol	ND	Kopitar and Lebez (1975)
mol. wt. 40,000–50,000	Extract from V_2 ascites cell carcinoma in rabbit muscle	75–90% saturated ammonium sulfate, ion-exchange chromatography	McCroskery *et al.* (1975)

mol. wt. 6000, 12,000	Embryonic chick skin culture media	3 M Nal, NaSCN	Shinkai *et al.* (1977)
mol. wt. 6000, 8000, 15,000	Pepsin-released type I procollagen peptides	ND	Nagai *et al.* (1978)
mol. wt. 33,000	Human skin fibroblast culture media	Spontaneous	Bauer *et al.* (1975), Welgus *et al.* (1978)
mol. wt. 30,000	Various mammalian tissues in culture	APMA	Sellers *et al.* (1977b), Murphy *et al.* (1977b), G. Murphy (unpublished observations)
mol. wt. 12,000–35,000	Rheumatoid synovial cell culture media	Mersalyl	Vater *et al.* (1978b)
mol. wt. 40,000	Human cartilage culture media	Plasmin	Harris *et al.* (1978)
mol. wt. 33,000 (25,000 by SDS gel electrophoresis)	Human tendon culture media	Plasmin	Vater *et al.* (1978c)

[a] Taken from Murphy and Sellers (1980).
[b] Molecular weights are those estimated from gel filtration chromatography.
[c] See Murphy and Sellers (1980) for references.
[d] ND, not described.

resorbing tissues (Fig. 8-4) (Usuku and Gross, 1965; Parakkal, 1969; Ten Cate and Deporter, 1974), fibrils occasionally appear to be embedded in the cytoplasm without separation by membrane. However, in most instances the fibrils appear to be enclosed in a narrow space bordered by plasma membrane suggesting an extracellular location within a deep pocket in the cell. Such an image has been described in developing tendon by Trelstad and Hayashi (1979), who interpret it to represent a biosynthetic process in which collagen is being added to the end of the embedded fibril. The morphologic picture may be very similar for both biosynthetic and some degradative processes; in the latter case, collagenase may be secreted within the narrow confines of the pocket, where it might attain very high concentrations. Immunoelectron microscopy may provide a definitive answer to this last possibility.

In an effort to understand the binding characteristics of collagenase to collagen fibrils, Welgus *et al.* (1980), in a detailed kinetic study of the interac-

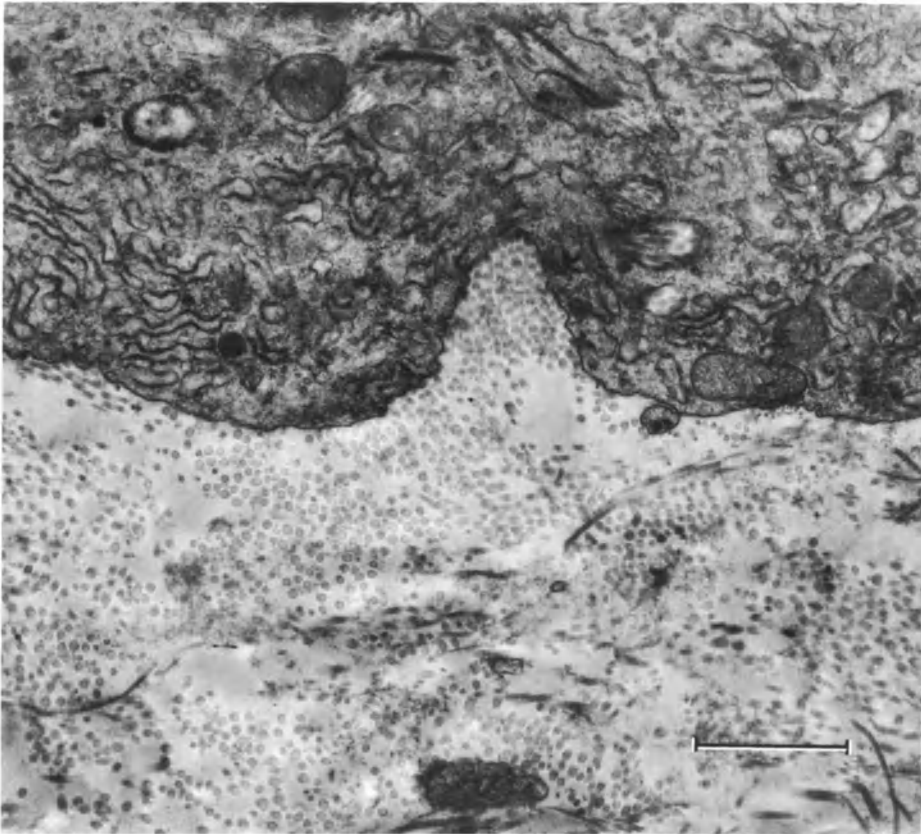


Figure 8-4. Electron micrograph of metamorphosing bullfrog tadpole tail fin treated 9 days with thyroxin. The mesenchymal cell contains engulfed collagen bundles. Note the disarray of extracellular collagen fibrils normally tightly packed in regular laminae. Bar = 1.5 μm.

tion of purified human skin collagenase with reconstituted type I fibrils, observed that only 10% of the molecules in the fibrils were available to the enzyme for binding at 25°C. The bound enzyme was not in equilibrium with unbound enzyme and was not readily displaced by added collagen. They also estimated a turnover number of 25 collagen molecules degraded per molecule of enzyme per hour at 37°C. Thus, it would appear as though the enzyme acts by stripping molecules off the surface of the fibrils very much as does bacterial collagenase (Gross, 1953). However, there may be differences that are not apparent from the kinetic studies by Welgus and colleagues (1980), for Woolley *et al.* (1978) observed that human rheumatoid synovial collagenase added to insoluble collagen fibrils causes considerable fraying, unraveling, and fragmentation, rather than a uniform stripping of protein from the fibrillar surface. It would appear from these electron microscopic studies that the molecules do indeed get within the body of the fibril sufficiently to cause unraveling of the structure. However, the *in vivo* dismantling process may differ considerably from the simplified *in vitro* system. Unravelling fibrils were not seen in the rapidly resorbing tadpole tail fin—only pleomorphism of fibril diameter (Usuku and Gross, 1965). A more detailed analysis of this action is in order.

1.2. Healing and Scarring

In healing of a tissue defect where true regeneration does not occur, finely tuned control of degradation, as described above for constructive remodeling, may not be required and the process of removal and replacement of collagen may be much less related to the physiologic requirements of the tissue. In a simple excision wound of normal skin, maximum collagenolytic activity was detected at the wound edge, falling off in the direction of central granulation tissue and normal skin. Evidence for an epithelial–mesenchymal regulatory interaction was also detected (Grillo and Gross, 1967). Studies to date on collagenase activity in scarring have not been particularly revealing, and a new approach is needed.

Pathologic or indiscriminant degradation resulting from invasion by outside organisms or neoplasms of the lytic type may invoke very little of the physiologic control mechanisms regulating collagenolysis. More will be said about this later.

1.3. Influence of Tissue Structure on Collagen Removal

Much is known about one of the key enzymes, collagenase, that plays a prime role in the turnover of collagen. Other enzymes probably involved in other phases of collagenolysis have not yet been well characterized, nor has specificity for these aspects of the process been established. Much less is known of the degradative machinery for proteoglycans and glycosaminoglycans (see Chapters 2 and 9) and even less relating to resorption of elastin (Chapter 3).

There are, in addition, other more recently described constituents of the extracellular matrix, such as fibronectin (see Chapters 4 and 10) and laminin (Chapter 4), the turnover mechanisms for which are still unexplored. No doubt there are still other constituents, perhaps lipid, nucleotide, sugar and combinations thereof, waiting to be found. An additional complicating and intriguing factor is the increasingly apparent heterogeneity, both genetic and posttranslational, of essentially all the molecular species of the extracellular matrix, which increases the complexity of the dismantling process.

Removal mechanisms depend, in part, on spatial and temporal relationships among structural components and on the resident and transient cells of the connective tissue: fibroblasts, chondrocytes, osteocytes, macrophages, mast cells, and others. These relationships vary in important specific ways from tissue to tissue although there are a few simple common patterns. In general, collagen fibrils are found in close association with proteoglycans or glycosaminoglycans in various combinations depending on the age of the animal, the physiologic state, the specific tissue structure and function, and on whether the matrix is laid down in response to normal growth, injury, repair, or regeneration. It is not known exactly what type of association exists between collagen and these mucosubstances. There is little evidence yet for covalent bonding, and it is likely that the association is one of geographic proximity perhaps modulated by any of several types of noncovalent linkages (Chapter 7).

In the case of cartilage, the proteoglycan component appears to embed the narrow collagen fibrils characteristic of cartilage in a fairly tight embrace; this may well be due to the mechanical properties of the cartilage proteoglycan complex, for here again there is no good evidence for anything beyond noncovalent associations. The relationship between elastin fibers in tissues such as skin, ligament, and aorta with both collagen and proteoglycan appears to be a mechanical association, very tight in some cases as where the collagen fibrils course directly through the elastin fibers. Any chemical type of binding among these constituents has not yet been discerned.

Basement membranes, which separate epithelial cells from mesenchymal tissue in essentially all regions of the organism, consist of variable amounts of particular collagenous proteins, proteoglycans, and at least in several organs the recently discovered glycoprotein laminin (Timpl *et al.*, 1979). In some unusual instances such as the early developing salivary gland, collagen appears to be missing from the newly deposited basal laminae in the rapidly growing regions of the lobules (Banerjee *et al.*, 1977). Considerable variation in the distribution of components and perhaps even in the types of macromolecules in basement membranes from one tissue to another is likely and may greatly influence the enzymatic requirements for dissolution.

The relationship between the mesenchymal cells responsible for synthesis, organization, and degradation of the extracellular matrices, and the surface and internal structural elements is an intimate and functional one, although at the moment we are barely beginning to visualize and understand the relationship. The manner in which degradative enzymes are secreted into the extracellular

space, their specific localization temporally as well as spatially, and the regulation of these activities are probably determined to a large extent by the associations between the extracellular components themselves. It is important to note that the connective tissues with very few exceptions are in the solid, not liquid, state. Diffusion processes for enzymes and other molecules must be under considerable constraint. Substrate concentrations and accessibility to susceptible sites must vary greatly along microregions of tissues, probably in many cases changing from time to time. Movement of materials from cell to matrix to cell may be directional and polarized due to orientations of the matrix structural elements.

2. Characteristics of the Animal Collagenases

For in-depth reviews, see Woolley and Evanson (1980), Harris and Cartwright (1977), Gross (1970, 1974, 1976), and Harris and Krane (1974).

2.1. General Mode of Action

Native collagen fibrils at physiologic temperature and pH are resistant to cleavage within the helical molecular domains by any of the known digestive proteases. Essentially all of the tissue collagen is in the fibril form; at body temperature, there is little or no extractable collagen or gelatin (Gross, 1969). If collagen is denatured by heat or other methods that randomize the triple-helical structure, it is then susceptible to multiple cleavages along the polypeptide chain by essentially all proteolytic enzymes. However, collagen fibrils from mammalian sources do not denature below 60°C, whether cross-linked or not (Gross, 1964). The pH of the extracellular spaces rarely drops below 6.6 (Hutchins and Sheldon, 1973), which is still in the isoelectric range of this protein. Conceivably, collagen fibrils within intracellular vacuoles if exposed to lysosomal enzymes at low pH (i.e., 3–4) could be susceptible to catheptic attack.

The enzymatic mechanism whereby collagen is dismantled, other than by the attack of bacterial collagenases, remained a puzzle until the early 1960s. Experiments at that time dealing with connective tissue resorption in anuran metamorphosis and regeneration led to the detection, in media from cultured bullfrog tadpole tail tissues, of a secreted collagenolytic enzyme capable of degrading native and reconstituted collagen fibrils under physiologic conditions of pH and temperature (Gross and Lapiere, 1962; Lapiere and Gross, 1963). Specific degradation is characterized by products of a single peptide-bond cleavage at the same locus in each of the three polypeptide chains, one quarter the distance from the COOH-terminal end of the molecule (Gross and Nagai, 1965; Sakai and Gross, 1967; Fig. 8-5).

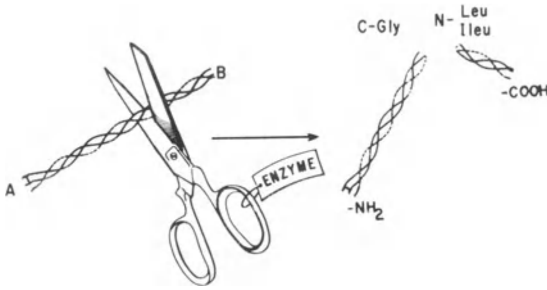


Figure 8-5. Model of the specific action of animal collagenase on the native collagen molecule. (From Gross, 1974.)

Since then, similar enzymes have been obtained from the culture media of a wide range of cultured amphibian and mammalian tissues and cells (Table II). Essentially all of these enzymes, although different as molecular species, have many prominent features in common. They all have neutral pH optima and cleave the collagen molecule at the same locus as does the tadpole collagenase. All of the vertebrate collagenases examined to date are metalloproteases, dependent upon calcium (Seltzer et *al.*, 1976), and in at least several instances where there has been enough to analyze, contain bound zinc. They are inhibited by chelating and sulfhydryl-binding agents, $\alpha 2$ macroglobulin and β_1 anticollagenase of the serum, and as yet not well-characterized tissue inhibitors (see Berman and Dohlman, 1975; Murphy and Sellers, 1980; and Table I). They are not blocked by inhibitors of serine proteases.

The highest concentrations of collagenases have been obtained in the medium of cultures of connective tissues capable of rapid resorption and from various fibroblast cultures. Protein synthesis is required for their production/secretion, indicating *de novo* synthesis rather than storage in subcellular organelles, such as lysosomes, for subsequent release. Cultured tissue explants or isolated cells killed by freeze-thawing or exposed to inhibitors of protein synthesis do not secrete collagenase. Collagenase may be extracted in variable, usually small, amounts from fresh tissues known to secrete the enzyme in larger amounts when cultured as explants.

Essentially all of the enzymes isolated from cell culture medium and a significant portion of the enzymes from tissue culture and tissue extracts are in an inactive form. Activation is accomplished by exposure to low concentrations of proteases, such as trypsin and plasmin, or to certain mercurial compounds, such as mersalyl and aminophenyl mercuric acetate, the function of which is not yet clearly ascertained (Sellers et *al.*, 1977). In some instances, long-term storage at low temperature or gel filtration alone results in activation. Because both cells and tissues are known to secrete inhibitors of collagenase into culture media, it is believed that the inactive form represents a complex of active enzyme and inhibitor (Sellers et *al.*, 1977; Murphy and Sellers, 1980). The interesting possibility has been raised by Nagai et *al.* (1978) that the extension peptides of procollagen when released may inhibit collagenase.

The work of others supports the picture of an inactive precursor or zymo-

gen that, through the action of a protease either prior to or subsequent to secretion from the cell, is converted to the active form (Harper and Gross, 1972; Vaes, 1972; Birkedal-Hansen et *al.*, 1976; Stricklin et *al.*, 1978). This may represent a conformational change or peptide-bond cleavage and removal of a fragment.

Perhaps the actual sequences of events include both these series of actions in a complex regulatory process whereby production of the zymogen, activated intracellularly or extracellularly through specific enzymatic attack, is the earliest step, the timing and place of which may vary among the different tissues, cell types, and physiologic conditions. Subsequent to activation, the free enzyme may then be bound by an inhibitor either prior to its function on its natural substrate or subsequent to it; again, the timing and locale might vary depending on circumstances. The inhibitor complex may even represent a step on a disposal route for an enzyme whose function is already accomplished. In cell culture, in the absence of much substrate, complexing with inhibitor may proceed rapidly and completely.

We do not yet know whether there is competition between substrate and inhibitor for the enzyme. It is claimed by some (Harper et *al.*, 1971; Stricklin et *al.*, 1977) that latent enzyme is not bound to the substrate, but this idea is disputed by Vater et *al.* (1978) and Vaes (1980). This important point needs further clarification.

Although the characteristic collagenases described above are most efficient in cleaving peptide bonds in the helical region of the collagen molecule in a specific manner, other nonspecific proteases such as trypsin (Olsen, 1964; Davison and Schmitt, 1968; Burleigh, 1977), and thermolysin (Wang et *al.*, 1978) have this capability to a limited degree on collagen in solution under physiologic conditions.

Lysosomal cathepsins at low pH (i.e., 3–4) and body temperature will attack collagen, which under these conditions is structurally unstable, and certain tissue proteases functioning at or near neutral pH will readily remove the telopeptide ends. Leukocyte elastase can slowly degrade the helical regions, at least in the test tube (see reviews by Weiss, 1976; Burleigh, 1977). There is no indication as yet that cellular proteases, other than plasminogen activator, leave the cell or can function under the conditions of the extracellular milieu. Plasminogen activator, however, is secreted by cells in culture and *in vivo* (Reich, 1978). In inflammatory conditions, its natural substrate, plasminogen, may be present in the extracellular spaces where it can be activated to plasmin, which in turn can have two effects: (1) cleavage of telopeptides in the fibrillar substrate, thus dissociating the cross-links from the molecules, and (2) activation of latent collagenase (Werb et *al.*, 1977).

The widely held consensus that the action of neutral collagenases is a key feature of collagen degradation is based on the observations that collagen is the specific substrate, that these enzymes operate under physiologic conditions, and that in tissue culture their activity correlates directly with the release of collagen breakdown products into the culture medium of a tissue undergoing resorption (Gross and Bruschi, 1971; Jeffrey et *al.*, 1975). It has been claimed

Table II. Summary of Reported Latent Forms of Vertebrate Collagenases and Methods of Enzyme Activation[a,b]

Source	Latent enzyme	Latent enzyme activators	Key references[c]
Tadpole tail extracts, culture media	mol. wt. 120,000[d]	Proteinase from tadpole tail culture media	Harper *et al.* (1971), Harper and Gross (1972)
Mouse bone culture media	mol. wt. 62,000[d] (105,000 in the presence of heparin)	Trypsin, plasmin, lysosomal proteinases, kallikrein, 3 M NaSCN mediated by activation of a latent endogenous proteinase activator	Vaes (1972a), Vaes and Eeckhout (1975), Eeckhout and Vaes (1977)
Various mammalian tissue culture media		Trypsin, dental plaque	Birkedal-Hansen *et al.* (1975a,b)
Gingival fibroblast, alveolar macrophage culture media	mol. wt. 79,000[d]	Mast cell serine proteinase mol. wt. 90,000	Birkedal-Hansen *et al.* (1976b)
Rabbit alveolar macrophage culture media	mol. wt. 49,000[d]	Metalloproteinases mol. wt. 11,000 and 20,000 in culture media	Horwitz *et al.* (1976)
Osteoarthritic human cartilage culture media		Trypsin, heparin–Sepharose chromatography	Ehrlich *et al.* (1977)
Human skin fibroblast culture media	mol. wt. 55,000–60,000[d] by (SDS gel electrophoresis)	Autoactivation (purified collagenase)—no mol. wt. change; trypsin	Stricklin *et al.* (1977, 1978)
Rheumatoid and osteoarthritic synovial fluid, embryonic chick skin culture media	mol. wt. 45,000–50,000[d]	3 M NaSCN 3 M Nal	Nagai *et al.* (1975), Shinkai *et al.* (1977)

Embryonic human skin culture media	mol. wt. 50,000[d]	3 M Nal, trypsin	Shinkai and Nagai (1977)
Embryonic chick bone culture media	mol. wt. 54,000[d]	3 M NaSCN, trypsin	Sakamoto *et al.* (1978)
Rabbit tissues, other mammalian tissue culture media	mol. wt. about 40,000[d]	Trypsin, APMA, and other thiol-binding reagents	Sellers *et al.* (1977b); Murphy et *al.* (1977b), A. Sellers and G. Murphy (unpublished observations)
Human rheumatoid synovial cell culture media		Trypsin	Dayer *et al.* (1977),
Human rheumatoid synovial cell culture media	mol. wt. 45,000–49,000[d]	Plasmin	Werb *et al.* (1977).
		Mersalyl, PCMB, APMA	Vater *et al.* (1978b), Harris *et al.* (1978)
Crude human PMN leukocyte extract	mol. wt. less than 80,000	Activator in rheumatoid synovial fluid and in rheumatoid synovial tissue culture media (serine proteinases?)	Kruze and Wojtecka (1972), Wize *et al.* (1975)
Human PMN leukocyte extracts, secretion during phagocytosis		Trypsin, rheumatoid synovial fluid	Oronsky *et al.* (1973)
Human PMN leukocyte-specific granule extract	mol. wt. 60,000	APMA	G. Murphy (unpublished observations)
PMN leukocyte granule extracts, alveolar macrophage extracts		Microbial plaque extracts	Robertson et *al.* (1974)
Involuting rat uterus extracts	mol. wt. 77,000[d]	Endogenous serine proteinase, trypsin	Woessner (1977)
Rat Graafian follicle homogenates		Trypsin, APMA	Morales *et al.* (1978)
Human gingival extracts		Trypsin	Uitto *et al.* (1978)

[a] Taken from Murphy and Sellers (1980).
[b] Molecular weights are those estimated from gel filtration chromatography.
[c] See Murphy and Sellers (1980) for references.
[d] A fall in molecular weight of 10,000–20,000 was observed on activation.

that typical collagen–collagenase reaction products, namely TC^A and TC^B, have been recovered in extracts of resorbing tissues (Dresden, 1971; Perez-Tamayo, 1973; Kuboki et *al*., 1973). These latter observations need further confirmation.

It has been argued that cross-linked fibrils are not readily degraded to soluble fragments under physiologic conditions by animal collagenases that have been highly purified and freed of other proteolytic or peptidase activity (Leibovitch and Weiss, 1971). Small numbers of cross-links introduced into reconstituted fibrils either by formaldehyde (Harris and Farrell, 1972) or through the action of the enzyme lysyl oxidase (Vater et *al*., 1979) resulted in very low yields of reaction products as compared with non-cross-linked reconstituted fibrils. These experimental observations have been used to support the hypothesis (Weiss, 1976) that a multienzyme system is required to solubilize and degrade a mature collagen fibril in the sense that the specific protease first cleaves in the telopeptide region at the NH_2 and COOH terminals, releasing adjacent molecules from the cross-link. This then loosens the structure, permitting access to the enzyme, which makes its specific attack in the helical region of the molecule within the fibril. The released fragments then are free to float away and lose their helical conformation spontaneously, as the two fragments are soluble and denature well below body temperature in solution (Sakai and Gross, 1967). The randomized polypeptide fragments can now be degraded by other relatively nonspecific endopeptidases and exopeptidases to amino acids.

This important point with regard to the need for a prior proteolytic attack is not yet firmly established; several other laboratories have observed that purified collagenases under physiologic conditions are capable of extensively degrading naturally insoluble fibrils obtained directly from several different mammalian tissues (Woolley et *al*., 1978; Sakamoto et *al*., 1973; Gross and Harper, unpublished). These enzymes are not demonstrably contaminated by peptidases capable of cleaving the telopeptides of the collagen molecule. The action of the animal collagenases on native insoluble fibrils may be different from that on experimentally cross-linked collagen. The explanations for these observational differences remain to be resolved.

It should be noted that most of the work to date deals with the mode of action of the animal collagenases and that still relatively little is known about the enzyme molecule itself. In general molecular weights have been determined by gel filtration and SDS gel electrophoresis, the former, unreliable in many instances, and the latter, providing a measure of the denatured protein. Stricklin et *al*., (1977, 1978), however, using mass culture of human skin cells have accumulated relatively large amounts of purified enzyme in sufficient quantity to determine molecular weight by sedimentation equilibrium, and have obtained amino acid analysis and peptide maps. No doubt the amino acid sequence will soon be available and isolation of the collagenase gene is in progress in several laboratories at this writing.

We know collagenases are metalloproteins with a calcium requirement and probably carrying zinc as a prosthetic group (Berman and Dohlman, 1975). Some represent different antigens, others cross-react. The problem in characterization has been the relatively small chemical amounts obtained by tissue and cell culture and in extracts.

3. Substrate and Enzyme Specificity

3.1. Properties of the Cleavage-Site Region

Essentially all of the known vertebrate collagenases appear to cleave the native collagen molecule at the same locus and indeed in all well-documented cases at the same peptide bond, i.e, Gly-Ile in the $\alpha 1$ chain or type I collagen and Gly-Leu in the $\alpha 2$ chain (positions 775–776 in the amino acid sequence). Where there has been a substitution in the $\alpha 2$ chain of isoleucine for leucine at position 776 in chick skin, the three COOH-terminal residues at the cleavage site are Ile (Dixit et *al*., 1979). The evidence for the single cleavage and its particular locus in the molecule was first obtained by electron microscopic examination of SLS crystallites produced by precipitation of the reaction products with ATP, and is supported by evidence for very limited cleavage as detected by gel electrophoresis (Gross and Nagai, 1965).

The amino acid sequences of the region around the cleavage site in both the $\alpha 1$ and the $\alpha 2$ chains are presented in Fig. 8-6. The characteristic repeating collagen triplet sequences are intact throughout the region and there are no obvious anomalies. Arbitrarily selecting a sequence of 21 residues with the cleavage site in the middle, there is no identical sequence anywhere else in the polypeptide chain; however, there are extremely few repeating sequences of more than 6 residues anywhere in the collagen molecule. It should be noted that there are 3 Gly-Ile and 18 Gly-Leu bonds in the $\alpha 1$ chain; those particular dipeptides are necessary but not sufficient for cleavage.

There is good evidence that conformation plays an important role, for gelatin (i.e, denatured α chains) is a poor substrate as compared with the native helical molecule (McCroskery et *al*., 1973). It may be that the slow rate of cleavage of nonhelical α chains and the isolated CNBr fragment, α1-CB7, reflects a limited ability of these peptides to renature, transiently perhaps, thus allowing an equally limited rate of enzyme activity. We suspect that helical

1. **Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Gln-Gly- Leu-Leu-Gly-Ala-Hyp-Gly-Phe-Leu-Gly-Leu-Hyp**
2. **Gly-Pro-Hyp-Gly-Thr-Hyp-Gly-Pro-Gln-Gly- Ile-Leu-Gly-Ala-Hyp-Gly-Ile-Leu-Gly-Leu-Hyp**
3. **Gly-Ala-Hyp-Gly-Thr-Pro-Gly-Pro-Gln-Gly- Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Hyp**
4. **Ile-Ala-Gly-Gln-Arg-Gly-Leu-Val-Gly-Leu-Hyp**
5. **Leu-Ala-Gly-Leu-Arg-Gly-Ala-Arg-Gly-Leu-Ala**

Figure 8-6. Sequences of 21 residues of the $\alpha 1$ and $\alpha 2$ chains around the collagenase cleavage site. Only the 10 residues on the COOH-terminal side of $\alpha 1$(II) and $\alpha 1$(III) are available at this time. 1. Calf skin, $\alpha 2$ (I); 2. chick skin $\alpha 2$ (I); 3. chick skin, $\alpha 1$ (I); 4. human cartilage, $\alpha 1$ (II); 5. human leiomyoma, $\alpha 1$ (III). (We wish to thank Drs. Glanville and Fietzek for making available to us the as yet unpublished sequence in this region of the $\alpha 2$ chain of calf skin.) (From Gross et *al*., 1980.)

conformation adjacent to the cleavage-site region is needed for binding of the enzyme to the substrate although this information is not yet available.

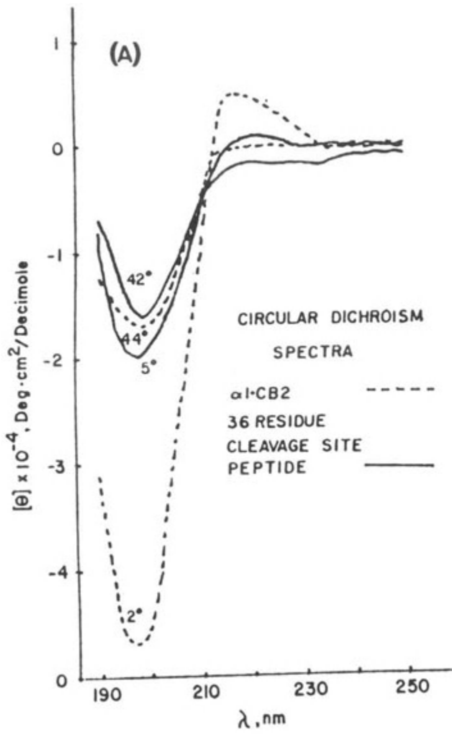
Of some interest is the ability of purified collagenases to cleave synthetic peptide substrates of no more than eight residues including the cleavage site (Masui et *al.*, 1977). The rate of cleavage, however, is much less than that of helical collagen. Our studies on 21-residue synthetic peptides duplicating the $\alpha 1$(I) cleavage site, with 10 residues on each side of Gly–Ile, indicate an extremely low rate of cleavage.

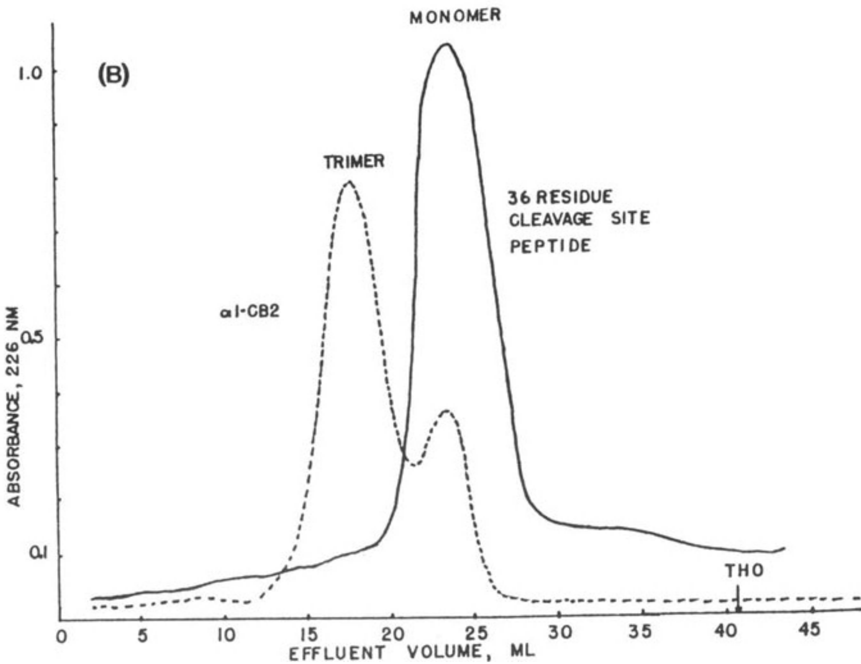
We have used these larger synthetic peptides and also a native 36-residue fragment (including the cleavage site near the center) isolated from $\alpha 1$-CB7 to examine the conformational characteristics of this region, by exploring the capacity for renaturation (Highberger et *al.*, 1979). This should be some index of the helical stability of the region. A similar-sized peptide from the NH_2-terminal end of the $\alpha 1$ chain, $\alpha 1$-CB2, will renature readily to the triple-helical conformation on warming to room temperature at acid pH (Piez and Sherman, 1970). As shown in Fig. 8-7, the 36-residue cleavage-site peptide is incapable of renaturing to the helical state as compared with $\alpha 1$-CB2. The cleavage-site peptide is unusually low in the number of imino acids, proline and hydroxyproline, which act as a driving force for helix formation as in the case of $\alpha 1$-CB2 and two other regions of the $\alpha 1$ chain containing non-cleavable Gly-Ile bonds (Fig. 8-8).

As a tentative model, we propose that the cleavage-site region, at least 36 residues in length, is resistant to triple-helix formation because of the low imino acid content. Collagenase probably cannot effectively cleave this peptide because of the absence of the adjacent helical binding site that exists in the native molecule on one side or other of this unstable region. We suggest that the enzyme actually has a requirement for the sensitive bond to be located in a nonhelical environment, and thus none of the other potentially susceptible Gly-Ile bonds are available. Possibly, this region is forced by the adjoining helical domains into an oscillating random coil–helix configuration, the duration and extent of either state being determined by environmental conditions. The rate and extent of fluctuation between an enzyme-susceptible (nonhelical) and a non-enzyme-susceptible (helical) configuration of this local region could be an additional determining factor of degradation rate.

Because we are proposing that a region of helix adjacent to a helically unstable region containing the necessary susceptible bond may specify the locus of collagen cleavage, we examined several related parameters along the length of the amino acid sequence of the $\alpha 1$ chain (Gross et *al.*, 1980). These

Figure 8-7. Comparison of triple-helix-forming capacity of the 36-residue cleavage-site peptide ⟶ from chick skin $\alpha 1$-CB7 and that of $\alpha 1$-CB2, the sequences of which are shown in Figure 8-8. (A) circular dichroism; (B) gel filtration (chromatography on Biogel P-10 at 5°C; 0.15 M Na acetate, pH 4.8). (From Gross et *al.*, 1980.)





CHROMATOGRAPHY ON BIOGEL P-10 AT 5°
0.15 M NA ACETATE PH 4.8

[1]Cleavage site sequence (Chick skin α1(I))
(Residues 758–793)

Ser-Hyp-Gly-Ala-Asp-Gly-Pro-Ile-Gly-Ala-Hyp-Gly-Thr-Pro-Gly-Pro-Gln
Gly↓Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Hyp-Gly-Gln-Arg-Gly-Glu
Arg-Gly

[2]Sequences around other Gly-Ile bonds in Chick skin α1(I)
(residues 209–244)

Asn-Hyp-Gly-Ala-Asp-Gly-Gln-Hyp-Gly-Ala-Lys-Gly-Ala-Thr-Gly-Ala-Hyp
Gly÷Ile-Ala-Gly-Ala-Hyp-Gly-Phe-Hyp-Gly-Ala-Arg-Gly-Pro-Ser-Gly-Pro
Gln-Gly

(residues 305–340)

Pro-Ala-Gly-Glu-Arg-Gly-Ala-Hyp-Gly-Ser-Arg-Gly-Phe-Hyp-Gly-Ala-Asp
Gly÷Ile-Ala-Gly-Pro-Lys-Gly-Pro-Hyp-Gly-Glu-Arg-Gly-Ser-Hyp-Gly-Ala
Val-Gly

[3]Sequence of α1-CB2 (chick α1(I))

(residues 4–39)

Gly-Pro-Ala-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Pro-Hyp-Gly-Ala-Hyp-Gly-Pro
Gln-Gly-Phe-Gln-Gly-Pro-Hyp-Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Ala-Ser-Gly
Pro-Met

Figure 8-8. Comparison of the sequences of a 36-residue peptide of chick skin α1-CB7 (top) containing a collagenase-susceptible Gly-Ile bond in the middle vs. two similar-sized peptides containing noncleavable Gly-Ile bonds elsewhere in the α1(I) chain. These are all compared with the 36-residue peptide α1-CB2 (bottom), which is readily renatured into the triple helix and has no collagenase-susceptible bond. [1]From Highberger et *al.* (1975, 1977); [2]from Highberger et *al.* (unpublished); [3]from Kang and Gross (1970) (cited in and figure taken from Gross et *al.*, 1980).

include the distribution of Gly-X-Y triplets in terms of their relative contribution to helical stability as determined by an analysis of synthetic bloc peptides (data summarized by Traub and Piez, 1971) and the relative distribution of the residue volumes in segments of 10 triplets along the helical region of the α1 chain. Fig. 8-9 graphically summarizes the data.

The cleavage site appears to be located in a region of low helical stability, and although there are other loci of even lower stability these do not carry the sensitive Gly-Ile bond. Another unusual aspect of the structure is that immediately adjacent to the NH_2-terminal side of the low-stability segment (containing the cleavage site), we find the longest segment of high stability along the entire α chain, with the exception of the extreme COOH-terminal region. This could be the enzyme-binding site. By charting the relative bulkiness (weighted molal volume) of groups of residues, we observed yet another unusual feature of the environment of the cleavage site as compared with the rest of the chain. Just adjacent to the sensitive bond on one side is a region of the lowest residue volume for the entire molecule and on the other side is located a region of the highest residue volume. This very steep gradient may also contribute to helical instability or may relate to steric accessibility of the enzyme to the sensitive bond in the middle. Thus, it is likely that the particular specific susceptibility

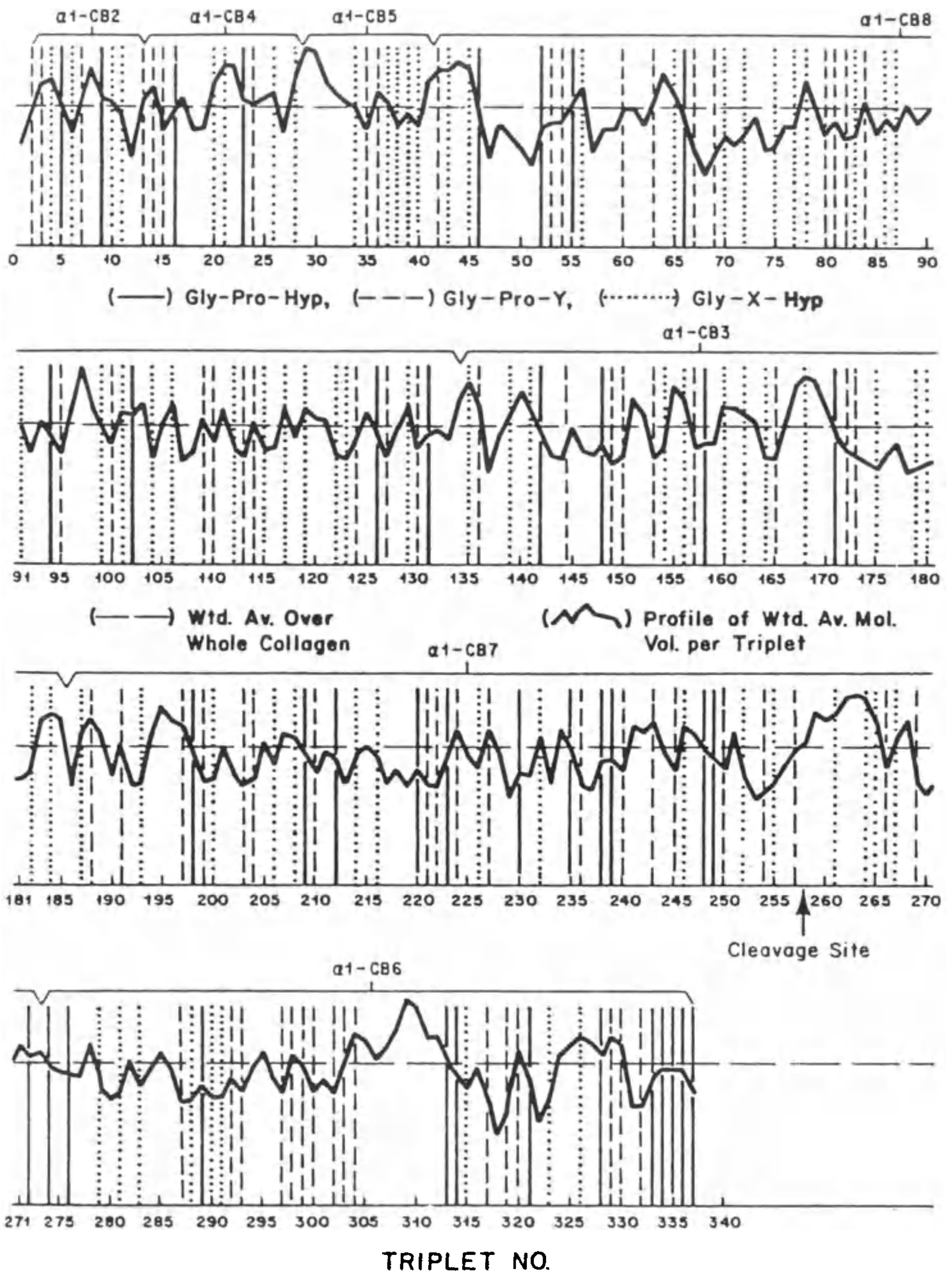


Figure 8-9. Schematic representation of the distribution of helix-stabilizing factors along the length of the α1 chain utilizing the relative helix-forming ability of successive Gly-X-Y triplets and the distribution of relative triplet volume compared to the mean along the chain. In order of decreasing helix-forming effectiveness: Gly-Pro-Hyp(-Pro) > Gly-Pro-Y > Gly-X-Hyp(Y). (From Gross et *al.*, 1980.)

of collagen to the animal collagenases is dependent upon a constellation of characteristics of the substrate molecule that confer upon a single locus a high degree of susceptibility.

Of interest here is the previously mentioned fact that this region is suceptible to attack by at least two serine proteases, namely trypsin and thermolysin. Trypsin at relatively high concentrations is capable of removing about one quarter of the molecule from the NH_2-terminal end (the TC^B region of type I collagen as indicated by SLS crystallites; Olsen, 1964; Davison and Schmitt, 1968). Curiously, trypsin also cleaves type III collagen at the Arg-Gly bond, eight peptide bonds away toward the COOH-terminal end of the molecule from the Gly-Leu, collagenase-specific site (Miller et *al.*, 1976), but not at another Arg-Gly bond several residues away. Most if not all endoproteases are capable of rapidly hydrolyzing denatured collagen (gelatin) at numerous sites characteristic of the particular enzymes' specificities.

3.2. Substrate Accessibility

Regulation by blocking and unblocking access to the collagenase cleavage site by any of a number of connective tissue constituents is a distinct possibility. The potential of proteoglycans serving such a function has been examined in a model system by Woolley and Evanson (1977) wherein mixtures of collagen and cartilage proteoglycan were exposed to active collagenase; no obvious protection in this model system was found. However, the use of normal cartilage and the same tissue divested enzymatically of its proteoglycan through the action of hyaluronidases (rather than proteases, which might affect the collagen structure) would be a more direct way of checking the question. Thus, this possibility is not yet ruled out.

Because fibronectin is known to bind to the region of the collagen cleavage site in gelatin or isolated peptides, it was plausible that fibronectin might block access to the enzyme. It should be noted that there is relatively little evidence to suggest that fibronectin will bind to native collagen molecules or fibrils, and experiments by Engvall and Ruoslahti (1977) using collagen and gelatin affinity columns indeed indicate very low binding by the native protein, as compared with more than 100-fold greater binding by gelatin. Even that low level exhibited by native collagen could represent a small fraction of denatured material produced while preparing the Sepharose–collagen complex. However, if there is some fluctuation between helical and nonhelical states in the collagenase cleavage-site region in the native molecule at body temperature, there is a possibility of low-level affinity of fibronectin in this region.

Although fibronectin isolated from fibroblast cultures or from serum by gelatin affinity chromatography proved strongly inhibitory to the action of collagenase on native collagen (Biswas, Gross, and Hynes, unpublished), it was inactive after additional purification by gel filtration. A low-molecular-weight contaminant seemed to be the inhibitory factor. If fibronectin does indeed bind

to native collagen at the cleavage site, it is surprising that it would not block the action of collagenase.

3.3. Enzyme and Substrate Heterogeneity

Both collagens and collagenases are families of molecular types, the members of which have the same fundamental characteristics but differ in their specific attributes. There are at least five different types of collagen, each a different gene product (see Chapter 1), characterized by small but significant variations in amino acid composition and sequence. These collagens vary in their distribution in different tissues, have different fibrillar widths, and undergo different degrees of posttranslational modification such as hydroxylation, glycosylation, and cross-linking. We know that there are significant differences in the susceptibility of the different substrates to collagenolytic attack (see reviews by Gross, 1976; Gross et *al.*, 1980). For example, type II collagen is much more resistant to fibroblast collagenase than is type I (Woolley et *al.*, 1975a), and type III is more resistant to human leukocyte collagenase than is type I (Horwitz et *al.*, 1977). Of interest is the selective cleavage of type III collagen by human leukocyte elastase, which does not attack type I collagen (Gadek et *al.*, 1980). The susceptible bond is just a few residues away from the collagenase cleavage site (Mainardi et *al.*, 1980).

Basement membrane type IV collagen seems to be specifically susceptible to an enzyme produced by certain malignant tumors and resistant to the enzyme produced by the normal connective tissue cells (Liotta et *al.*, 1979, 1981a). Recently, Mainardi et *al.* (1980) and Uitto et *al.* (1980) have isolated a type IV-specific collagenolytic serine protease from human polymorphonuclear leukocytes. The more recently characterized type V collagen, found in a variety of tissues in relatively small amounts and perhaps more specifically in certain basement membranes, is cleaved by an enzyme isolated from rabbit pulmonary alveolar macrophages (Mainardi et *al.*, 1980), and another apparently type V-specific degrading enzyme has been isolated from the culture medium of several different tumors (Liotta et *al.*, 1981b). None of these type IV- and V-cleaving enzymes appear to be active against type I and III collagens.

4. Regulation of Collagenase

4.1. Stimulation and Inhibition of Active Enzyme

Potential mechanisms for regulating the function of collagenase itself may be found at different levels of complexity. The simplest is direct stimulation or inhibition of the fully formed and functional enzyme molecule, as for example the formation of enzyme–inhibitor complexes. As yet we have no thoroughly documented examples of enhancement of enzyme activity by complexing with substances, although it has been suggested that heparin might function in this

manner (Sakamoto *et al.*, 1975, 1978). A stimulatory substance, termed "enhancer," for skin collagenase has been reported to be present in serum (Seltzer *et al.*, 1978). However, its function depends on coincident binding of enzyme and enhancer to collagen substrate.

As shown in Table I a broad range of inhibitors of collagenase may be extracted from a variety of tissues, from cells or tissue explants in culture medium, or may be obtained from blood. These all seem to be of polypeptide nature although there may exist non protein inhibitors as yet undetected. Collagenase inhibitors range greatly in molecular weight and specificity. It would appear that the very large $\alpha 2$ macroglobulin of the serum is a general scavenger for proteases which apparently functions by "grasping" the enzyme in such a way as to block access of macromolecular substrates (but not small peptides) to the active site. β_1 anti-collagenase of sera found in some animals, appears to be specific for animal collagenases. The degree to which these tissue inhibitors are specific for different tissue collagenases is not yet apparent.

We do not yet know whether the same cell secretes both the enzyme and its inhibitor together or sequentially, or whether each is produced separately by different subpopulations of cells (Section 1.1).

4.2. Activation of Latent Enzyme

On a somewhat more complex level of control, the conversion from inactive to active form of the enzyme may play an important role. The hypothesized (but likely) conversion of zymogen to active collagenase would require, in all likelihood, some selective function whereby a portion of the precursor molecule is either removed or altered through the cleavage of one or more peptide bonds (Harper *et al.*, 1971; Harper and Gross, 1972; Stricklin *et al.*, 1977, 1978). This process might occur either inside, at the surface, or outside the cell. In a similar manner, proteolytic activity might also be involved in reactivation of an enzyme–inhibitor complex, perhaps at the site of enzyme function, by a specific proteolytic event. There is little doubt but that such mechanisms would be under close cellular control. At the moment there is no understanding as to how these processes occur *in vivo*, or *in vitro* for that matter. As mentioned earlier, the addition to cell culture of plasmin generated from serum plasminogen by fibroblast-secreted plasminogen activator will activate latent collagenase (Werb *et al.*, 1977). Mast cells can release proteases *in vitro* that also activate latent collagenase (Birkedal-Hansen *et al.*, 1976). These may be useful models for *in vivo* activation.

4.3. Regulation of Collagenase-Producing Cells in Culture by Added Substances

Naked cells with collagenase-producing potential in culture can be "turned on" to manufacture and secrete collagenase by a wide range of diverse

substances, including collagen (Biswas and Dayer, 1979), proteases (Werb and Aggeler, 1978), latex beads (Werb and Reynolds, 1974), cytochalasin B (Harris *et al.*, 1975), endotoxin (Wahl *et al.*, 1974), phorbol esters (Brinckerhoff *et al.*, 1979), prostaglandin E_2 (Wahl *et al.*, 1977), and lymphokines (Dayer *et al.*, 1980). No doubt the list will lengthen.

Similarly, a large range of agents can "turn off" cultured cells already producing collagenase, including glucocorticoids (Werb *et al.*, 1978), progestins (Jeffrey *et al.*, 1975; Newsome and Gross, 1977), inhibitors of protein synthesis (Eisen and Gross, 1965), retinoids (Brinckerhoff *et al.*, 1980), and cyclic AMP (Koob and Jeffrey, 1974).

The key question is whether any of these agents, or physiologic analogues, act similarly *in vivo*. Probably hormones and others of the above-mentioned substances known to affect cell function *in vivo* do function in an analogous but perhaps not identical manner to their action in cell culture. Even the "non-physiologic" agents are likely to have physiologic analogues—witness the morphine-endorphin paradigm.

4.4. Regulation by Cellular Interactions

Specific interactions between different cell types probably represent one of the more important morphogenetic control systems in development and remodeling. Such interactions are known to regulate the synthesis of intracellular enzymes of the metabolic pathways (Morris and Moscona, 1977) as well as the formation and deposition of extracellular matrix macromolecules (Merrilees and Scott, 1980). We now know that synthesis/secretion of collagenase is also controlled by such interactions between different cell types. Grillo and Gross (1967) in a study of collagenolytic activity, using a semiquantitative analytical procedure, observed that in an excision wound in guinea pig skin, the intact wound edge containing both epithelium and mesenchyme displayed a relatively high level of activity, whereas the separated epithelium and stroma were only weakly active. Recombination of these two tissue compartments reinstated the capacity to produce enzyme at the level of the intact tissue. Parenthetically, it might be noted that in recent years, the production of collagenases by epithelium from any tissue has not been confirmed. Whether wound epithelium and tadpole skin epithelium are special cases requires further study.

Wahl *et al.* (1975) reported that lectin-stimulated mammalian cells release substances that increase collagenase production by macrophages. Newsome and Gross (unpublished) observed that epithelium grown from explants of alkali-burned rabbit cornea conditioned culture medium with the capability of stimulating "turned-off" corneal stromal cells to produce relatively large amounts of this enzyme. The epithelial cultures may have included macrophages, a possibility that was not ruled out at the time. These investigators (Newsome and Gross, 1979) reported the ability of mononuclear cells of blood from rabbits with burned corneas to condition culture medium so that it, too, could stimulate collagenase production by stromal cells from either normal or

alkali-burned cornea. Apparently, the injury itself was sufficient to "activate" both the stimulating cell type and the responder. In these experiments, either one or the other had to come from the injured animal. Maximum response was obtained if both derived from that source.

In studies directed at an understanding of regulation of collagenase in inflammatory processes in rheumatoid synovium, Dayer et *al*. (1977, 1980) described the release of large amounts of enzyme to the culture medium upon stimulation by a factor isolated chromatographically from medium conditioned by a mixture of lymphocytes and monocytes from the blood. This substance, called mononuclear cell factor, has an apparent molecular weight of about 14,000 and could be characterized as a lymphokine. Although prostaglandin E_2 influences the regulation of collagenase production by macrophages (Wahl et *al*., 1977) and human rheumatoid synovial cells (Dayer et *al*., 1980), this relationship remains complex. Of considerable interest are the observations of Dayer et *al*. (1980) that an interaction between T lymphocytes and a mononuclear component (macrophage) is needed for the production of mononuclear cell factor.

In a further effort to understand the conditions involved in regulating collagenase function in a normally stable connective tissue subject to remodeling consequent to injury, studies were initiated on interactions between epithelial and stromal cells isolated enzymatically from normal adult rabbit cornea (Johnson-Muller and Gross, 1978). Epithelial and stroma cells, examined separately or mixed, failed to produce any collagenolytic enzyme over long periods of culture in the presence or absence of serum. Based on the hypothesis that an additional stimulus was required, we tested the action of cytochalasin B (CB) on this system. Although primary cultures of normal adult rabbit epithelial and stromal cells from cornea fail to produce collagenase in the presence or absence of CB, the addition of this agent to a mixture of the two cell types resulted in enzyme production and release into medium. This effect is concentration dependent with regard to CB and stromal cell concentration. However, the response to changes in epithelial cell concentration is complex; at relatively low cell densities, there is active enzyme production showing linear dependence on cell density, but beyond a certain level of epithelial cell density, progressively less enzyme is produced (Fig. 8-10).

Epithelial cells alone, at low density in the presence of CB, condition the medium with stimulatory properties for corneal stromal cells; molecular sieve chromatography of such stimulatory media isolated two reproducible peaks of activity with apparent molecular weights of 19,000 and 54,000 (Fig. 8-11A). The same experiment repeated at higher epithelial cell densities (in the presence or absence of CB) endowed the medium with inhibitor properties for the stromal cells with respect to producing collagenase; molecular sieve chromatography here revealed two sharp peaks of inhibitory activity at 19,000 and 7000 molecular weight (Fig. 8-11B). CB was not required for release of the inhibitor (Johnson-Wint, 1980).

We surmise that in a normal and morphogenetically inactive tissue, there is a relative insensitivity of stromal cells to stimulation. Similarly, the relatively

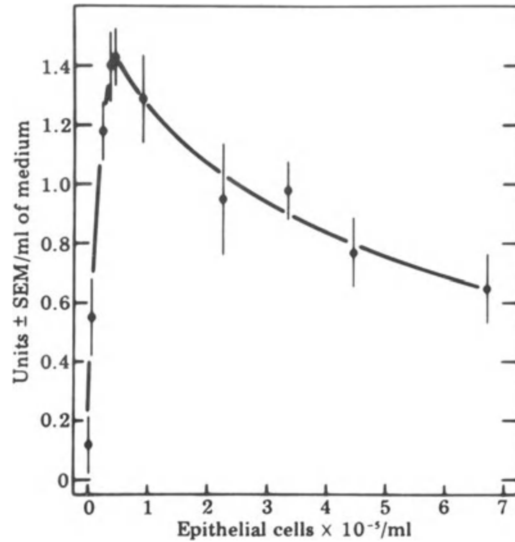


Figure 8-10. Effect of epithelial cell concentration on cumulative collagenase production by mixed epithelial–stromal cell cultures plus CB at 9 days. Stromal cells = 3×10^5 cells/ml; CB = 5 μg/ml; n = 5 replicate cultures for each datum point. (From Johnson-Wint, 1980.)

inactive epithelial cell population, under stable conditions, is unlikely to be elaborating stimulatory agents, and in fact may be releasing low levels of inhibitor, thus maintaining the stromal cells in a suppressed or insensitive state. With injury, however, as in the case of the alkali-burned cornea, where both epithelial and stromal cell types are proliferating and active in remodeling a damaged tissue, an additional stimulus (analogous to the effect of CB used in the above experiments as an artificial stimulator) induces the epithelial cells to manufacture and release stimulating materials. The repair fibroblasts of the stroma are sensitive to these substances and respond by producing and releasing collagenase. We suggest that the epithelial cells may always be producing suppressor substances, but these are overwhelmed by stimulators newly released by the repair epithelium. This balance may regulate the function of the collagenase-producing stromal cells.

Although we are able to obtain substances produced by cells that appear to have specific regulating effect on other cells *in vitro* (the turning on and off of a specific enzyme in this case), it seems unlikely that this isolated, stripped-down, and contrived biological system, cell culture, is doing anything more than telling us what the cells can do—not what they actually do *in vivo*. It seems unlikely that these stimulatory and inhibitory substances, which probably are produced in low concentrations in a functioning tissue, diffuse in a random manner for any appreciable distances from their source. Intuitively, it seems more likely that transfer by direct contact across short distances from cell to cell is the mode of transfer. If appreciable distances are involved in certain cases, perhaps polarized organization of structural elements of the extracellular matrix constrains the interchange of chemical information and orients and restricts pathways that maintain concentration and direction of flow.

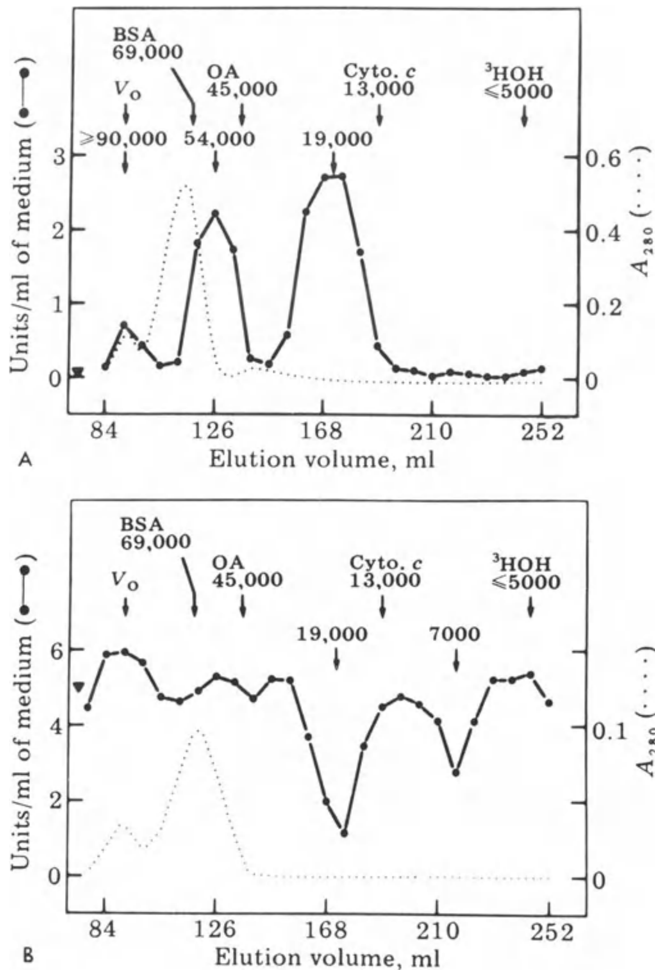


Figure 8-11. Ultrogel AcA 54 gel filtration chromatography of (A) low-density epithelial cell culture medium and (B) high-density epithelial cell culture medium. In (A), the stimulatory effect of elution fractions on cumulative collagenase production by stromal cells plus CB at 6 days is shown. In (B), the inhibitory effect of elution fractions on cumulative collagenase production by mixed corneal cell culture plus CB at 9 days is shown. The chromatographed medium was conditioned by 0.6×10^5 (A) or 6×10^5 (B) epithelial cells/ml in the presence of CB. Stromal cells = 3×10^5 cells/ml (A); for assaying inhibitory activity in (B), collagenase producing mixed epithelial and stromal cell cultures were used, stromal cells = 3×10^5/ml, epithelial cells = 0.6×10^5/ml; CB = 5 μg/ml; n = 3 replicate cultures for each datum point. Control stromal cell (A) or epithelial–stromal cell (B) cultures in complete medium plus CB. (●–●) Collagenase activity of stromal cells (A) or mixed cells (B) in response to 1 ml of eluate; (·····) $A280$ of fractions; V_0, void volume; BSA, bovine serum albumin; OA, ovalbumin. (From Johnson-Wint, 1980.)

In the case of epithelial–mesenchymal cell communication, an intact basement membrane may be a significant barrier established to prevent communication during periods of morphologic stability. When the need for remodeling arises, the basement membrane may be pierced or disintegrated, probably by enzymatic mechanisms, which allow closer approximation of cell types, or at least a freer passage of chemical information. There are a number of such examples: limb regeneration in the salamander (Salpeter and Singer, 1960), the involuting mouse mammary gland (Martinez-Hernandez *et al.*, 1976), and the developing tooth germ (Slavkin and Bringas, 1976). The specific collagenases selective for basement membrane collagen type IV produced by some tumor cells (Liotta *et al.*, 1979, 1980), mast cells (Sage *et al.*, 1979), and polymorphonuclear leukocytes (Mainardi *et al.*, 1980) may also be manufactured by epithelial and endothelial cells when required.

Interactions between different cell types may be important in tumor invasion if collagenase is shown to play a significant role in this process. Involvement of a type I-selective collagenase is indicated by morphologic evidence of collagen dissolution in the collagenous stroma surrounding a malignant neoplasm, associated with the production of the appropriate collagenase by tumor explants (Hashimoto *et al.*, 1972; Gross *et al.*, 1981).

The rabbit V2 carcinoma, which produces considerable type I-selective collagenase (McCroskery *et al.*, 1975; Biswas *et al.*, 1978), when introduced into the rabbit cornea grows rapidly in three dimensions (Gimbrone *et al.*, 1974). We (Gross *et al.*, 1981) used this system to examine the morphologic changes in the densely packed collagen lamellae of the cornea implanted with the V2 carcinoma. In the absence of steroid hormones, the tumor cells penetrated and destroyed the collagenous stroma (Fig. 8-12) and released collagenase in culture. Local application of medroxyprogesterone or dexamethasone in a sustained-release polymer blocked three-dimensional growth of the tumor and disruption of collagen layers. Neovascularization was totally inhibited and collagenase production greatly reduced (Fig. 8-13).

Because the blood supply and tumor cell proliferation were diminished, the reduction of collagenase could not directly be related to an action of the hormone on the tumor or host cells. Capillary invasion of the collagenous stroma may reflect "induction" of endothelial cell collagenase by a tumor secreted factor. However, the hormones blocked collagenase production by tumor explants in culture, and incidentally, blocked vessel proliferation induced by an angiogenic extract alone, indicating two separate activities. The experiments do not tell us whether the host or tumor cells make collagenase or whether an interaction between the two are required. Several groups have proposed a tumor cell–host cell interaction whereby the former stimulates the latter to produce the enzyme (Bauer *et al.*, 1977; Gross *et al.*, 1981). The use of tumor cell lines and host cells in culture and, in addition, *in vivo* studies utilizing antibodies specific for host and tumor collagenases should clarify the issue.

Liotta *et al.* (1980) have been able to show a relationship between the amount of collagenolytic activity released per cell and the metastatic potential

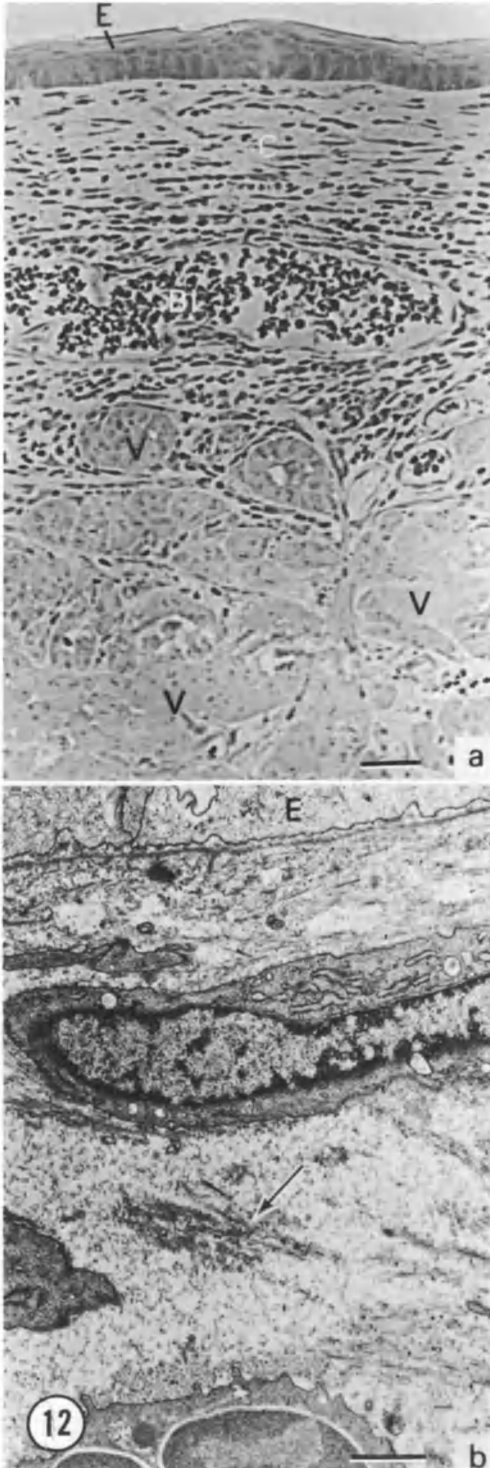


Figure 8-12. Twenty-two-day V2 tumor implanted in rabbit cornea. (a) Photomicrograph of section cut perpendicular to surface of the corneal epithelium (E) showing fibrous stroma infiltrated with V2 tumor cells (V), blood vessels (BL), and small unidentified cells (C). Phase-contrast optics, epoxy section. Bar = 40 μm. (b) Electron micrograph showing disruption and apparent fragmentation (arrow) of normal collagenous structure of stroma near corneal epithelium. Bar = 1 μm. (From Gross *et al.*, 1981.)

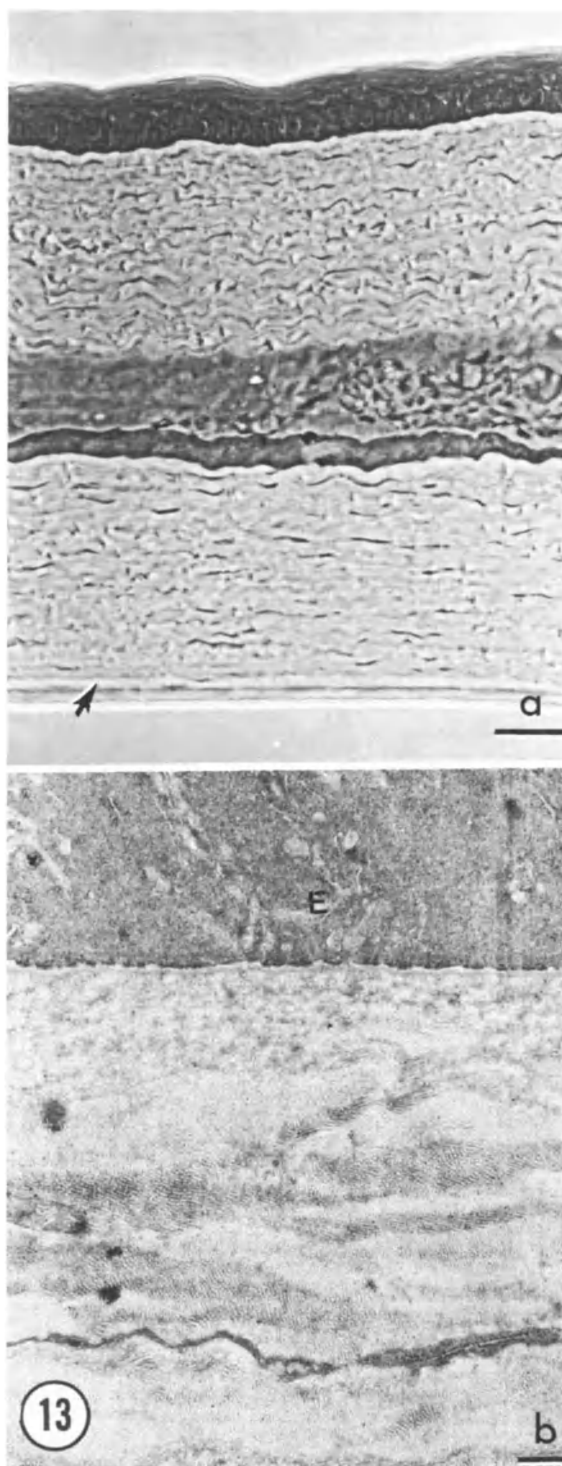


Figure 8-13. Seventeen-day V2 tumor implanted in rabbit cornea with medroxyprogesterone pellet. (a) Photomicrograph of section cut perpendicular to surface of corneal epithelium showing V2 tumor cells implanted in corneal pocket near polymer containing 450 μg medroxyprogesterone. Arrow, Descemet's membrane. Bar = 40 μm. (b) Electron micrograph showing ordered stroma near epithelium (E) in area similar to that shown in Figure 8-12. Section prepared as in Figure 8-12. Bar = 500 nm. (From Gross et al., 1981.)

of two different malignant mouse lines, the B-16 and the T-241 sarcoma, and also a culture of human breast carcinoma cells. They (Paranjpe et *al*., 1980) have reported that a human breast carcinoma cell line produced collagenase that was selective for type I collagen. At least some neoplastic cells with metastatic capability manufacture the type IV-specific collagenase that facilitates their passage through the capillary wall and may also have the capacity to degrade stromal collagen or induce the host fibroblasts, via secreted products, to secrete a type I-degrading collagenase.

5. Tissue Localization of Collagenase

Of considerable interest is the whereabouts of collagenase in tissues undergoing connective tissue resorption. Can the enzyme be found within all the local cells? Only associated with certain cell populations? At the cell surface? In the connective tissue stroma, associated with collagen fibers? At a distance from cells or only at regions of contact between the cells and the extracellular matrix? In situations where connective tissue is invaded by tumor cells or blood vessels, is collagenase found in association with the host cells or the invaders? Finally, with the availability of the different types of collagenases, i.e, those specific for types I, IV, and V collagen, can we demonstrate cooperative activity or completely separate functions and regulatory mechanisms?

Immunologic approaches should help to localize active and latent enzyme and establish spatial and temporal relationships with activating proteases. Anti-collagenase antibodies have been used in immunofluorescence studies in preliminary efforts to answer some of the above questions (see Table III for summary of immunohistochemical localization efforts to date). Early studies of the distribution of collagenase as detected by conventionally produced antibodies in rabbits or goats, using as antigens highly purified collagenases, localized the enzyme to connective tissue stromal elements, particularly the collagen fibers. There are several reports of immunofluorescence within fibroblasts, macrophages, endothelial cells, osteoblasts, osteoclasts, and cartilage cells of mouse bone. Woolley and colleagues, in carefully controlled immunofluorescence studies of the joint lesions in human rheumatoid arthritis (summarized in their 1980 review), using purified human synovial collagenase as antigen and isolated anti-collagenase IgG as monospecific antibody, have reported well-localized immunofluorescence at the very edge of the invading pannus and resorbing cartilage with which it is in close contact (Fig. 8-14).

In a study of a series of invasive gastric tumors examined immediately after surgery, the majority were negative for immunoreactive collagenase, both in the tumor and in the surrounding normal tissue. In three gastric adenocarcinomas, fluorescence labeling was found around individual cells in various regions of adjacent connective tissues. These cells were not identified. Similarly, with breast carcinomas, when immunoreactive collagenase could be detected by fluorescence, it appeared to be localized to the collagenous element around the tumor cells but not to the cells. Such interactions have been described for

Table III. Summary of Collagenase Immunolocalization Studies[a]

Authors[b]	Antibody	Tissue	Summary
Reddick *et al.* (1974)	Anti-human skin collagenase	Human skin and skin fibroblasts	Enzyme localized to collagen fibers of upper or papillary dermis and in cytoplasm of skin fibroblasts
Bauer *et al.* (1977)	Anti-human skin collagenase	Basal cell carcinoma	Enzyme demonstrated in stromal elements surrounding tumor cells. No staining of epithelial cells
Montfort and Perez-Tamayo (1975a,b, 1978)	Anti-rat uterus collagenase	Involuting rat uterus and normal rat tissues	Enzyme demonstrated on connective tissue structures of all organs examined
		Rat liver cirrhosis	Enzyme associated with connective tissue septums of cirrhotic liver. Cellular origin of enzyme not indicated
Abramson and Huang (1977)	Anti-human skin collagenase	Middle ear cholesteatoma	Enzyme appeared in granulation tissue and dermis of canal skin; also within fibroblasts, macrophages, and endothelial cells
Woolley *et al.* (1977)	Anti-human rheumatoid synovial collagenase	Rheumatoid joint tissue	Enzyme confined to cartilage–pannus junction; generally very little enzyme observed
Woolley *et al.* (1978, 1979)	Anti-human rheumatoid synovial collagenase	Adherent rheumatoid synovial cells	Enzyme produced by a relatively small population of "dendritic" cells *in vitro*
Sakamoto *et al.* (1978b)	Anti-mouse bone collagenase	Mouse bone and normal tissue	Enzyme associated with all connective tissues and within fibroblasts, endothelial cells, osteoblasts, osteocytes, osteoclasts, and cartilage cells

[a] Taken from Woolley *et al.* (1980).
[b] See Woolley *et al.* (1980) for references.

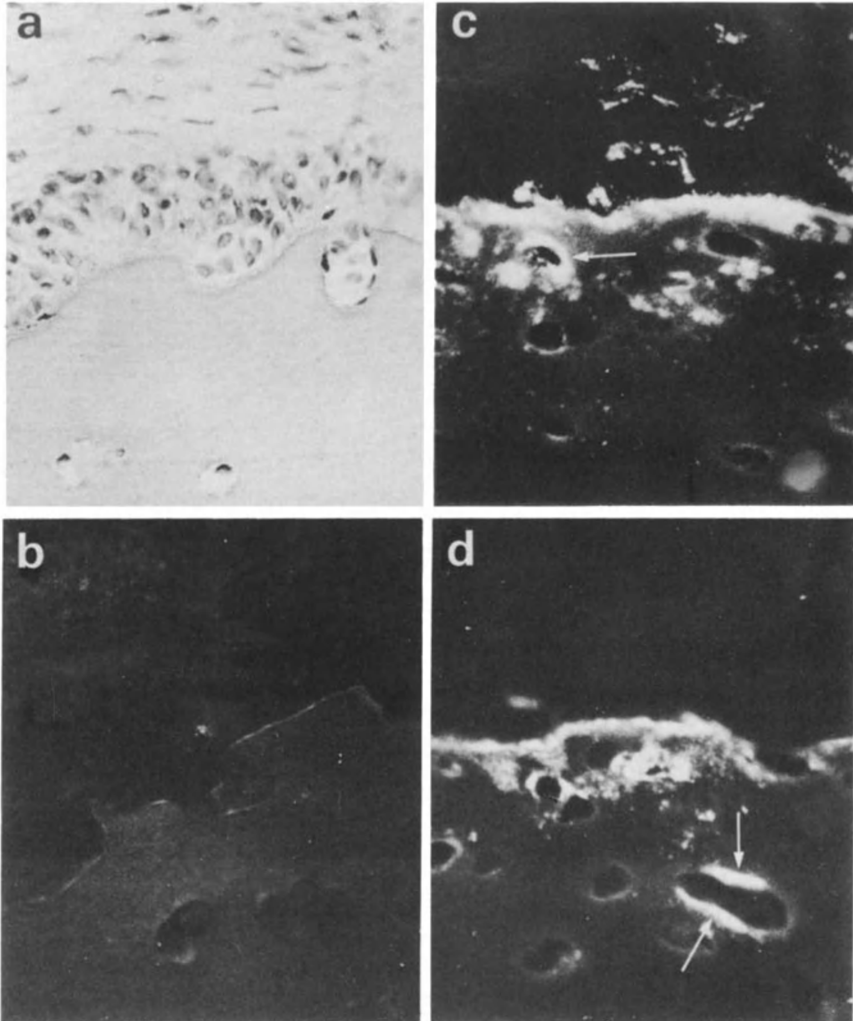


Figure 8-14. Immunolocalization of collagenase at the cartilage–pannus junction of a rheumatoid joint. (a) Frozen section of cartilage–pannus junction stained with hematoxylin and eosin. Note that the distribution and morphology of the pannus cells at the junction differ from those of the subjacent tissue. (b) Control section treated with the indirect FITC-antibody technique using nonimmune sheep IgG in place of the collagenase IgG antibody. (c) FITC fluorescence showing immunoreactive collagenase at the resorbing cartilage front. Enzyme is not observed in chondrocytes (arrow), lacunae, deeper regions of the cartilage matrix, or most of the pannus tissue. (d) FITC fluorescence localized to the cartilage matrix at the junction with pannus tissue and also to the cartilage walls of infiltrating pockets of synovial cells (arrowed). Note absence of demonstrable enzyme in chondrocytic lacunae, deeper cartilage matrix, and contiguous pannus tissue. ×100 (From Woolley *et al.*, 1980.)

nontumor collagenase-producing systems as described elsewhere in this chapter. However, Woolley et *al*. (1980) favor the idea that collagenase is produced by tumor cells transiently, depending on physiologic conditions, and not by all the cells in the area. In this connection the observations of Gordon and associates (1980) are of interest in that they detected no immunoreactive collagenase (by immunofluorescence) in normal human cornea *in situ*, whereas upon culturing it became abundant throughout the stroma. It was also found in ulcerated corneas *in situ*. The cultured tissue explant is evidently an injured tissue.

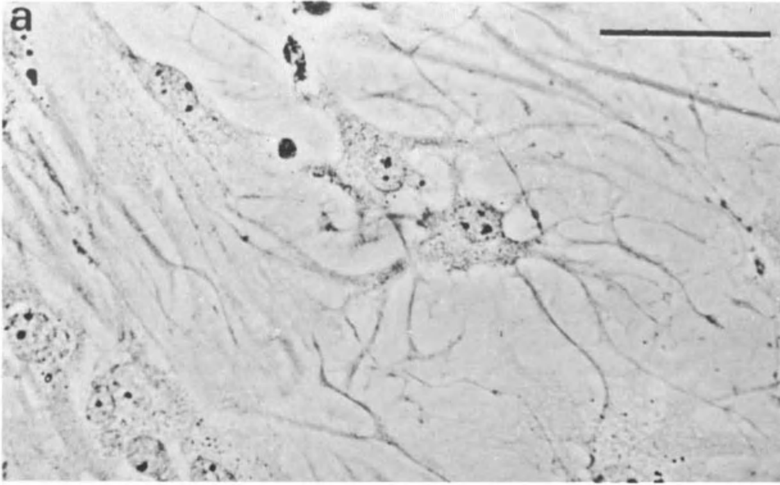
Woolley et *al*. (1980) have examined the adherent collagenase-producing dendritic cells from rheumatoid synovial tissue in culture, originally described by Dayer et *al*. (1976), using immunofluorescence. They found that only the dendritic type is positive for collagenase. Immunofluorescence was seen all over the cell, including the extended cytoplasmic processes, which stained in discrete, irregularly spaced nodular regions (Fig. 8-15).

Woolley et *al*. (1980) made the significant observation that the inactive form of collagenase does not bind to monospecific antibody although after tryptic or mercurial activation it is strongly reactive. Essentially all of the collagenase extractable from tissues is in the latent state, with some exceptions, whereas in tissue cultures after an initial lag period, active enzyme appears. It is under these conditions that diffuse labeling of tissues is seen as mentioned above. The monospecific antibody used by Bauer et *al*. (1977) is said to react equally with each of these proenzyme species, as well as with their respective active enzyme forms (Valle and Bauer, 1979). Quite possibly, in the experiment of Woolley and colleagues, exposure of the tissue to trypsin might have, through activation, greatly increased the amount of observable collagenase. The more general labeling of stromal and tumor tissue observed by others might be ascribed to the presence of latent collagenase in the immunogen, which would result in extension of specificity to include the inactive form of the enzyme, probably more frequently present and more widely distributed. This possibility could readily be checked by examining the cross-reactivity of antibodies to both the active and the inactive forms of the enzyme used as antigen.

6. Some Puzzles Remaining

The following is a partial list of questions worth asking and which now are or will be in the near future amenable to rigorous experimentation: How are the collagenases controlled extracellularly so that breakdown is selective and limited? Are the degradative enzymes operating at any significant distance from the cells of origin?

Are the degradative enzymes always released extracellularly or in some cases are the matrix constituents drawn into the cells by engulfment or some process of phagocytosis where intracellular digestive systems come into play? In the case of collagen, are both extracellular and intracellular processes in-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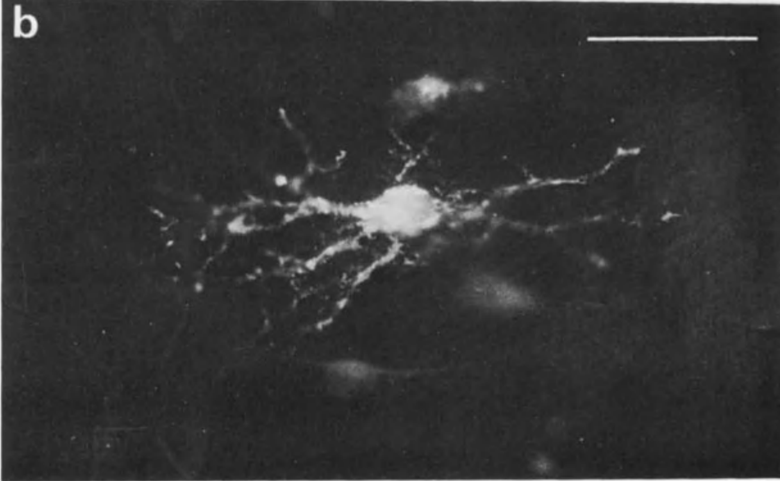
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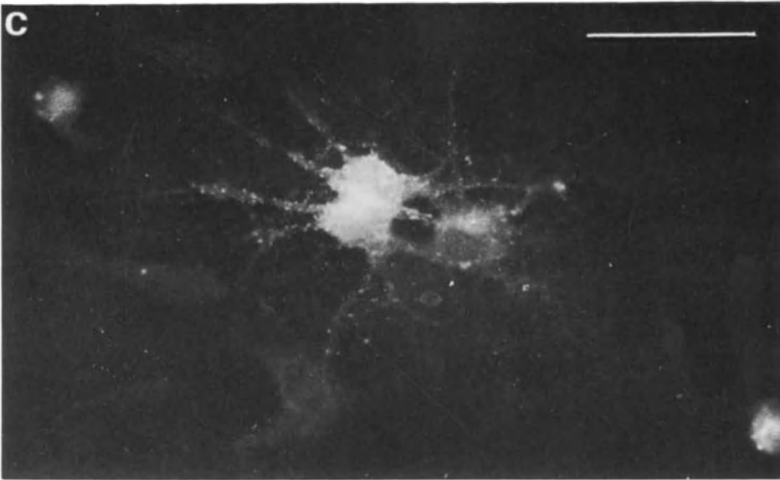
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volved? Why is a significant amount of newly synthesized collagen degraded intracellularly, and is it regulatory in morphogenesis?

Is the dismantling of supramolecular constituents, such as collagen fibrils, a stepwise process involving a series of specific enzymes operating in consort, and if so, how are these regulated? How are stimulators and inhibitors of enzyme production, activators of latent enzymes, and inhibitors of active enzyme synchronized in their distribution, concentration, and function so as to permit orderly dismantling?

How are biosynthetic, architectural, and degradative activities synchronized by the cells so that specific macroorganization emerges on the tissue and organ level, as in the development and growth of a bone?

To date we have barely the beginnings of answers to some of these questions. We have isolated and characterized some of the enzymes responsible for degrading or activating particular extracellular constituents. We are just learning to identify enzyme inhibitors and potentiators and to think about their function. Understanding the role and mechanisms of cell to cell interactions and the activity of hormones in the regulation of extracellular remodeling is on the horizon.

Because of the remarkable progress in molecular genetics with the use of recombinant DNA techniques and their application to the collagen genes (see Chapter 6), the characterization of one or more collagenase genes lies not too far ahead. Understanding the molecular regulation of these genes by cell to cell interactions, and other extracellular factors, can be seriously contemplated for the foreseeable future.

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← **Figure 8-15.** Collagenase immunolocalization studies of dissociated rheumatoid synovial cells adherent to collagen-coated coverslips. (a) Phase-contrast micrograph of a mixed population of adherent synovial cells showing a "dendritic" cell with long multibranched cytoplasmic extensions. (b, c) FITC fluorescence associated with the cytoplasmic extensions of a mononucleate and a binucleate "dendritic" cell, respectively. These cells were fixed after 4 days of culture in serum-free Dulbecco's modified Eagle's medium. Note the absence of immunoreactive enzyme in the surrounding cells. Bars = 25 μm. (From Woolley *et al.*, 1979, courtesy of the British Medical Association.)

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Chapter 9

Glycosaminoglycans in Morphogenesis

BRYAN P. TOOLE

1. Introduction

Formation of a precisely organized, functional tissue or organ is the culmination of a complex series of specific cellular events, usually termed morphogenesis. These events involve several common types of cell behavior, notably movement, proliferation, shape change, recognition, and adhesion. At each stage of morphogenesis of an organ or tissue, the macromolecules present in the extracellular matrix or associated with the external cell surface are important in providing structural support for and environmental signals to the cells involved. These contributions in turn exert a considerable influence on the course of morphogenesis.

Characteristic components of all extracellular matrices are the glycosaminoglycans (GAG). As explained in detail in Chapter 2, GAG are almost always present *in situ* as covalently bound side chains of proteins to form complexes termed proteoglycans. However "GAG" will usually be used as a general term in this chapter, because in most of the studies to be discussed, the existence and the nature of putative proteoglycans in each particular system have not been examined.

Three types of investigation form the basis of evolving ideas concerning the role of GAG in morphogenesis. These are: (1) descriptive studies correlating changes in GAG type and concentration with morphogenetic events *in vivo*; (2) experimental studies of the relationships of GAG to specific types of cell behavior *in vitro* and biochemical analysis of mechanisms controlling this relationship; and (3) experimental manipulations *in vivo*, based on the above, that probe directly the role of GAG in cellular events. This chapter begins by summarizing some of the transitions known to occur in the GAG composition of extracellular matrices during several morphogenetic sequences (Section 2). Then, Section 3 discusses interactions of GAG with cell surfaces, and Sections 4–8 the information currently available concerning the relationship of GAG to several specific types of cell behavior *in vitro* and *in vivo*.

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2. Transitions in Glycosaminoglycans during Morphogenesis and Differentiation

2.1. Hyaluronate Synthesis and Removal

Studies of hyaluronate metabolism at discrete stages of early development of tissues undergoing striking morphogenetic events have revealed close correlations between synthesis of hyaluronate and cell movement or proliferation, as well as between removal of hyaluronate and differentiation.

A particularly illustrative case is the chick embryo cornea, the morphogenesis of which is described in detail by Hay and Revel (1969) and Hay (1980). The initial corneal epithelium forms at about stage 18 (3 days) of chick embryo development as a result of formation and detachment of the lens from the ectoderm (Fig. 9-1). This epithelium is responsible for production of the primary stroma, the extracellular matrix that lies in the space between this epithelium and the lens (Hay and Dodson, 1973). In the chick cornea, this stroma is comprised of approximately 30 layers of orthogonally arranged collagen fibrils with attached chondroitin sulfate proteoglycan. In the primate, a primary stroma also develops, but is less organized (Ozanics et *al.*, 1977). Shortly after formation of the primary stroma in the chick embryo, at 4–5 days of development, mesenchymal cells from the neural crest migrate underneath the stroma to form a posterior epithelial layer, usually termed the corneal endothelium (Figs. 9-1 and 9-2). Junctions are established between these cells at stage 27 (5½ days), and then immediately the stroma swells and is invaded by another wave of neural-crest-derived mesenchymal cells (Johnston et *al.*, 1979). These cells, which will become the corneal keratocytes, continue to migrate and proliferate until they occupy all the stroma except for a narrow region beneath the epithelium, which becomes Bowman's membrane (Figs. 9-1 and 9-2). The mesenchymal cells acquire the prominent Golgi apparatus and rough endoplasmic reticulum of actively secreting cells and produce the components of the mature secondary stroma, which are also organized into a highly ordered orthogonal array. Between 10 and 14 days of avian corneal development, when cell migration and proliferation have virtually ceased, the stroma loses water and condenses due to the action of thyroxine, thus leading to the onset of transparency (Coulombre and Coulombre, 1964) (Fig. 9-2).

Subsequent to migration of the endothelium posterior to the primary stroma, hyaluronate is secreted by this cell layer into the stroma (Toole and Trelstad, 1971; Trelstad et *al.*, 1974). Hyaluronate is the major GAG component of the cornea until stage 35 (9 days). Subsequently, hyaluronidase activity increases in the cornea and hyaluronate levels decrease (Fig. 9-2). Thus, there is a close correlation between the presence of hyaluronate and the phase of corneal mesenchyme migration and proliferation within the hydrated primary stroma. The removal of hyaluronate by hyaluronidase corresponds to the period (Fig. 9-2) when the tissue loses water and condenses (Toole and Trelstad, 1971); at the same time, the mesenchymal cells differentiate to corneal keratocytes, which are then the major producers of extracellular macromolecules in the cornea. Keratocytes secrete large amounts of type I collagen (Trelstad et *al.*,

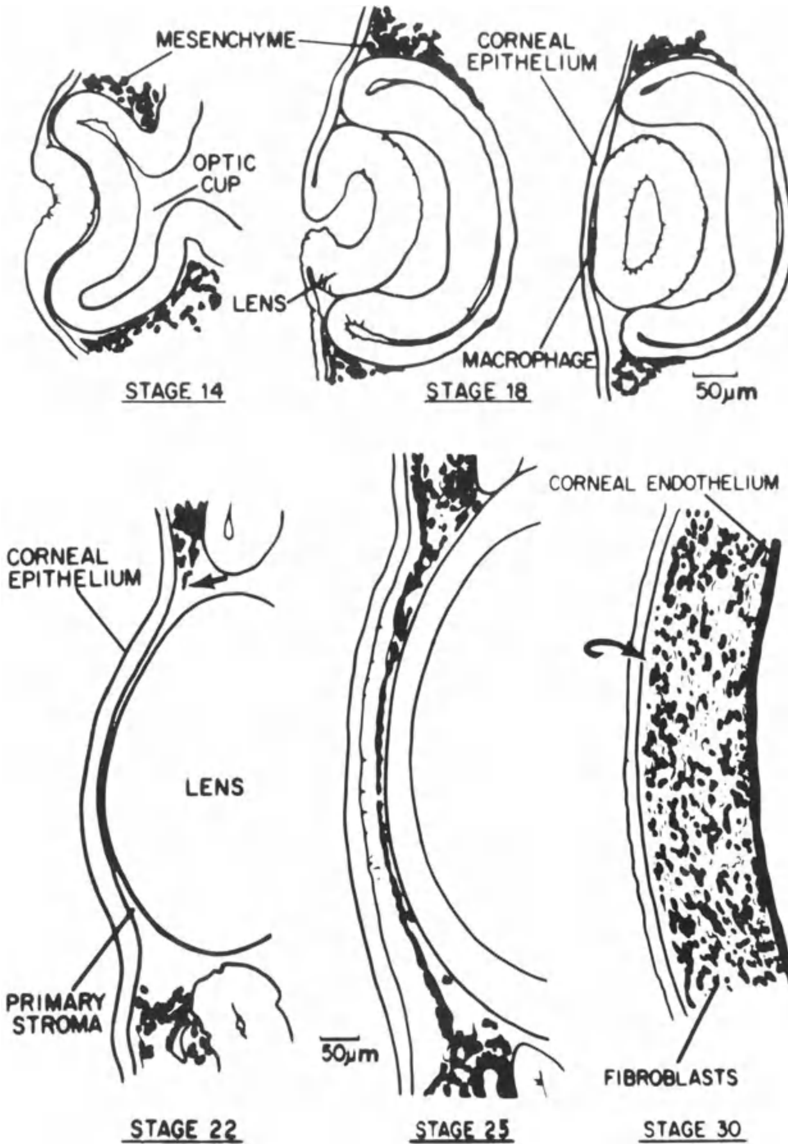
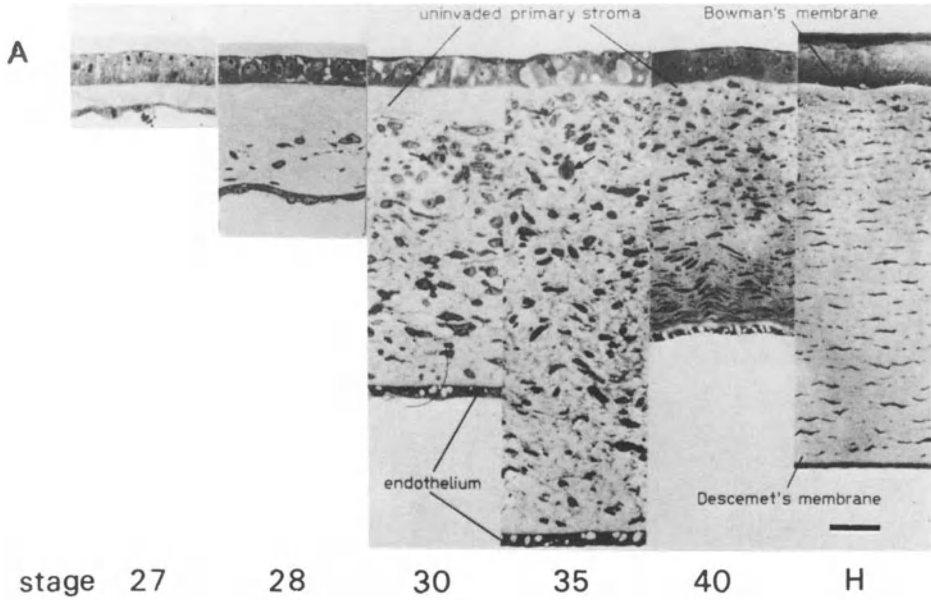


Figure 9-1. Camera lucida drawings showing the early stages in development of the avian cornea. At the time that the lens placode begins to invaginate (stage 14), the presumptive corneal epithelium lies over the lip of the optic cup. At 3 days of incubation, the lens vesicle pinches off and the overlying ectoderm becomes the corneal epithelium (stage 18). Macrophages clean up debris associated with lens vesicle formation. By stage 22 (4 days), the corneal epithelium has secreted the primary corneal stroma, and the mesenchymal cells destined to become the corneal endothelium have started to invade the area (straight arrow). Endothelial cell migration is almost complete at stage 25 (4½–5 days). During stage 27 (5–5½ days), junctions between the endothelial cells are established and the primary stroma swells (not shown). It is then immediately invaded by the corneal fibroblasts. By stage 30 (6½–7 days), the fibroblasts occupy all layers of the stroma except for a narrow juxtaepithelial zone (curved arrow). (Reproduced with permission of Karger from Hay and Revel, 1969.)



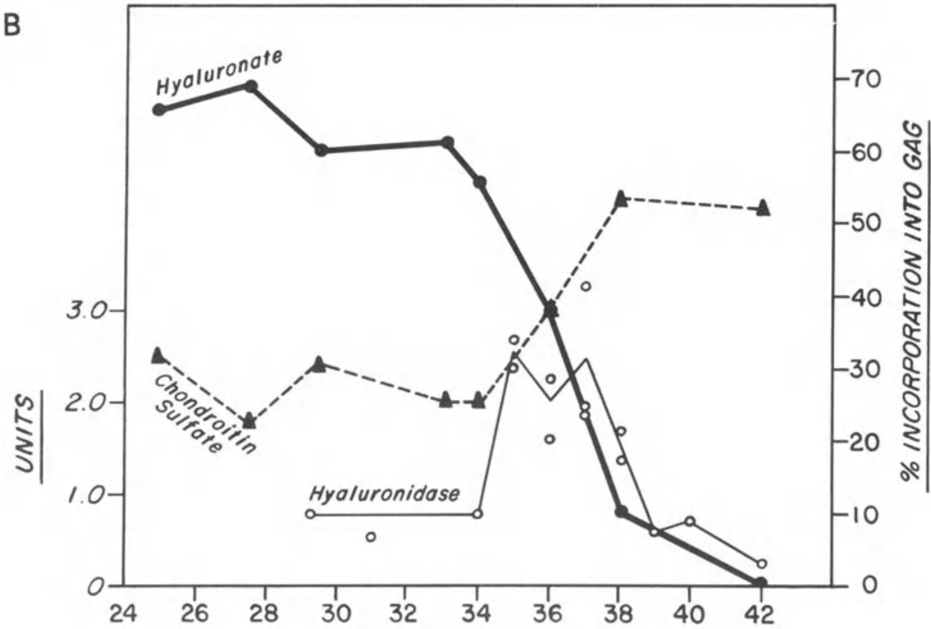


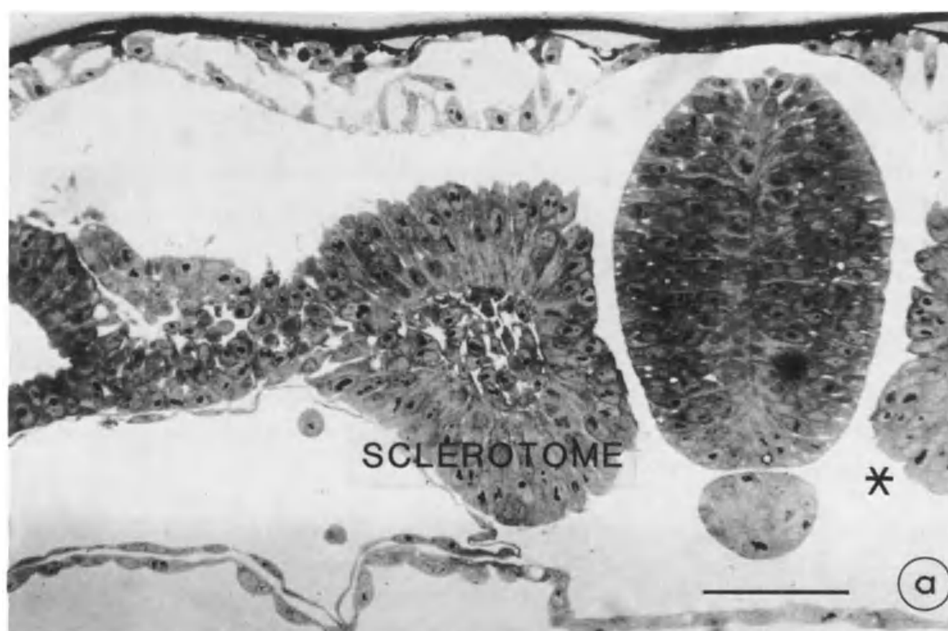
Figure 9-2. Comparisons of (A) morphology (Hay and Revel, 1969) and (B) GAG metabolism (Toole and Trelstad, 1971) in the embryonic chick cornea. Hyaluronate synthesis is predominant before and during stromal swelling and the migration of mesenchymal cells into the stroma (stages 27 through 35). Hyaluronidase activity appears at the end of the migratory stage, concomitant with stromal deswelling (stages 35 through 40). Bar = 50 μm. [Reproduced with permission of Karger (part A) and Academic Press (part B).]

1974; von der Mark *et al.*, 1977), chondroitin sulfate proteoglycan, and keratan sulfate proteoglycan (Hart, 1978; Hassell *et al.*, 1979), which rapidly become the major components of the secondary stroma that characterizes adult life.

A second example is the dissociation of sclerotomal cells from the chick embryo somite and the translocation of these cells to the notochord (Fig. 9-3). These cells surround the notochord and form the cartilagenous precursor of the vertebral bodies. Kvist and Finnegan (1970a,b) examined this phenomenon histochemically and chemically with respect to the types of GAG present and to their location. They found that hyaluronate was the major GAG in the matrix surrounding the migrating sclerotomal cells. At the site of cartilage formation, around the notochord, the amount of chondroitin sulfate, but not hyaluronate, increased dramatically. In addition, Toole (1972) found decreased synthesis of hyaluronate and increased hyaluronidase activity to occur precisely at the time that the cells began to produce cartilage matrix after concluding their migration around the notochord.

The ultrastructure of the hyaluronate-rich area between the notochord and the neural tube at the time of migration of the sclerotome can be preserved by ruthenium red (Fig. 9-4a) or by adding cetylpyridinium chloride to the fixative (Pratt *et al.*, 1975). The leading edge of a migrating mesenchymal cell at the time of dispersal of the sclerotome change is shown in Fig. 9-4a. This cell is entering the space indicated by the asterisk in Fig. 9-3a. This mesenchymal cell, which is moving to the left in Fig. 9-4a, is surrounded by GAG granules and filaments that are susceptible to testicular hyaluronidase digestion and thus, presumably, are composed of hyaluronate and/or chondroitin sulfate (Hay, 1978). The GAG-rich structures are closely associated with the cell surface, and it is possible that the mesenchymal cell plasmalemma contains receptors (Section 3) that temporarily bind the matrix as the cell migrates through. In the cornea, invading mesenchymal cells show a similar cell coat (Fig. 9-4b) that is continuous with the surrounding GAG-rich matrix. Both the cornea and the perinotochordal area contain fibronectin and collagen at this stage (Mayer *et al.*, 1981), which are located in fibrils (F) (Fig. 9-4; Fig. 4-1) and on the cell surface, and probably contribute, together with the hyaluronate and proteoglycan, to the migration-promoting properties of these matrices (See Chapter 10).

Similar correlations between the presence of hyaluronate and morphogenetic movements have been made during the development of several other embryonic tissues. Ectodermal cells at gastrulation (Solursh and Morriss, 1977), endocardial cushion cells (Markwald *et al.*, 1978), neural crest cells (Pratt *et al.*, 1975; Pintar, 1978; Derby, 1978), and kidney mesenchyme (Belsky and Toole, 1981; Fig. 9-5) all move and proliferate in a hyaluronate-rich matrix. In most of these cases, subsequent differentiation has been shown to be associated with decreased hyaluronate levels (e.g., see Fig. 9-5). In addition to embryonic development, cell movement, and proliferation taking place during salamander limb regeneration (Toole and Gross, 1971; Smith *et al.*, 1975), tendon remodeling (Reid and Flint, 1974), bone fracture repair (Maurer and Hudack, 1952), and skin wound healing (Bertomali and Donoff, 1978) are also associated with high levels of hyaluronate and subsequent differentiation with decreases in hyaluronate.



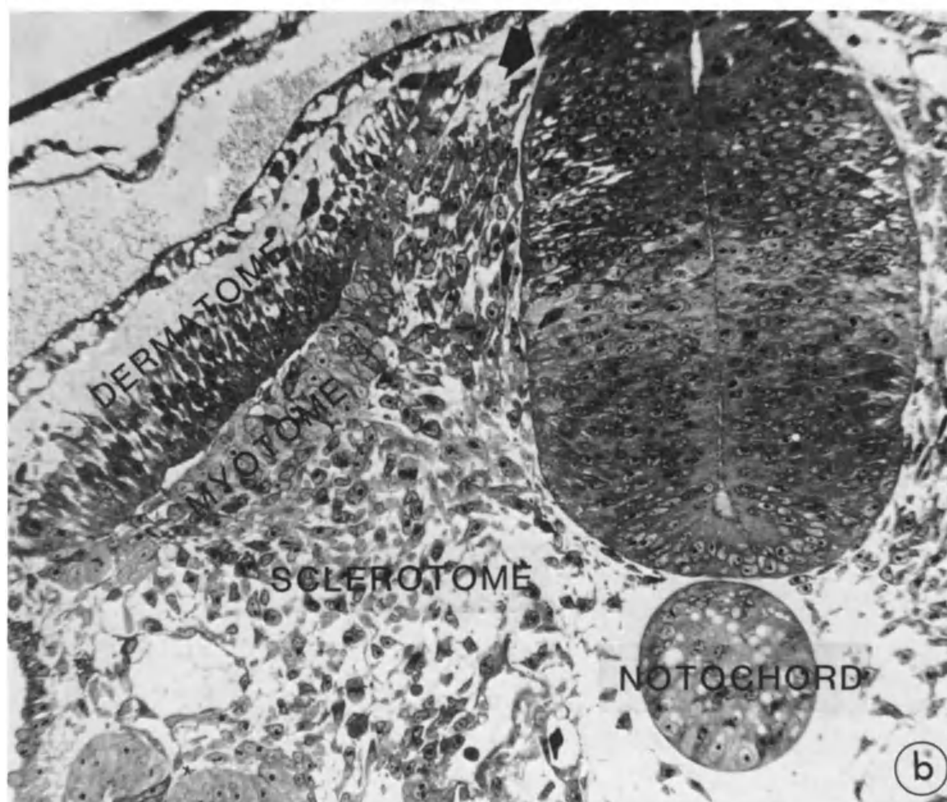


Figure 9-3. Axial region of the chick embryo (a) prior to and (b) during migration of the sclerotomal cells from the somite to the perinotochordal region. The asterisk in (a) indicates the region in which mesenchymal cells derived from the sclerotome will migrate. Bar = 75 μm. (Reproduced with permission of R. L. Trelstad and McGraw–Hill from Hay, 1966.)

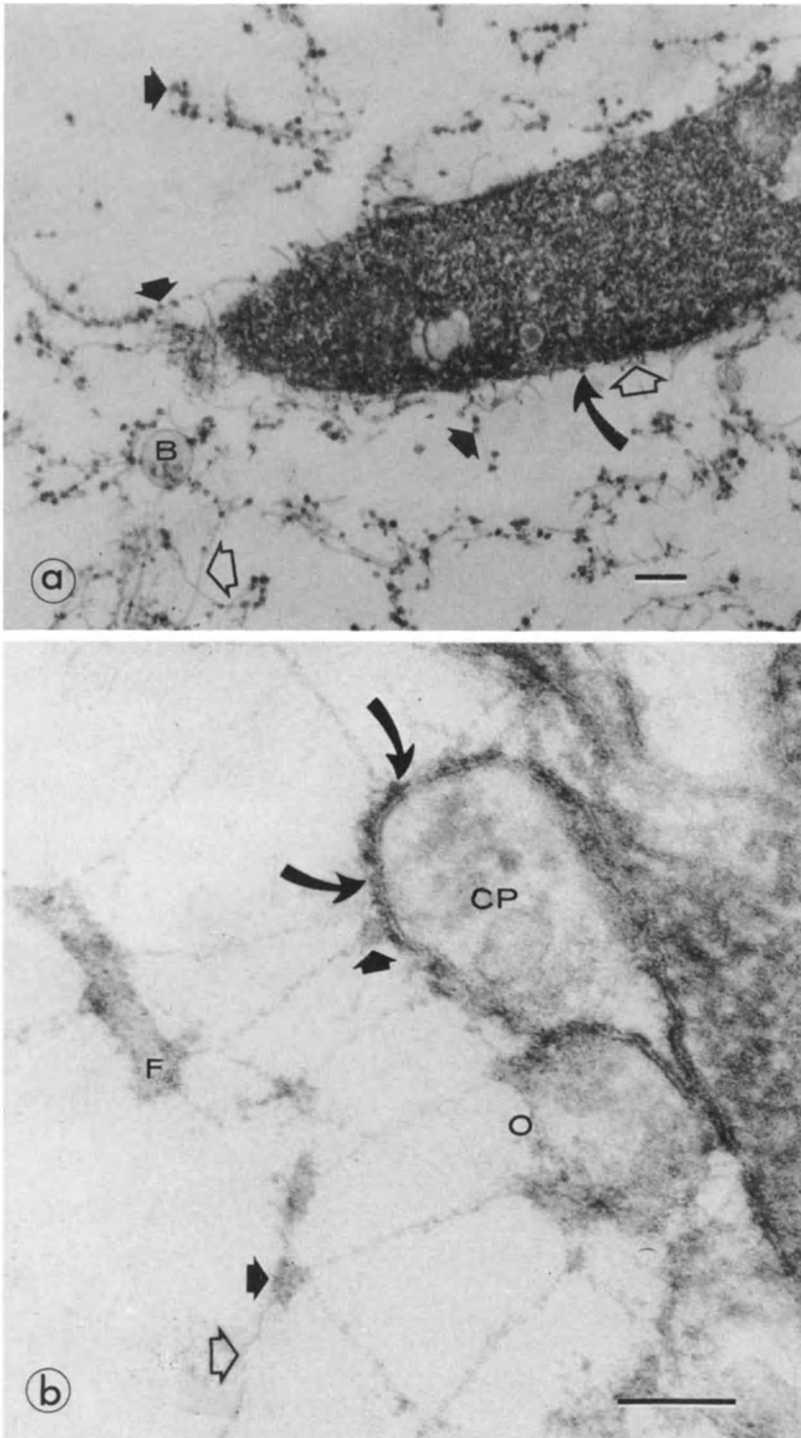


Figure 9-4. Electron micrographs of cell processes and surrounding extracellular matrix of migrating mesenchyme from (a) chick embryo sclerotome and (b) stage 35 cornea. Proteoglycan granules (short, solid arrows) and filaments (open arrows) are abundant in the matrix and associated with the plasmalemma in both tissues. GAG-rich materials associated with the plasmalemma are labeled by curved arrows. O, obliquely sectioned plasmalemma; F, collagen fibril; CP, cell process; B, bleb. (a) × 35,000, bar = 200 nm; (b) × 180,000, bar = 90 nm. (From Hay, 1978.)

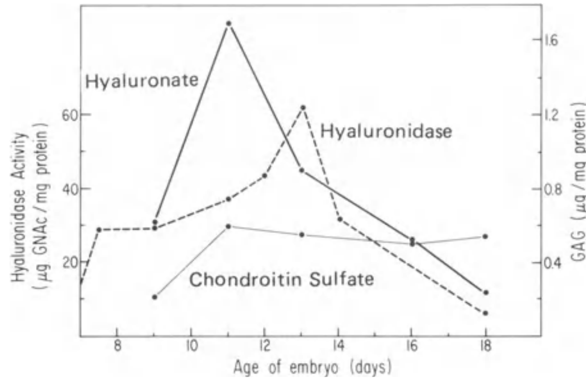


Figure 9-5. Changes in GAG and hyaluronidase activity during chick embryo kidney development (Belsky and Toole, 1981). The concentration of hyaluronate is greatest 2 days prior to the height of differentiation to tubules in the metanephros and is localized in the mesenchymal tissue. Subsequently, as the tubules reach maximum differentiation (13 days), the level of hyaluronidase activity increases markedly and the concentration of hyaluronate decreases. A peak of hyaluronidase also occurs at the time of maximum tubule formation in the mesonephros (5 days).

These correlations have led to the hypothesis that a hyaluronate-rich extracellular matrix is suitable for cell migration and proliferation and may prevent precocious differentiation (Toole and Gross, 1971; Toole, 1973, 1976a).

2.2. Transitions in Structure and Source of Sulfated Proteoglycans

2.2.1. Tissue Specificity of Proteoglycan Composition

It is clear that the final differentiated tissues resulting from various morphogenetic sequences vary not only in their GAG composition but also in the structure and properties of native proteoglycans with which the GAG are associated (see Chapter 2). In addition, it is probable that each stage or morphogenesis of a particular tissue has a characteristic complement of sulfated proteoglycans. Changes in these proteoglycans add another level of complexity to the changes in hyaluronate (see above) and collagens (see Chapter 10). Such changes are best illustrated in the development of the chick embryo limb.

Limb bud outgrowth from the flank of the embryonic chick begins at stage 17 (3 days) of development. Until stage 22, the limb bud or mesoblast consists of a uniform-appearing population of mesenchyme-derived cells surrounded by ectoderm. At stage 22 (day 3½), a "condensation" occurs in the core of the limb, delineating the presumptive cartilage from soft tissue areas. However, histological characteristics of cartilage do not appear until stage 25 on day 4 (Searls, 1973). Stage 25 is critical in that the precartilage cells of the condensation become stabilized in their commitment to forming cartilage and changes occur in the cellular environment of the limb bud that affect its ability to influence the phenotypic expression of the cells (Searls and Janners, 1969). Bone formation begins around day 7–8 in the perichondrium at the center of the future shaft

and gives rise to a sleeve surrounding the cartilage anlage. This is followed by calcification of the central part of the anlage, blood vessel invasion into the calcified cartilage, and division into two epiphyseal zones of endochondral ossification, in addition to the continued periosteal membranous ossification in the diaphysis.

The transition from mesenchyme to cartilage involves not only a large increase in chondroitin sulfate synthesis, but also a change in the type of proteoglycan of which the chondroitin sulfate chains are part. Cartilage proteoglycan monomer (Chapter 2) is a very large macromolecule comprised of a core protein to which is attached covalently many chondroitin sulfate and keratan sulfate chains. The chondroitin sulfate proteoglycan of the limb mesoblast, however, is of smaller molecular weight and presumably less complex construction (Goetinck et *al.*, 1974; Levitt and Dorfman, 1974). In addition, its reactivity with collagen is considerably less than that of cartilage proteoglycan (Toole et *al.*, 1977), suggesting that its function may not be directly concerned with matrix deposition. It is interesting to note that at the same point as the transition in proteoglycan type occurs, there is a concomitant increase in type II collagen (Linsenmayer et *al.*, 1973) and hyaluronidase (Toole, 1972) activity (Fig. 9-6), all of which occur at the time of stabilization of the cartilage phenotype in the mesenchymal cells that form chondroblasts (Searls and Janners, 1969).

The mesenchymal cells that form the osteoblasts produce a matrix that is strikingly different in proteoglycan concentration from that of cartilage. In osteoid, the uncalcified seams of matrix that are the immediate precursors of calcified bone matrix, a dramatic decrease in chondroitin sulfate occurs precisely at the mineralization front (Baylink et *al.*, 1972). Thus, mature bone contains only minute amounts of GAG. In the case of epiphyseal cartilage that is being replaced by endochondral ossification, the amount of chondroitin sulfate proteoglycan undergoes a complex series of changes. Some studies have suggested that there is an abrupt loss of proteoglycan in the calcified cartilage zone (Campo, 1970; Lohmander and Hjerpe, 1975), but recent immunohistochemical studies do not support this conclusion (Poole et *al.*, 1981). There is a considerable increase in proteoglycan concentration, however, in the hypertrophic zone of the epiphyseal growth plate as compared to the resting or columnar zones (Matukas et *al.*, 1967; Larsson et *al.*, 1973). The configuration and organization of proteoglycan as well as the amount present in these zones may also change. For example, the extractability (Larsson et *al.*, 1973) and morphological organization (Eisenstein et *al.*, 1973) of the proteoglycans are altered during the progression from resting to hypertrophic to calcifying zones. It is thought that these changes in proteoglycans are important in the control of calcification of cartilage (Howell and Pita, 1976; Blumenthal et *al.*, 1979), but this is not yet established (Poole et *al.*, 1981).

2.2.2. Changes in Cellular Origin of Proteoglycan

In addition to alterations in the nature of GAG present in extracellular matrices during morphogenesis, changes often occur in the cellular source of

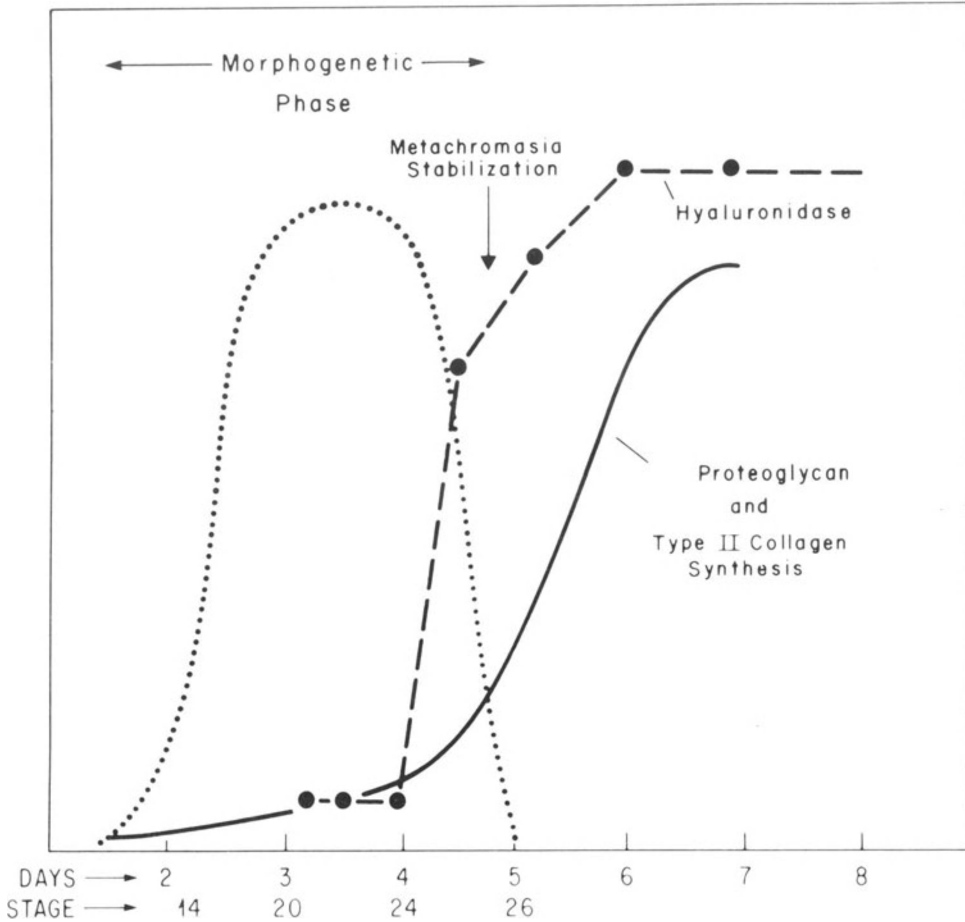


Figure 9-6. Correlation of hyaluronidase activity in the developing chick embryo limb with the phase of cytodifferentiation in the scheme of Zwilling (1968). (From Toole, 1973.)

these components. The most dramatic example is the chondroitin sulfate of the chick embryo vitreous body, which is derived from the neural retina between day 6 and day 8 of development and from the vitreous cells themselves at later stages (Smith and Newsome, 1978). An analogous situation applies to the collagenous component, which is type II derived from the neural retina at the earlier stages but changes to type I subsequently when it is derived from the vitreous cells (Newsome *et al.*, 1976).

In the case of the avian cornea, the proteoglycan associated with the stromal collagen fibers is at first derived from the corneal epithelium, but subsequent to invasion of the corneal keratocytes, it is produced largely by these latter cells (Meier and Hay, 1973; Trelstad *et al.*, 1974; Hart, 1978). In a similar fashion, ectodermal cells produce considerable amounts of GAG early

in development (Pintar, 1978; Solursh *et al.*, 1979a), but neighboring mesenchymal cells subsequently become the major source. A common pattern in development then is the initial production of GAG (and collagen) by epithelial cells, followed later by mesenchymal cells.

3. Glycosaminoglycan-Containing Cell Surface Coats

3.1. Glycosaminoglycan-Cell Surface Interactions

Numerous studies have shown that GAG are associated with the surface of many different types of cells, and that the level of these GAG vary under different physiological conditions *in vitro*. Much of the pioneering work in this area has been conducted by Kraemer (1971a,b; reviewed in Kraemer, 1979), who showed that heparan sulfate is tightly associated with the surface of many different cultured cell lines. Because of its very widespread distribution, Kraemer (1971b) hypothesized that heparan sulfate is a ubiquitous component of cell surfaces and therefore presumably plays a fundamental role in cellular function. The role of this GAG is still not clear, but some possibilities are discussed in Sections 5.1 and 6 of this chapter and in Chapter 11. In addition, heparan sulfate may have a significant function in hemostasis due to its similarity to heparin in structure (Lindahl *et al.*, 1977), its anticoagulant action (Radhakrishnamurthy *et al.*, 1980; Khoory *et al.*, 1980), and its presence on the surface of endothelial cells (Buonassisi and Root, 1975).

Recent work has shown that specific binding sites for heparan sulfate and heparin are present on the surface of hepatocytes (Kjellen *et al.*, 1977). The existence of such sites was confirmed by the observation that a portion of the endogenous cell surface heparan sulfate proteoglycan of both hepatocytes (Kjellen *et al.*, 1980) and Chinese hamster ovary cells (Kraemer, 1977) can be displaced from the cell surface by addition of large concentrations of exogenous heparin. In addition to this mode of attachment of the heparan sulfate polysaccharide chains to binding sites on the cell surface, however, it seems that the core protein of a separate population of endogenous cell surface heparan sulfate proteoglycan is attached directly to the plasmalemma. Several investigators (Kraemer and Smith, 1974; Oldberg *et al.*, 1979; Mutoh *et al.*, 1980) have isolated cell surface heparan sulfate proteoglycan complexes, but Kjellen *et al.* (1981) have separated two distinct species by treating hepatocyte plasmalemmae first with heparin to remove the fraction bound to the sites mentioned above and then with detergent. The detergent-extracted proteoglycan has a higher molecular weight than the heparin-displaced species and its protein core exhibits marked hydrophobic properties. These workers conclude that the larger proteoglycan is attached directly to the hepatocyte plasmalemma and that the smaller molecule may be derived from it by proteolytic cleavage. A similar proposal has also been made by Kraemer (1979).

The interaction of hyaluronate with the cell surface has recently been studied along similar lines to that above because of the likely relevance of such interactions to the morphogenetic events described in previous sections. Several investigations have shown that hyaluronate partially inhibits chondroitin sulfate synthesis by cartilage-forming cells *in vitro* (Toole, 1973; Wiebkin and Muir, 1973; Handley and Lowther, 1976; Solursh *et al.*, 1980). It seems probable that hyaluronate causes this effect by interacting with the surface of the cartilage-forming cells, rather than by entering the cells. Consequently, direct evidence has been sought for the presence of receptors for binding hyaluronate on cell surfaces. To date, most of this work has been done with cell lines because of convenience in obtaining and handling large numbers of these cells.

When [^{3}H]hyaluronate is added to suspensions or monolayers of certain types of cells, e.g., simian virus 40-transformed 3T3 (SV3T3) cells, reproducible binding can be measured (Underhill and Toole, 1979, 1980). The extent of binding of [^{3}H]hyaluronate is found to be a function of both the concentration of labeled hyaluronate and the cell number. Most of the binding takes place within the first 2 min of the incubation, is not influenced by the presence or absence of divalent cations, and can be prevented by the addition of an excess of unlabeled hyaluronate. High-molecular-weight preparations bind more effectively than low, but binding of [^{3}H]hyaluronate can be inhibited by high concentrations of hyaluronate oligosaccharide fragments of six sugars or more. Labeled hyaluronate bound to the cells can be totally removed by incubating the intact cells with *Streptomyces* hyaluronidase or trypsin. Scatchard plot analyses of binding data generated in these experiments demonstrated that, for hyaluronate of molecular weight approximately 10^6, a single SV3T3 cell binds at saturation approximately 3×10^3 molecules with a dissociation constant of $1–2 \times 10^{-9}$ M. This high affinity appears to be due to interaction of a single molecule of hyaluronate with multiple receptors (Fig. 9-7A) resulting from the repetitive structure of this polysaccharide (Underhill and Toole, 1980). The interaction with an individual binding site may be relatively weak, but the sum for the hyaluronate polymer would give rise to a strong interaction. Binding of this type has been shown to occur in several types of cell lines (Underhill and Toole, 1981a) and embryonic cells (Orkin *et al.*, 1981), but to vary quantitatively for different cell types.

The above binding of hyaluronate to the cell surface appears to involve a noncovalent reaction between a protein receptor and the carbohydrate moieties of hyaluronate. There may in addition exist a second type of binding, which retains newly synthesized hyaluronate at the cell surface and which involves a protein covalently bound to hyaluronate (Mikuni-Takagaki and Toole, 1981). This protein may serve to anchor hyaluronate directly to the plasmalemma (Fig. 9-7B) in a similar fashion to that discussed above for heparan sulfate proteoglycan.

The physiological function of these two types of binding of hyaluronate (or heparan sulfate) to the cell surface is not yet established. One possibility is that the protein covalently bound to hyaluronate (Fig. 9-7B) is involved in mediat-

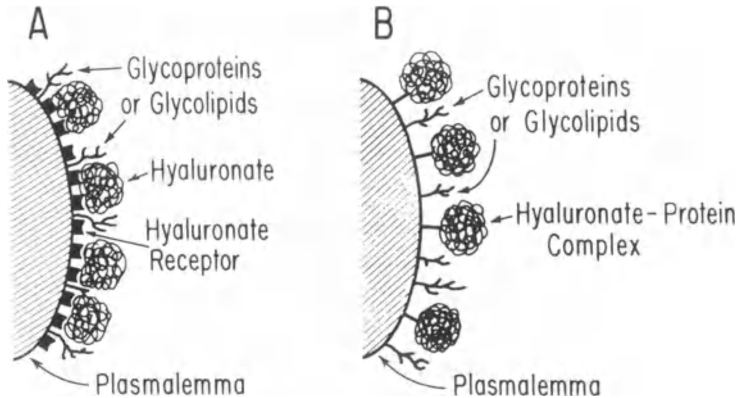


Figure 9-7. Two possible modes of binding of hyaluronate to the cell surface. (A) Hyaluronate interacts noncovalently with specific receptor sites on the cell surface, each molecule binding to multiple receptors (Underhill and Toole, 1979, 1980). (B) Hyaluronate is covalently bound to a peptide that in turn interacts with the plasma membrane (Mikuni-Takagaki and Toole, 1981). These two modes of binding may coexist on the cell surface, and in both cases the hyaluronate retained at the cell surface may block access of adjacent cells or substrata to other cell surface receptors, due to its negative charge or to its ability to occupy an extended molecular domain and exclude other large structures (see Fig. 9-9).

ing synthesis, transport, and secretion of hyaluronate. It may act as a primer for elongation of hyaluronate while embedded in the membranes of the Golgi apparatus, then facilitate transport to the cell surface in exocytotic vesicles. Prelabel–chase studies have indicated the possibility that once the hyaluronate–protein is assembled at the cell surface, the protein is cleaved and the hyaluronate shed into the medium (Mikuni-Takagaki and Toole, 1979, 1981). The binding of exogenous hyaluronate to cell surface receptors (Fig. 9-7A), on the other hand, may mediate endocytosis of extracellular hyaluronate leading to its degradation by lysosomal hyaluronidase (Orkin and Toole, 1980a,b; Orkin *et al.*, 1981). Thus, these two binding phenomena may be intimately associated with the regulation of hyaluronate metabolism.

A second alternative, which does not necessarily exclude the above,is that binding of hyaluronate to the cell surface contributes to the formation of a hyaluronate-rich coat around certain cells (Section 3.2). This coat may facilitate cell movement by masking receptors on the cell surface that otherwise would interact with other macromolecules, substrata, or cells to bring about precocious immobilization or differentiation. These latter ideas are further discussed in the following sections.

3.2. Structure of Hyaluronate-Rich Cell Surface Coats

Clarris and Fraser (1968) demonstrated a hyaluronate-rich pericellular zone around synovial fibroblasts *in vitro* that excludes particles such as

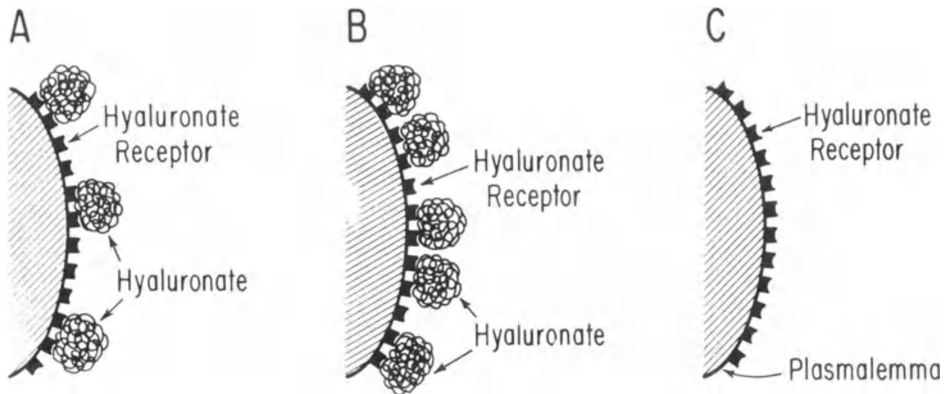


Figure 9-8. Three configurations of hyaluronate and hyaluronate-binding sites at the cell surface. (A) Both occupied and unoccupied hyaluronate receptors are present. This configuration would be representative of SV3T3 cells and would allow hyaluronate molecules to cross-bridge cells, causing aggregation (see Fig. 9-11A). (B) Virtually all receptors are occupied, preventing aggregation (see Fig. 9-11C). This may depict the surface of embryonic mesenchymal cells or of 3T3 cells, which are rich in cell surface hyaluronate but poor in receptors (Underhill and Toole, 1981a,b). Another possibility is that for both 3T3 and SV3T3 cells, the endogenous hyaluronate is bound directly via a covalently linked peptide (see Fig. 9-7B) and that the binding sites for exogenous hyaluronate are more sparse in 3T3 than in SV3T3 cells. (C) No endogenous cell surface hyaluronate is present but unoccupied receptors are available for binding hyaluronate. This configuration may be representative of nonaggregating lymphoma cells (Pessac and Defendi, 1972; Wasteson *et al.*, 1973) or macrophages (Love *et al.*, 1979) where addition of small amounts of exogenous hyaluronate induces them to aggregate.

molybdenum disulfide or fixed erythrocytes. This zone was destroyed by digestion with streptococcal or testicular hyaluronidase but not with nucleases, neuraminidase, or EDTA. Similar hyaluronate-containing zones have been noted around some tumor cells (McBride and Bard, 1979).

Underhill and Toole (1981b) have shown that a similar coat is present around many types of cells in culture and that its thickness varies greatly according to cell type (Figs. 9-8 and 9-9). The coat is removed by *Streptomyces* hyaluronidase in all cases tested so far, suggesting that hyaluronate is a major structural component of the coat. However, because the coat is too thick (5–25 μm) to be composed entirely of hyaluronate directly bound to the cell surface by the mechanisms describe above (Section 3.1), we propose that further hyaluronate is held within the coat by secondary interactions (Fig. 9-10). For example, specific interactions are known to occur between hyaluronate and proteoglycans (Norling *et al.*, 1978; Coster *et al.*, 1979; see also Chapter 2) or fibronectin (Yamada *et al.*, 1980; see Chapter 4) obtained from cultured cells; both proteoglycans and fibronectin are closely associated with the surface of the cells exhibiting hyaluronate-containing coats. In addition, proteoglycan–collagen (Mathews, 1965; Toole, 1976b) and fibronectin–collagen (Kleinman *et al.*, 1979; Johansson and Höök, 1980; also see Chapter 4) interactions have been demonstrated. Possibly, then, the coat is stabilized by these various interactions (Fig. 9-10).

The proposed structure of the cell coat described above points to two interesting features of the interface between the cell surface and the extracellular matrix. The first is that both compartments share similar macromolecular components. The second is that these components are highly interactive with one another such that it is difficult to define where one compartment ends and the other begins. It seems reasonable to argue that extracellular matrix is in fact an extension of the cell surface. This point is well illustrated in electron micrographs of embryonic matrix fixed *in situ* with ruthenium red (Fig. 9-4). It should also be emphasized, however, that the extracellular matrix distant from the cell often has a composition different from that closely apposed to the cell surface, e.g., in cartilage (Poole *et al.*, 1980) and in basement membranes (see Chapter 12).

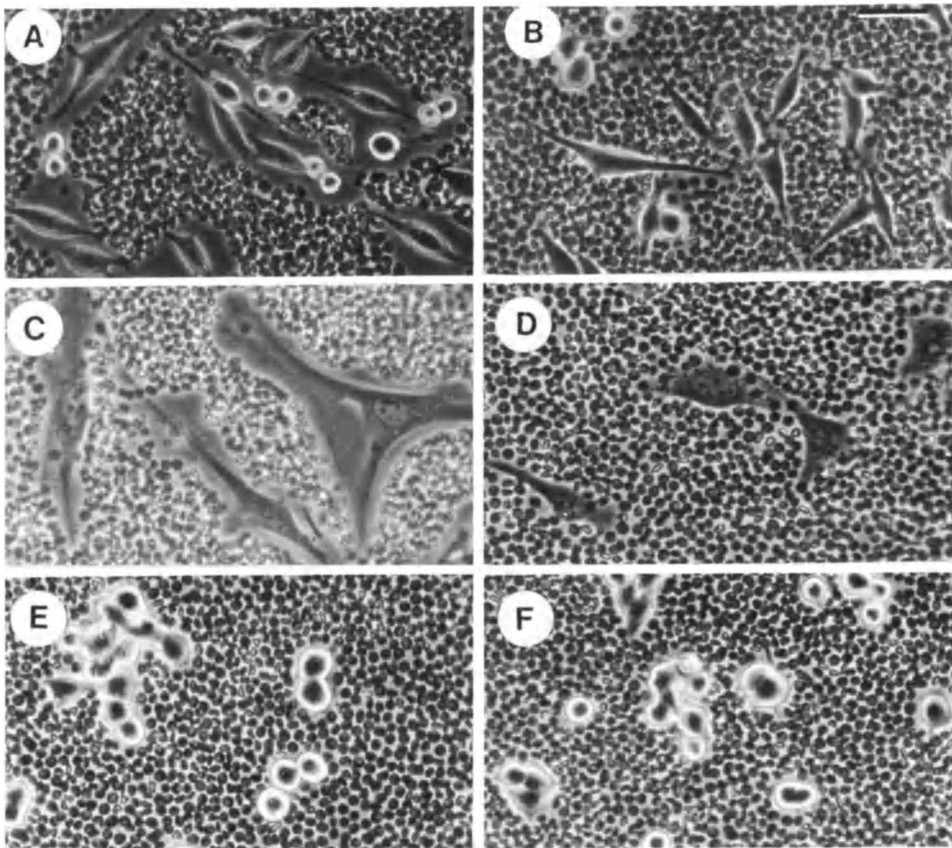


Figure 9-9. Visualization of the pericellular, hyaluronate-rich coat by exclusion of fixed red blood cells. (A) Rat fibrosarcoma cells; (B) rat fibrosarcoma cells and *Streptomyces* hyaluronidase; (C) 3T3 cells; (D) 3T3 cells and hyaluronidase; (E) SV3T3 cells; (F) SV3T3 cells and hyaluronidase. The rat fibrosarcoma cells have a larger coat than 3T3 cells. In both cases the coat is removed by small amounts of hyaluronidase. SV3T3 cells have virtually no discernible coat. The amount of hyaluronate associated with the surfaces of these cell types correlates with the thickness of the coats (Underhill and Toole, 1981b). Bar = 10 μm. (Micrographs kindly supplied by Dr. Charles B. Underhill.)

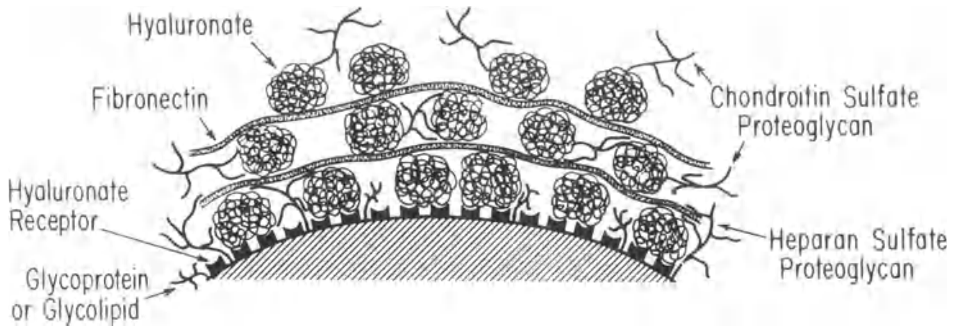


Figure 9-10. Hypothetical model for a hyaluronate-rich cell coat (see Fig. 9-9). A series of interactions between cell surface and extracellular macromolecules leads to formation of an extensive coat that is continuous with the extracellular matrix. Further discussion of the interactions depicted is provided in the text.

4. Glycosaminoglycans and Cell–Cell Interactions

It is to be expected that the cell surface coats described in the previous section would have profound effects on establishment of close contacts between cells displaying such coats because of the remarkable hydrodynamic properties of the GAG components. Their dense negative charge and ability to exclude other large macromolecules from their highly extended molecular domains (Laurent, 1966; Comper and Laurent, 1978; Kraemer, 1979) are two such properties that would restrict the approach of other cells. Little systematic investigation of the effect of GAG on cell adhesion and recognition has been published, but various isolated examples suggest that cell-surface-bound GAG can either mediate or inhibit cell interactions depending on the particular circumstances. Thus, in the case of macrophages (Love et *al.*, 1979) and lymphoma cells (Pessac and Defendi, 1972; Wasteson et *al.*, 1973), small amounts of exogenously added hyaluronate cause aggregation. On the other hand, GAG present around the surface of Rous sarcoma virus-transformed fibroblasts inhibits lectin-mediated agglutination of these cells (Burger and Martin, 1972). Likewise, cytotoxic interactions of lymphocytes with synovial fibroblasts (Fraser and Clarris, 1970) and tumor cells (McBride and Bard, 1979) are inhibited by the presence of hyaluronate-containing coats around the target cells. In both these cases, removal of the coat with hyaluronidase allowed interaction with the lymphocytes, followed by cytolysis. Exclusion of virus particles may also be effected by these coats (Clarris et *al.*, 1974; Patterson et *al.*, 1975).

Recently, Underhill and colleagues have attempted a more systematic approach to the effect of hyaluronate on cell aggregation. Underhill and Dorfman (1978) have shown that divalent-cation-independent aggregation of some transformed cell lines is dependent on endogenous cell surface hyaluronate, and Underhill and Toole (1981a) have shown that this dependence has very

similar characteristics to the binding of exogenous hyaluronate. It is hypothesized that endogenous cell surface hyaluronate on one cell binds to free receptor sites on an adjacent cell (Fig. 9-11A). In support of this idea, Underhill and Dorfman (1978) showed not only that enzymatic degradation of cell-surface-bound hyaluronate inhibited this aggregation (Fig. 9-11B), but also the addition of a large excess of exogenous hyaluronate inhibited aggregation. In the latter case, the exogenous hyaluronate would occupy all receptor sites, thus preventing cross-bridging (Fig. 9-11C). It is possible that a similar situation exists for embryonic cells *in vivo*. Thus, at the concentration of hyaluronate present in the pathways of migration of embryonic cells, these cells may also be prevented from adhering to one another. This effect might be expected to facilitate movement, and prevent cell recognition interactions leading to precocious immobilization or differentiation. In the case of the lymphoma cell and macrophage aggregation mentioned in the previous paragraph, it may be that these cells have receptors for binding hyaluronate, but no endogenous cell surface hyaluronate to cross-bridge between cells. Exogenous hyaluronate would react with the sites on adjacent cells causing aggregation (Fig. 9-8C).

5. Glycosaminoglycans and Cell Movement

Two possible roles for GAG in cell movement will be discussed. These are: (1) the involvement of various GAG in cell–substratum adhesion, a phenomenon essential for cell motility *in vitro* and thought to be necessary also *in vivo*; and (2) the contribution of hyaluronate to creation of an appropriate environment for cell movement *in vivo*.

5.1. Cell–Substratum Interactions

Observations *in vitro* indicate that cell movement requires sequential attachments and detachments of localized areas of the cell surface in relation to the substratum. The sites of attachment of cells to artificial substrata, sometimes termed footpads, are enriched in the glycoprotein fibronectin (Chapters 4 and 10) and in GAG (Culp, 1976; Culp *et al.*, 1979). The composition of the GAG components has been shown to vary according to the metabolic age of the footpads. Isotope incorporation experiments suggest that when 3T3 cells are in the process of attaching to an artificial substratum, the footpad material is highly enriched in labeled heparan sulfate, whereas more mature footpads and footprints, i.e., material left behind by migrating cells, contain more hyaluronate and undersulfated chondroitin 4-sulfate proteoglycan (Rollins and Culp, 1979). On the basis of these and other experiments, Culp and colleagues (1979) have proposed that interaction of cell surface heparan sulfate proteoglycan and fibronectin mediates substratum attachment but that the weakening of this initial adhesion, which is necessary for cell movement, involves accumula-

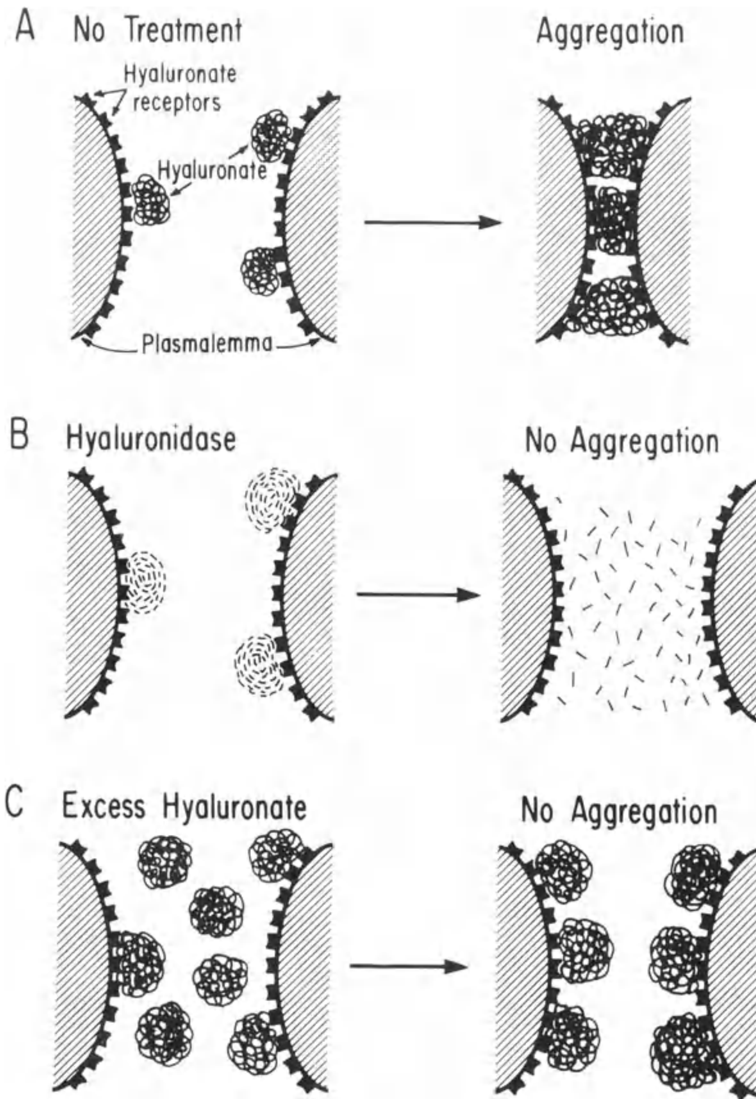


Figure 9-11. Hyaluronate-mediated, divalent-cation-independent aggregation of cell lines in suspension (Underhill and Dorfman, 1978; Underhill, 1981; Underhill and Toole, 1981a). (A) Endogenous cell-surface-associated hyaluronate cross-bridges cells by binding to receptors on adjacent cells. (B) Hyaluronidase treatment degrades the hyaluronate, inhibiting aggregation. (C) Addition of excess exogenous hyaluronate blocks all the receptors, inhibiting aggregation.

tion of hyaluronate and chondroitin sulfate proteoglycan. The latter GAG may compete with heparan sulfate for binding to fibronectin (see Chapter 4).

Unfortunately, little information is available with respect to levels of heparan sulfate during morphogenetic cell movements *in vivo*, but hyaluronate concentrations are clearly elevated (see Section 2). Consequently, the suspected role of hyaluronate in cell motility *in vitro* is of clear relevance to morphogenesis. Other observations in addition to those of Culp's group also point to the possibility that hyaluronate can form a bridge between cells and their substratum but that this interaction leads to weakened adhesion. Involvement of hyaluronate in cell–substratum interactions is indicated by the observations that: (1) SV3T3 cells plated on hyaluronate-derivatized substrata glycosylate the hyaluronate (Turley and Roth, 1979); (2) Chinese hamster ovary cells cultured on a solid substratum produce larger amounts of hyaluronate than when grown in suspension (Kraemer and Barnhart, 1978); (3) hyaluronate interacts in a specific manner with fibronectin (Yamada *et al.*, 1980; see Chapter 4), which is an important component of footpad material and which in itself is stimulatory to cell migration (Chapter 10); and (4) Rous sarcoma virus-transformed chondrocytes are detached from their substratum by treatment with *Streptomyces* hyaluronidase (Mikuni-Takagaki and Toole, 1980). That this involvement of hyaluronate in cell–substratum interactions leads to *weakened* adhesion can be concluded from the findings that: (1) loosely attaching variants of Chinese hamster ovary cells produce more cell-surface-associated hyaluronate than tightly attaching variants (Atherly *et al.*, 1977; Barnhart *et al.*, 1979); (2) the spreading of mesenchymal cells on collagen gels is retarded by the presence of hyaluronate in the gels (Fisher and Solursh, 1979); and (3) the Rous sarcoma virus-transformed chondrocytes mentioned above attach more weakly to their substratum than normal chondrocytes or fibroblasts, which are not detached by *Streptomyces* hyaluronidase (Mikuni-Takagaki and Toole, 1980).

5.2. Hydrated Space Formation

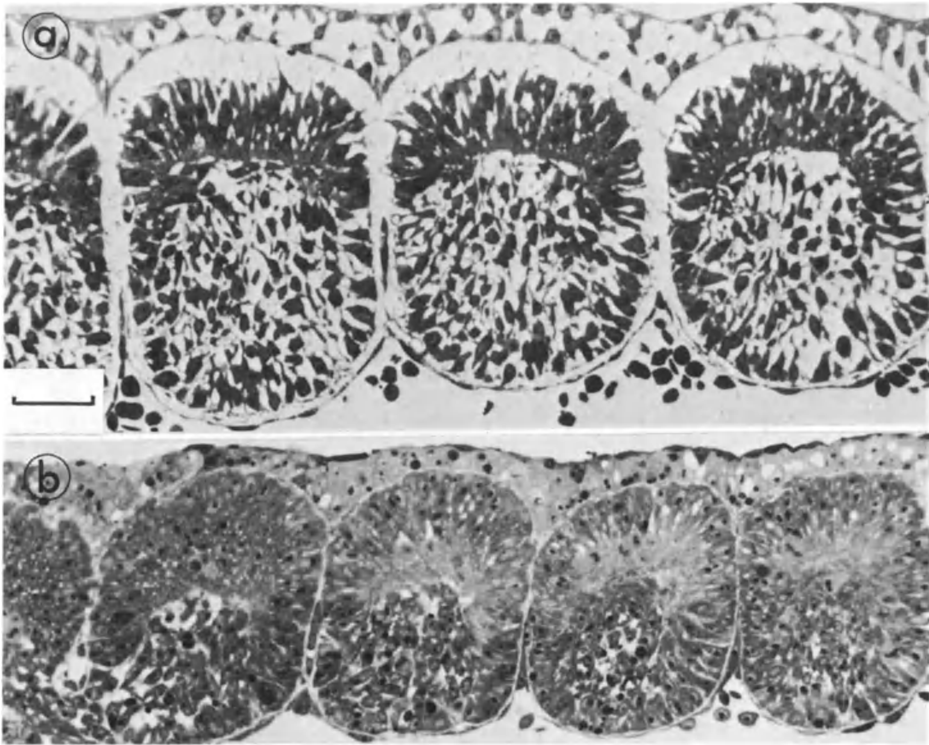
Mesenchymal cell movement within embryonic tissues has been shown to take place within hydrated extracellular matrices enriched in hyaluronate. A dramatic example is found in the invasion of the chick embryo cornea by mesenchymal cells between day 5½ and day 8 of development (see Section 2). The beginning of this migration coincides precisely with increased hydration and swelling of the hyaluronate-rich acellular stroma lying between the corneal epithelium and endothelium as well as with an increase in the space between the orthogonal layers of collagen fibers within the stroma. Subsequently, when the stroma loses water, condenses, and the migration ceases, hyaluronidase activity within the cornea increases and the hyaluronate content decreases (Toole and Trelstad, 1971). In a similar fashion, the movement of embryonic neural crest cells (Pratt *et al.*, 1975; Derby, 1978; Pintar, 1978), cardiac cushion cells (Markwald *et al.*, 1978), sclerotomal cells (Toole, 1972; Solursh *et al.*,

1979b), and primary mesenchyme (Fisher and Solursh, 1977; Solursh and Morriss, 1977) takes place in highly hydrated, hyaluronate-containing matrices. Also, cell movement during tissue remodeling and repair (Toole and Gross, 1971; Iwata and Urist, 1973; Reid and Flint, 1974) and, in certain cases, tumorigenesis (Toole *et al.*, 1979) has been shown to be associated with high hyaluronate concentrations in the extracellular matrix. It seems then that whenever mesenchymal cells move, they do so within a highly hydrated, hyaluronate-rich milieu.

Direct treatment of chick embryo tissues with specific hyaluronidases has demonstrated that hyaluronate (Fisher and Solursh, 1977; Solursh *et al.*, 1979b; Fig. 9-12) or hyaluronate plus chondroitin sulfate (Nakamura and Manasek, 1978) is responsible for maintaining the hydrated spaces through which mesenchymal cells move. In addition, it has been shown that hyaluronate added to collagen gels causes an increase in the rate of movement of chick embryo cardiac mesenchyme in these gels and the number of filopodia associated with the cells (Bernanke and Markwald, 1979).

Hyaluronate has been shown to cause a high degree of hydration in several biological (Rienits, 1960; Szirmai, 1966) and experimental systems (Fessler, 1960; Ogston, 1966; Comper and Laurent, 1978). This hydration is largely dependent on the polyanionic nature of hyaluronate, as mutual repulsion between the negatively charged carboxyl groups of the glucuronate moieties causes each molecule to occupy a large domain when in solution. At the concentrations of hyaluronate found in early embryonic and remodeling tissues, these molecular domains would enmesh, restricting the flow of water, which thus becomes entrapped. A common observation, alluded to above, is the occurrence of tissue swelling, which gives rise to formation of large cell-free spaces at the time of onset of cellular movement, e.g., between ectoderm and mesoderm ahead of the leading edge of migrating neural crest cells (Pratt *et al.*, 1975; Noden, 1978), between layers of collagen fibers in the corneal stroma prior to mesenchymal invasion (Hay and Revel, 1969), and between endocardium and myocardium prior to cushion cell invasion of the cardiac jelly (Markwald *et al.*, 1978). It seems likely that these spaces are avenues necessary for cellular migration to occur and that they form in response to dramatic changes of hyaluronate configuration or concentration.

Figure 9-12. Effect of removal of hyaluronate on the structure of the dispersing sclerotome of the chick embryo. (a) A section taken through somites wherein the sclerotome has begun dispersion under a dermamyotomal cap. (b) The effect of *Streptomyces* hyaluronidase treatment *in ovo*. The somites have become individually condensed and closer together. In addition, the overlying ectoderm and underlying endothelium of the dorsal aorta are closely applied. Sclerotomal cells are no longer dispersed and appear clumped together. This experiment indicates that hyaluronate is essential for the separation of these cells. Bar = 50 μm. (Reproduced with permission of the Company of Biologists from Solursh *et al.*, 1979b.) (c) A model for expansion of intercellular spaces. Prior to expansion, hyaluronate is secreted between epithelial cells and held in a compact configuration due to confinement by cellular junctions or the basal lamina. On breakage or rearrangement of these restraints, the swelling pressure exerted by the hyaluronate meshwork (represented by the tightly coiled springs) causes expansion of the intercellular spaces and separation of the cells. ⟶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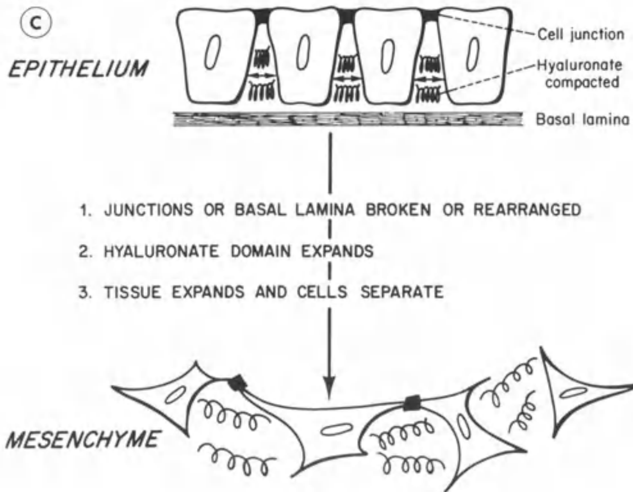
c
EPITHELIUM
Cell junction
Hyaluronate compacted
Basal lamina
1. JUNCTIONS OR BASAL LAMINA BROKEN OR REARRANGED
2. HYALURONATE DOMAIN EXPANDS
3. TISSUE EXPANDS AND CELLS SEPARATE
MESENCHYME

Although it is clear that high concentrations of hyaluronate are present at the sites of cell movement mentioned above, it does not seem likely that its presence alone would account for the suddenness of swelling observed prior to corneal (Hay and Revel, 1969) or neural crest cell migration (Pratt et *al.*, 1975). We would speculate that a rapid change in configuration of the hyaluronate meshwork already present in these tissues might cause the tissues to swell. One way in which this could occur is diagrammed in Fig. 9-13. At the high concentrations known to exist in the matrices prior to infiltration of corneal mesenchyme, cardiac cushion cells, and neural crest cells, the hyaluronate meshwork would be in a highly entangled or compacted configuration. If the glucuronate moieties of hyaluronate were charged, as would be expected under normal physiological conditions, this meshwork would be exerting a considerable pressure on adjacent cells and other structures. Thus, in order for the hyaluronate molecules to remain in this configuration, there must exist restraints capable of resisting this swelling pressure. These restraints could be: (1) physical cross-bridges holding tissue compartments at a constant volume and shape (e.g., see Ebendal, 1977; Lofberg and Ahlfors, 1978); (2) chemical interaction of cationic materials with the hyaluronate, thus blocking or reducing charge; or (3) chain interactions inducing ordered, compact configurations in individual molecules and close interactions between molecules (Winter and Arnott, 1977; Morris et *al.*, 1980). If these restraints were then broken or altered such as to be less restrictive, then the hyaluronate meshwork would expand until a new state of equilibrium was reached, resulting in tissue swelling. This swelling would thus open up spaces between collagen or cell layers, which may in turn facilitate migration of cells into these areas. Subsequent degradation of the hyaluronate by hyaluronidase would reduce the pressure, thus allowing recondensation as seen in the cornea (Figs. 9-2 and 9-13).

Cell movement or translocation appears to take place by two different morphogenetic strategies: first, actual migration of groups of cells, e.g., neural crest, cushion cells, and corneal mesenchyme, into a swollen matrix as discussed above; and second, displacement by expansion of intercellular spaces, e.g., during the epithelium to mesenchyme transition in the sclerotome (Solursh et *al.*, 1979b). The latter type of event has also been correlated with high hyaluronate production (Kvist and Finnegan, 1970a,b; Toole, 1972; Solursh et *al.*, 1979b). In this case, extracellular matrix produced by the epithelial cells appears to expand at the same time as intercellular junctional complexes are reduced in number or rearranged, thus resulting in larger spaces between the cells and their displacement from one site to another. Again, alteration in the configuration of a hyaluronate meshwork lying between these cells could act in an analogous way to that described for tissue swelling (see Fig. 9-12C). The hyaluronate may initially be held in an overlapping, collapsed, or compacted state due to the physical restraints imposed by cellular junctions or the basal lamina or to binding of cations such as Ca^{2+} (Kraemer, 1979). Then, on alteration in these restraints the hyaluronate meshwork would expand, causing larger intercellular spaces, and overall tissue expansion as described by

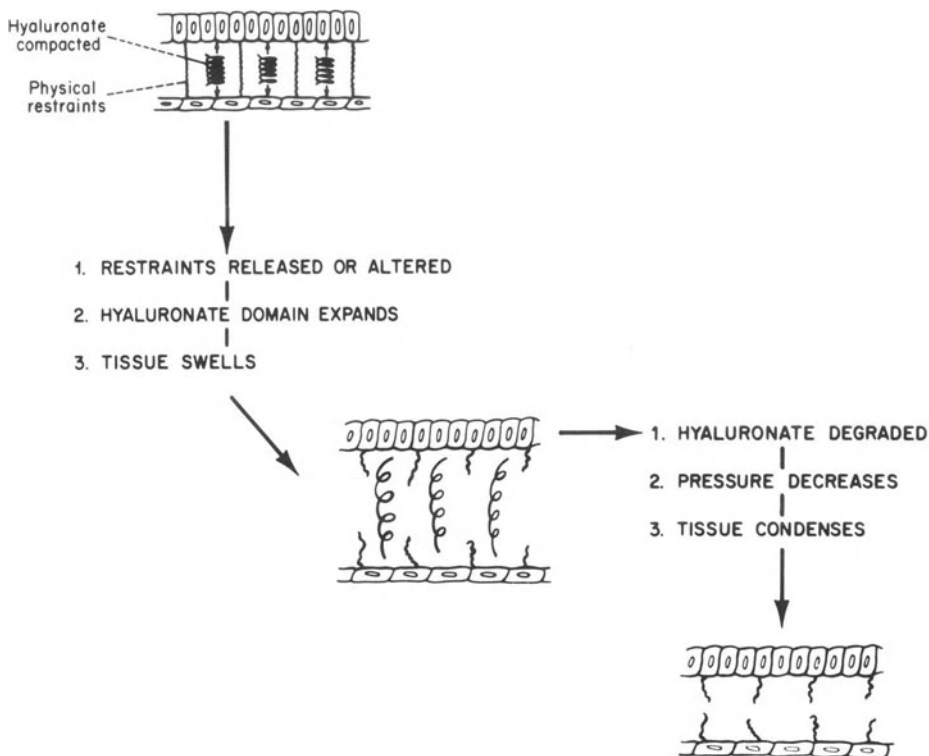

Figure 9-13. A model for tissue swelling and condensation in the embryonic chick cornea. Prior to swelling, the hyaluronate meshwork is held in a compact configuration due to hypothetical physical restraints (represented as cross-bridging fibrils) opposing expansion. Thus, the hyaluronate meshwork exerts a swelling pressure (represented by the tightly coiled springs), which on scission or rearrangement of the restraint causes separation of cell and fiber layers. This separation creates spaces that may act as avenues for the cell migration that follows tissue swelling. Degradation of hyaluronate by hyaluronidase would lead to reduced swelling pressure and thus allow recondensation of the corneal stroma to occur.

Solursh *et al.* (1979b) for the sclerotome. Another effect of hyaluronate on cell translocation may occur in the growing limb bud where hyaluronate appears to be localized beneath the apical ectodermal ridge (Sugrue, 1979). Possibly, the distal mesoderm can enter this zone and proliferate while the central area is depleted in hyaluronate and differentiates (Toole, 1972).

The physical effects of changes in hyaluronate configuration that have been discussed above could also contribute to changes in organ or tissue shape. For example, increases in hyaluronate concentration have been correlated with neural fold formation (Solursh and Morriss, 1977; Morriss and Solursh, 1978) and palate rotation (Pratt *et al.*, 1973; Wilk *et al.*, 1978). Alternatively, hyaluronate may contribute to a more complex skeletal framework whose shape is the primary determinant in tissue form (Nakamura and Manasek, 1978), and

this may remodel in response to stresses caused by physical interaction with neighboring structures (Gillard *et al.*, 1977).

6. Glycosaminoglycans and Cell Proliferation

It has frequently been observed that during the growth of cells *in vitro*, hyaluronate production is greater during proliferation than at confluency, while the opposite trend applies to the sulfated GAG (Tomida *et al.*, 1974; Cohn *et al.*, 1976; Underhill and Keller, 1976; Hopwood and Dorfman, 1977). The quantitative and qualitative pattern of these changes vary greatly according to the nature of the cells used in the cited studies. However, other observations suggest that these trends may have some significance.

Stimulated proliferation of cultured cells resulting from treatment with serum (Tomida *et al.*, 1975), insulin (Moscatelli and Rubin, 1975), or epidermal growth factor (Lembach, 1976) is accompanied by a marked increase in hyaluronate production. Also, extensive cell proliferation *in vivo* often takes place within a hyaluronate-enriched milieu (Toole and Gross, 1971; Hay, 1978). Thus, the presence of hyaluronate may in some way be involved in providing a facilitative environment for cell division.

In the case of heparan sulfate, Kraemer and Tobey (1972) showed that a specific shedding of this cell-surface-associated polysaccharide immediately preceded division of synchronized Chinese hamster ovary cells. They hypothesize that fluctuating levels of cell surface heparan sulfate may be involved in regulation of expression of other plasmalemma-bound, reactive sites (see Fig. 9-7). Of related interest is the recent report that heparan sulfate or heparin produced by confluent endothelial cells inhibits the growth of smooth muscle cells (Castellot *et al.*, 1980; Guyton *et al.*, 1980).

Many experiments suggest that, in certain cases, cell shape and anchorage to a substratum may be important in allowing cell division. Inasmuch as GAG may influence these parameters, they would also influence proliferation (for further discussion see Letourneau *et al.*, 1980).

7. Basal Lamina Glycosaminoglycans and Epithelial Morphogenesis

The interface between epithelium and mesenchyme has been established as a crucial site in the control of many morphogenetic sequences (Grobstein, 1967). The basal laminae underlying epithelia are thought to be important in this control. For many years, attention was focused mainly on the collagenous component of this structure (Kallman and Grobstein, 1965; Wessels and Cohen, 1968; Bernfield, 1970), but GAG are now recognized as a prominent component during morphogenesis (Bernfield and Banerjee, 1972; Trelstad *et al.*, 1974; Hay and Meier, 1974).

The GAG in the basal lamina of the corneal epithelium is chiefly chondroitin sulfate, organized in a regular array in two planes on either side of the lamina densa (see Fig. 2-12 and Chapters 2, 11). The GAG composition and arrangement of the basal lamina vary, however, from tissue to tissue. It seems to contain hyaluronate and chondroitin sulfate in the mouse embryo salivary gland (Cohn et *al.*, 1977), hyaluronate and heparan sulfate in the pregnant mouse mammary epithelia (Gordon and Bernfield, 1980), chondroitin sulfate and heparan sulfate in the chick embryo notochord and neural tube (Hay and Meier, 1974), and mainly heparan sulfate in the mature rat glomerular basement membrane (Kanwar and Farquhar, 1979) and chick embryo lens capsule (Hay and Meier, 1974). A possible function of the GAG of the glomerular basement membrane in plasma filtration is discussed in detail in Chapter 11. A second function may be to create an ordered pattern of fixed charge domains that could serve to orient the secretion and assembly of stromal collagen fibrils (e.g., see Trelstad et *al.*, 1974).

Preliminary studies by Kallman and Grobstein (1966) suggest that GAG at the epithelial–mesenchymal interface may also be involved in tissue interactions leading to morphogenesis of mouse salivary gland epithelium. Bernfield and his colleagues (Bernfield and Banerjee, 1972, 1978; Bernfield et *al.*, 1972; Banerjee et *al.*, 1977; Cohn et *al.*, 1977; Gordon and Bernfield, 1980) have studied in great detail the role of basal lamina GAG in branching morphogenesis of several types of mouse epithelia and the relationship of epithelial–mesenchymal interactions to basal lamina GAG turnover. During branching of the epithelia, the hyaluronate and chondroitin sulfate of these basal laminae accumulate preferentially in the relatively quiescent clefts between branches but turn over rapidly at the most active sites of branching (Fig. 9-14). Treatment of isolated mouse salivary gland epithelia with testicular hyaluronidase or chondroitinase ABC removed the basal lamina and led to loss of branching when the epithelia were cultured in combination with mesenchyme. Recombination of untreated epithelium with mesenchyme allowed continued branching. Also, if the hyaluronidase-treated epithelium was preincubated alone for 2 hr prior to recombination with mesenchyme, branching resumed in a similar fashion to untreated epithelium (Fig. 9-15). In this latter case, the GAG-containing basal lamina regenerated during the 2-hr preincubation. Thus, it was concluded that the basal lamina GAG was essential for maintenance of lobular morphology and that the mesenchyme inhibited its resynthesis. Because under normal circumstances the mesenchyme is essential for continued epithelial branching, it was postulated that this inhibition may represent exaggeration of a normal function, namely to induce loss of basal lamina GAG. Further experiments in which it was shown that recombination of prelabeled epithelium with mesenchyme caused the loss of label GAG from the basal lamina (Fig. 9-16) supported this idea. Banerjee and Bernfield (1979) have recently reported the presence of neutral and acidic hyaluronidase activities in the salivary gland, the former correlating in level of activity with epithelial branching. Thus, it seems probable that the role of the mesenchyme is to mediate controlled turnover of the basal lamina, which in turn acts as a flexible extracellular scaffold contributing to the changing shape of the epithelium.

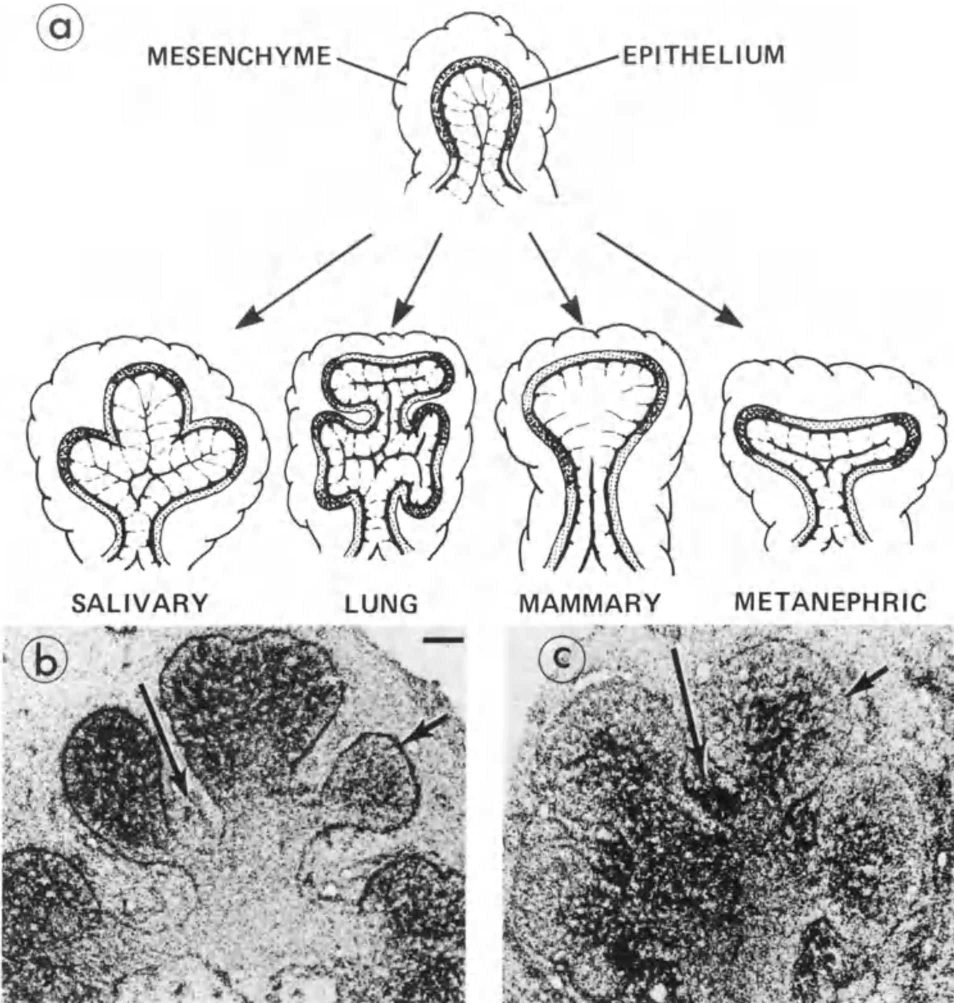

Figure 9-14. (a) Patterns of GAG distribution in several mouse epithelial organs. Areas of light stippling are the sites of greatest accumulation of GAG as revealed by Alcian blue staining. Areas of dark shading are the sites of greatest GAG label observed autoradiographically following 2 hr of [^{3}H]glucosamine incorporation. (b, c) [^{3}H]glucosamine autoradiography of 13-day-old mouse embryo submandibular glands. The label at the epithelial surface (arrows) is susceptible to testicular hyaluronidase; short arrows denote the distal aspects of lobules, the long arrows denote the clefts. The label is localized at the distal surface after 2 hr labeling (a), but has decreased at this site and increased in the clefts after 2 hr labeling plus 6 hr chase in medium containing nonradioactive glucosamine (c). Bar = 50 μm. (From Bernfield and Banerjee, 1978.)

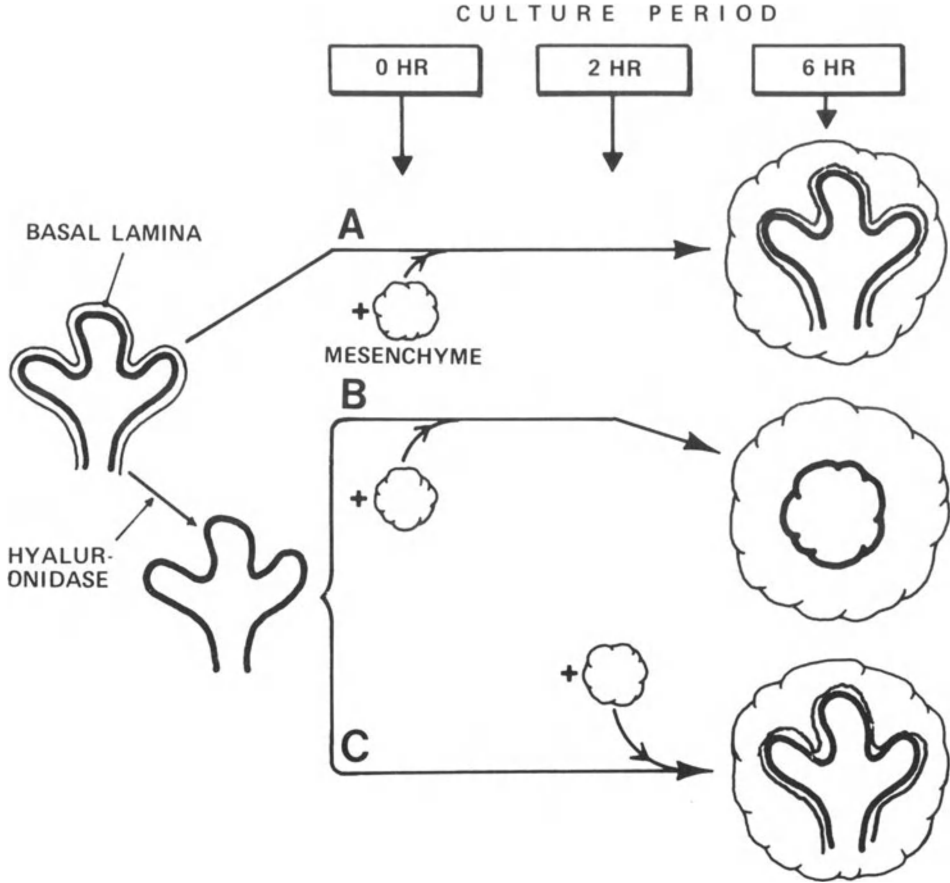

Figure 9-15. Morphogenetic effects of removal and replacement of the basal lamina from 13-day-old mouse embryo submandibular epithelia. (A) Recombination of intact epithelium and mesenchyme leads to correct morphogenesis *in vitro*. (B) Removal of the basal lamina from the epithelium by treatment with testicular hyaluronidase followed by immediate recombination with mesenchyme and incubation leads to loss of branching of the epithelium. (C) If the hyaluronidase-treated epithelium is preincubated for 2 hr prior to recombination, the basal lamina is restored and branching continues. See also Fig. 12-4. (From Bernfield and Banerjee, 1978.)

8. Stabilization of Phenotype by Extracellular Macromolecules

It is clear that GAG are involved in various aspects of cell behavior as summarized in the preceding sections. In addition, however, these macromolecules may interact with cells in such a way as to influence their metabolic state, specifically in regard to production of components of differentiated structural matrices.

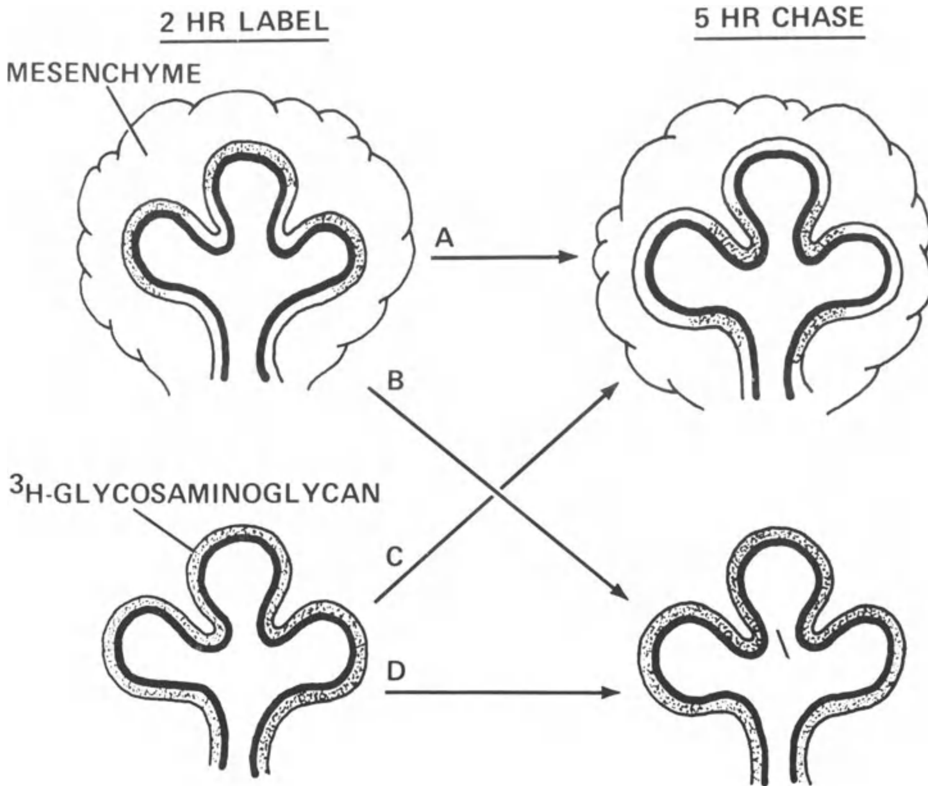

Figure 9-16. Effect of mesenchyme on the autoradiographic distribution of basal laminar GAG label. The GAG of epithelia with intact basal laminae were labeled for 2 hr before (A, B) and after (C, D) removal of mesenchyme with collagenase. These epithelia were then chased in the presence (A, C) or absence (B, D) of mesenchyme for 5 hr. The presence of mesenchyme during the chase period leads to loss of labeled GAG from the distal portion of the epithelium and accumulation in the clefts. (From Bernfield and Banerjee, 1978.)

In Section 2 it was pointed out that a common pattern in developing tissues is the transition from a hyaluronate-rich loose extracellular matrix in which cells are proliferating and migrating, to a structural matrix poor in hyaluronate but rich in other sulfated proteoglycans. Several studies suggest that the composition of these matrices influences the metabolism of the cells therein in such a way as to stabilize their existing phenotype. As mentioned, hyaluronate has been shown by several investigators to inhibit partially the synthesis of proteoglycan by chondrocytes (Toole, 1973; Wiebkin and Muir, 1973; Solursh *et al.*, 1974; Handley and Lowther, 1976). In the case of mature chondrocytes, its mechanism of action is at two levels, namely a direct effect on proteoglycan synthesis and displacement of proteoglycan from aggregated matrix (Solursh et *al.*, 1980). Thus, the presence of high concentrations of hyaluronate in matrices prior to chondrogenesis (Toole and Gross, 1971; Toole, 1972) may prevent the

precocious onset of active deposition of cartilage matrix, allowing continued cell assembly that would otherwise be inhibited by the presence of a highly structured matrix.

On the other hand, when hyaluronate is removed and the sulfated products of the differentiating cells begin to accumulate, it would seem that these latter products interact back with the cells and enhance further the production of extracellular macromolecules. This would serve to cause very sudden initial deposition of structural matrix and rapid stabilization of the differentiated phenotype, as seen, for example, in the chick embryo limb at 4 days of development (Searls and Janners, 1969).

The first evidence for such a positive-feedback effect was produced by Nevo and Dorfman (1972), who showed that cartilage proteoglycan and related sulfated polymers stimulated cartilage cells to accelerate their production of proteoglycan (see also Chapter 5). This basic observation has now been applied to two well-established developmental interactions. First, in the case of promotion of somite chondrogenesis by the notochord, it was found that in organ culture, the effect of the notochord was virtually eliminated by removal of proteoglycan from the notochord sheath (Kosher and Lash, 1975) and that the effect of intact notochord was mimicked by addition of exogenous sulfated proteoglycan (Kosher *et al.*, 1973). Further studies led to the finding that collagen (Kosher and Church, 1975) and proteoglycan–collagen complexes (Belsky *et al.*, 1980) also have a stimulatory effect on chondrogenesis. Second, it was shown in an analogous manner that the promoting or "inductive" effect of the lens on production of extracellular stroma by corneal epithelium in organ culture could be replaced by the lens capsule or other collagenous substrata (Hay and Dodson, 1973; Meier and Hay, 1974a). Moreover, purified collagenous substrata stimulated both collagen and sulfated GAG synthesis, and addition of the same type of sulfated GAG produced by the corneal epithelium stimulated further GAG synthesis by this epithelium (Meier and Hay, 1974a,b).

It has been concluded from the preceding studies that extracellular macromolecules are, in part, responsible for stabilizing the differentiated state of these tissues. Whether these materials also contribute to the initial induction of the differentiated state is not yet clear, for these studies employed tissues that had probably already undergone this event. This question will be considered in more detail in Chapter 12.

9. Concluding Remarks

Morphogenesis is accompanied by dramatic transitions in the composition of extracellular matrix, including the GAG or proteoglycan components. A common pattern in developing tissues is accumulation of hyaluronate along the pathways of migration of mesenchymal cells, followed by its removal and replacement by a sulfated proteoglycan-rich, structural matrix during subsequent differentiation. These correlations have suggested a role for GAG in these developmental sequences.

GAG have been shown *in vitro* to be associated in significant amounts with the cell surface as well as with the extracellular matrix. Specific interactions with the cell surface havebeen demonstrated for hyaluronate and heparan sulfate. These interactions may be mediated either by hydrophobic protein covalently attached to the GAG or by reaction of the polysaccharide moiety with protein receptors. It is proposed that these GAG participate, via interactions with other extracellular and cell surface macromolecules, in the formation of pericellular coats and substrata that may be of central importance in the regulation of cell behavior.

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Chapter 10

Fibronectin and Its Relation to Cellular Structure and Behavior

RICHARD O. HYNES

1. Introduction

Apart from proteoglycans and collagens, the best understood constituent of the extracellular matrix is fibronectin, a large glycoprotein. The structural properties of fibronectin are reviewed in Chapter 4 as well as in several recent reviews (Mosher, 1980; Yamada *et al.*, 1980; Hynes, 1981). In this chapter we will be concerned with functional aspects of the interaction of fibronectin with cells. Current research reviewed here and in Chapter 4 places fibronectin at the interface between cells and the matrix and implicates it in several features of cellular behavior. Because almost all the information concerning functions of fibronectin comes from studies *in vitro*, this chapter will discuss first the distribution of fibronectin in cell culture and its effects on cellular functions *in vitro*. We will then consider the distribution of fibronectin *in vivo* and end with a rather speculative discussion of the possible functions of fibronectin *in vivo*.

2. Occurrence of Fibronectin *in Vitro*

2.1. Distribution

Cellular fibronectin was initially reported as a cell surface component of cultured fibroblastic cells (see Hynes, 1976, for review). Since then, it has been found on many different cell types (see Yamada and Olden, 1978; Vaheri and Mosher, 1978; Hynes *et al.*, 1979; Mosher, 1980). Detection has been by a variety of methods: surface labeling procedures, immunofluorescence, and metabolic labeling are the most commonly used. The characteristic expression of fibronectin is as a fibrillar matrix around the cells. The exact pattern of fibrils varies with the cell type and the culture conditions. Fibronectin is also secreted by many cells and found in the culture medium as a soluble protein. Figure 10.1

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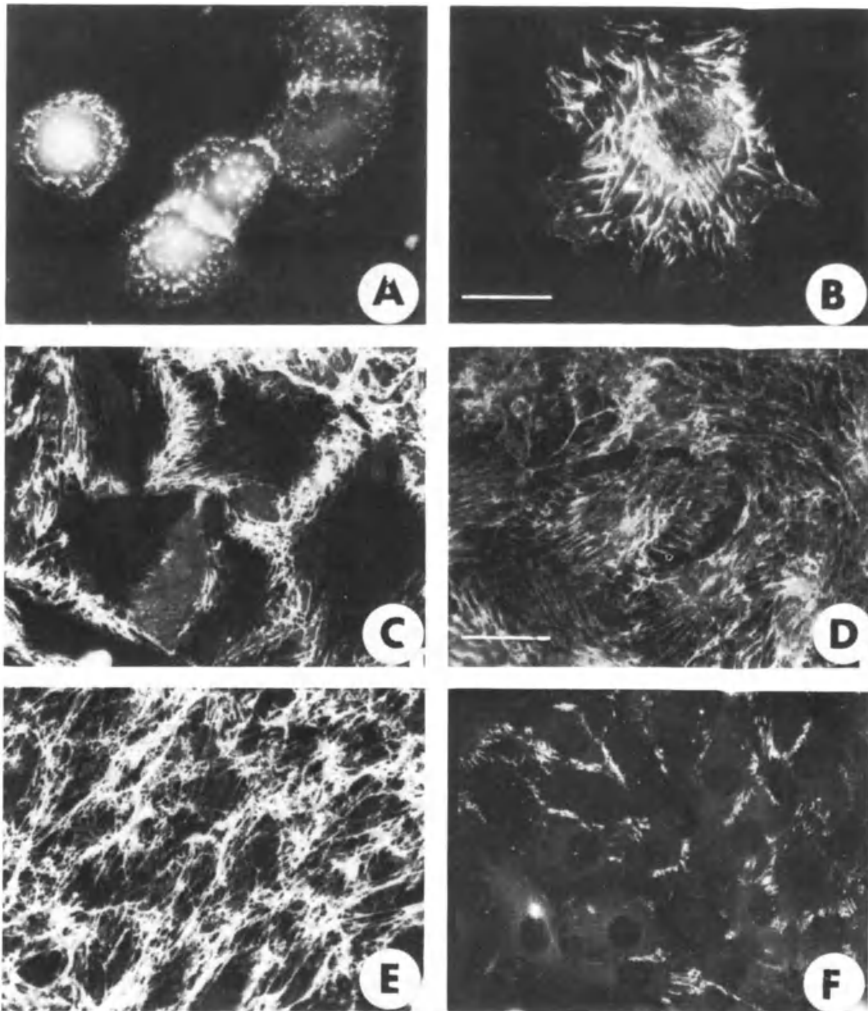

Figure 10-1. Characteristic patterns of fibronectin distribution as detected by immunofluorescence. All panels except F show patterns of fibronectin of a normal hamster cell line, NIL8. F shows the pattern on a virally transformed cell line derived from NIL8.

A and B show subcellular fibronectin beneath cells in the course of spreading: A, 2 hr after seeding, fibronectin is in punctate pattern frequently arranged in a circle; B, 5 hr after seeding, fibronectin is now arranged in fibrils beneath the cell.

C, D, and E show confluent or near-confluent cultures of cells cultured and stained in different ways. C, D. Low-serum-arrested cells are very flat and fibronectin is present as fibrils between adjacent cells (C), as a diffuse layer on the substratum (C, top center), and between the cells and the substratum (D). Subcellular fibronectin is not seen in C because cells were stained while live and the antibody does not penetrate beneath the cells. In D, cells were fixed and permeabilized prior to staining. E. Cells were grown to confluence in 5% serum. Fibronectin is present in a complex fibrillar array above and around the cells. See also Fig. 4-2.

F. Transformed NIL8.HSV cells have very little fibronectin. Some patches of cells have short intercellular "stitches" between the cells as shown in this panel.

shows some characteristic patterns of fibronectin as detected by immunofluorescence, and Table I lists most of the cultured cell types that have been shown to produce fibronectin, as well as comments on detection procedures and arrangement of fibronectin.

If immunofluorescence is the only procedure used to detect fibronectin, doubts can remain as to the exact source of the fibronectin, because serum fibronectin can bind to the cell layer and form fibrils (Hayman and Ruoslahti, 1979). Thus, care must be taken either to eliminate serum fibronectin from culture medium or to use antibodies that do not recognize the serum fibronectin. Serum fibronectin can be removed by gelatin affinity chromatography (Engvall and Ruoslahti, 1977), or antisera can be absorbed with serum fibronectin to remove antibodies reacting with it. If the cells are from a species different from the serum, antibodies that recognize the cellular fibronectin will generally remain (see Hayman and Ruoslahti, 1979). Similar problems can arise with surface labeling protocols, although careful analysis of gels can distinguish cellular from plasma fibronectin. Analysis of fibronectin production by metabolic labeling (with or without antibodies) does not suffer from the problem of contamination by serum fibronectin. However, it is important to ensure purity of the cultures for a given cell type, as fibronectin secreted by one cell can bind to another; a small number of contaminating cells that are high producers of fibronectin could lead to the erroneous conclusion that the majority cell type produces fibronectin.

It is clear that fibroblasts, myoblasts, undifferentiated chondrocytes, endothelial cells, and amniotic epithelial cells produce large amounts of fibronectin *in vitro* (see Table I). These results are consistent with distributions of fibronectin antigenicity *in vivo* (see Section 4). Certain other reports of *in vitro* expression of fibronectin are not consistent with the distribution *in vivo*. For example, some glial cell lines have been reported to be fibronectin positive (Vaheri *et al.*, 1976; Kurkinen and Alitalo, 1979), but glial cells appear to be negative *in vivo* (Schachner *et al.*, 1978). The explanation for this apparent contradiction is unknown, however. It could represent an artifactual response to culture, as several cells that are negative *in vivo* have been shown to turn on production of fibronectin *in vitro*. These include certain tumor cells (Chen *et al.*, 1980), as well as cultured chondrocytes that are caused to dedifferentiate (Dessau *et al.*, 1978; Hassell *et al.*, 1979; Pennypacker *et al.*, 1979). Alternatively, some glial cell types may synthesize fibronectin *in vivo*, for example during embryogenesis.

The patterns shown in this figure can be seen in a variety of cell types. E is characteristic of dense cultures of fibroblasts. The arrangement shown in C and D is characteristic of endothelial cells. Many cells that have little fibronectin (e.g., tumor cells) display intercellular "stitch" pattern like that in F. Isolated cells frequently show patterns like A or B with most or all the fibronectin beneath the cells. However, although certain patterns are characteristic of certain types of culture, as shown in this figure, a given cell type can display very different patterns of fibronectin depending on the culture conditions.

Bar in B represents 25 μm for A and B. Bar in D represents 50 μm for C–F. (A and B, from Hynes and Destree, 1978; C–F, from Mautner and Hynes, 1977.)

Table I. Distribution of Fibronectin *in Vitro*

Cell type	Species/source	Detection procedure[a]	References	Arrangement	Evidence for: Synthesis	Evidence for: Secretion	Other comments
Fibroblasts	Human, mouse, rat, hamster, chicken	SL, IF, IEM, B, RIPA	Numerous; see review articles	Fibrillar	+	+	Can be up to 1–3% of total cell protein. Reduced on oncogenic transformation.
Myoblasts	Rat	SL, IF, IEM	Hynes *et al.* (1976), Chen (1977), Furcht *et al.* (1978)	Fibrillar	ND[b]	ND	Viral transformation of rat myoblasts causes loss. Myotubes have less than myoblasts in both rat and human
	Human	IF, RIPA	Walsh *et al.* (1981)	Fibrillar	+	ND	
Chondrocytes	Chicken	IF, RIPA, SL, B, IF	Dessau *et al.* (1978) Hassell *et al.* (1979)	Fibrillar	+	+	Fibronectin correlates negatively with differentiation
Endothelial cells	Cow	IF, B	Macarak *et al.* (1978), Birdwell *et al.* (1978)	Fibrillar	+	+	Large proportion is secreted. Cell-bound fibronectin is all beneath the cells
	Human	B, IF	Jaffe and Mosher (1978)		+	+	
Amniotic cells	Human	B, IF	Crouch *et al.* (1978), Alitalo *et al.* (1980b)	Fibrillar	+	+	Large amount secreted
Glial cells	Human (astroglia)	IF	Vaheri *et al.* (1976)	Fibrillar	ND	+	Tumor lines have less fibronectin
	Rat (Schwann cell)	IF, B	Kurkinen and Alitalo (1979)	Fibrillar	+	+	Majority is secreted
Epithelial cells	Rat (small intestine)	IF, B	Quaroni *et al.* (1978)	Short intercellular fibers	+	+	Small amounts only
	Human (kidney glomerular epithelium)	B	Killen and Striker (1979)	ND	+	+	
	Rodent (kidney/liver)	IF	Chen *et al.* (1977)	Fibrillar	ND	ND	Small amounts only
	Human (kidney/liver)	IF	Mosher *et al.* (1977)	Various	ND	ND	
	Human (various)	IF	Smith *et al.* (1979)	Fibrillar	ND	ND	
	Rat (liver)	IF, B	Voss *et al.* (1979)	Fibrillar	+	+	
	Human (mammary)	IF	Asch *et al.* (1981)	Fibrillar	ND	ND	Fibronectin lost on malignant transformation (Human and Rat mammary)
	Rat (mammary)	IF, B	Warburton *et al.* (1981)	Fibrillar	+	+	
Macrophages	Mouse (peritoneal)	IA, B	Colvin *et al.* (1979), Johansson *et al.* (1979)		+	+	
	Mouse (peritoneal)	B, RIPA	Van De Water *et al.* (1981)		+	+	Some cell lines negative
	Human (blood)	IF, B	Alitalo *et al.* (1980a)	Sparse subcellular fibrils	+	+	Very little retained by cells
Teratocarcinomas	Mouse	IF	Zetter and Martin (1978), Wartiovaara *et al.* (1978)	Short intercellular fibrils or patches			Present in both embryonic carcinoma and endodermal cultures
		IF, RIPA	Wolfe *et al.* (1979)	Fibrillar	+	+	
		RIPA	Hogan (1980)		+	+	

[a] Abbreviations used for detection procedures: B, biochemical; IA, immunoassay; IEM, immunoelectron microscopy; IF, immunofluorescence; RIPA, radioimmunoprecipitation assay; SL, surface labeling.

[b] ND, not done.

Less work has been done on fibronectin production by epithelial cells other than endothelial and amniotic cells. Most or all basement membranes (basement laminae) underlying epithelial cells *in vivo* contain fibronectin antigen, but in most cases the source of the fibronectin is not known. Probably the clearest description of fibronectin production *in vitro* by well-defined epithelial cells is that of cultured intestinal epithelial cells by Quaroni *et al.* (1978). These cells accumulate matrix fibronectin around them and also secrete fibronectin *in vitro; in vivo* basal lamina contains fibronectin. Cultured kidney glomerular epithelial cells have also been shown to secrete fibronectin (Killen and Striker, 1979), and fibronectin is present in the glomerulus *in vivo* (Section 4 and Chapter 11). Although one study failed to detect any fibronectin in mammary epithelial cultures (Yang *et al.*, 1980), others report production of fibronectin by normal mammary epithelial cells (Smith *et al.*, 1979; Asch *et al.*, 1981; Warburton et *al.*, 1981).

Purified preparations of peripheral blood cells have also been studied. Erythrocytes and lymphocytes have been found to be fibronectin negative in numerous investigations. Most reports also conclude that platelets do not have fibronectin on their surfaces (Hynes *et al.*, 1978; Plow *et al.*, 1979; Santoro and Cunningham, 1979), although Bensusan et *al.* (1978) suggest that they do. Others have suggested that fibronectin is present in the α granules inside platelets (Zucker *et al.*, 1979; Plow *et al.*, 1979; Ginsberg *et al.*, 1980) and can be released on stimulation of the platelets (Ginsberg *et al.*, 1980).

Although Pearlstein *et al.* (1978) found mouse peritoneal macrophages to be negative for surface fibronectin, Johansson et *al.* (1979) and Van De Water *et al.* (1981) report that mouse peritoneal macrophages are fibronectin positive. Van De Water *et al.* (1981) also observed synthesis of fibronectin by one cultured macrophagelike cell line (RAW Cr 1), but not by another (P388D_1). Alitalo *et al.* (1980a) have investigated synthesis and accumulation of fibronectin by cultured human peripheral blood monocytes. They observed production and secretion of fibronectin only after several days in culture. At all times very little fibronectin was found in a matrix around the cells. Therefore, it seems that some, but not necessarily all, macrophages can produce fibronectin under certain conditions. Given the adhesive properties of fibronectin (see Section 3.1), it seems unlikely that circulating blood cells would bear it on their surfaces, although under certain conditions some blood cells might use fibronectin to adhere to substrata. More work is necessary to establish clearly if and when blood cells do bind or produce fibronectin.

2.2. Arrangement

The fibronectin secreted by cells *in vitro* is dimeric and soluble, like plasma fibronectin. However, plasma fibronectin apparently consists of equimolar amounts of two different-sized but very similar subunits. In contrast, fibronectin secreted by cells characteristically migrates as a single broad band on reducing gels. A major reason for the broadness of the monomeric fibro-

nectin band is apparently carbohydrate heterogeneity (Crouch et *al.*, 1978; Wagner and Hynes, 1979). The subunits of secreted fibronectin have not been distinguished from those of cell- or matrix-bound fibronectin retained by the same cells. Hence, secreted and bound fibronectin produced by cells are very similar and differ electrophoretically from plasma fibronectin. Plasma fibronectin can also participate in fibril formation under some conditions (Hynes et *al.*, 1978; Hayman and Ruoslahti, 1979).

It is unclear what determines which molecules of fibronectin are secreted into the medium and which are retained in the cell layer. The fibronectin in the cell layer is insoluble, and much of it is in high-molecular-weight aggregates held together by disulfide bonds (Hynes and Destree, 1977; Keski-Oja et *al.*, 1977; Ali and Hynes, 1978a). Exogenously added secreted fibronectin can also participate in such disulfide-bonded aggregates (Wagner and Hynes, 1979, 1980). The insolubility of the bound fibronectin probably arises either from the intermolecular disulfide bonding or from noncovalent interactions between fibronectin molecules and with other molecules, or a combination of these. Few, if any, covalent bonds that are stable to SDS and reducing agents occur in most cultures. However, fibronectin can be cross-linked by exogenous transglutaminase (Keski-Oja et *al.*, 1976; Mosher et *al.*, 1979), and under some conditions, cross-linking may occur naturally.

Fibronectin is known to bind to collagen and glycosaminoglycans (see Chapter 4) and has been shown to be associated with proteoglycans at the cell surface (Graham et *al.*, 1978; Hedman et *al.*, 1979; Perkins et *al.*, 1979; Culp et *al.*, 1980). In some cases, fibronectin and collagen are present in the same fibrils (Vaheri et *al.*, 1978; Dessau et *al.*, 1978), but in others, fibronectin forms fibrils in the absence of detectable collagen (Mautner and Hynes, 1977; Chen et *al.*, 1978). The mechanism of fibril formation is not yet clear. Fibrils can be formed from fibronectin *in vitro*. Fibrils will form spontaneously (Vuento et *al.*, 1980), but their formation is promoted by the addition of heparin (Jilek and Hörmann, 1979) or polyamines (Vuento et *al.*, 1980). An attractive model is that fibronectin associates noncovalently with itself and with other constituents such as proteoglycans to form fibrils that are subsequently stabilized by disulfide or other covalent bonding. The secretion of fibronectin *in vitro* could be an artifact caused by disruption of the process of matrix formation *in vitro*. Most studies of fibronectin secretion *in vitro* have not included additions of ascorbic acid to ensure adequate production of collagen, and, in general, the correct culture conditions for producing a "normal" balance of matrix components are not known.

The arrangements of fibrils are characteristic of cell type and culture conditions (Fig. 10-1) so the cells obviously control the process of fibrillogenesis in some way (Chapter 7). In sparse cultures of cells, most of the bound fibronectin is between the cells and the substratum (Mautner and Hynes, 1977; Hynes and Destree, 1978), appearing first in punctate patches and later in fibrils that increase in length with time (Hynes and Destree, 1978; Heggeness et *al.*, 1978). Fibronectin frequently accumulates in the regions of cell–cell contact (Chen et *al.*, 1976; Mautner and Hynes, 1977; Quaroni et *al.*, 1978; Dessau et *al.*, 1978)

and, as cultures increase in density, appears on top of the cell layer in a complex fibrillar network (e.g., Mautner and Hynes, 1977; Chen *et al.*, 1978). However, confluent normal endothelial cells have no fibronectin on their upper surface; all the fibronectin is in a matrix beneath the cells (Birdwell *et al.*, 1978; Vlodavsky *et al.*, 1979). Other places where fibronectin accumulates *in vitro* are: in trails left behind cells that have migrated (Ali and Hynes, 1978b); in subcellular materials left behind when cells detach (Mautner and Hynes, 1977; Chen *et al.*, 1978; Culp *et al.*, 1980); and in nonfibrillar layers bound to the substratum some distance from the cells (Grinnell and Feld, 1979; Hynes and Destree, unpublished). Some cells produce aggregates of fibrillogranular extracellular matrix containing fibronectin and proteoglycans (Graham *et al.*, 1978; Hedman *et al.*, 1978) that are similar to the interstitial bodies found in embryos (Mayer *et al.*, 1981; Section 5.2.3). The differing arrangements of fibronection have to be taken into account when considering the functions of fibronectin.

3. Functions of Fibronectin *in Vitro*

3.1. Adhesion and Morphology

The best established functions of fibronectin are its roles in the adhesion of cells to substrata and in cell spreading and morphology. Because these phenomena are probably related, they will be discussed together. Two independent lines of research converged to produce our understanding of these functions of fibronectin. The first was the study of the role of serum in cell cultures, in particular in adhesion of cells; the second followed from the observation that surface fibronectin was lost on transformation.

Until the recent development of some defined media, all cultured cells required serum for propagation *in vitro*. Serum provides hormones or growth factors, but also promotes adhesion of cells to plastic or other substrata such as collagen. In tissue culture dishes, cells actually adhere to a layer of serum proteins attached to the plastic (Grinnell, 1978). Investigation of the factors in serum responsible for cell adhesion led to identification of a high-molecular-weight glycoprotein that promoted adhesion either to plastic or to collagen or fibrin substrata (see Table II and review by Grinnell, 1978). In each case, it was found that the adhesion factor was identical with the previously discovered plasma protein, cold-insoluble globulin, now known as plasma fibronectin. These initial studies used fibroblastic cells (BHK and CHO) which are actually transformed and will also grow in suspension. Further investigation has shown that many cells require fibronectin in the serum for efficient attachment; Table II lists those that require or respond to fibronectin. Many cells do not require exogenous fibronectin, for example fibroblasts or fibroblastic cell lines, which themselves produce fibronectin. Yet other cells apparently employ different proteins for cell–substratum adhesion. For example, differentiated chondrocytes apparently require a different protein, chondronectin, for adhesion to type II collagen (Hewitt *et al.*, 1980). Other large glycoproteins that are

Table II. Effects of Fibronectin on Cell Adhesion and Morphology

Cell type	Substratum	Source of fibronectin	Response	Reference
SV3T3	Denatured collagen	Calf serum	Attachment	Klebe (1974)
PyBHK	Denatured collagen	BHK cells	Attachment	Pearlstein (1976)
CHO	Collagen	Bovine serum	Attachment	Kleinman *et al.* (1979a,b)
CHO	Plastic	Fetal calf serum	Attachment, spreading	Grinnell and Hays (1978)
BHK	Plastic	Human plasma or serum	Attachment, spreading	Grinnell and Hays (1978)
BHK, human fibroblasts	Plastic	Human fibroblasts	Attachment, spreading	Grinnell and Feld (1979)
BHK	Fibrin(ogen)	Human plasma	Attachment, spreading	Grinnell *et al.* (1980)
16C rat fibroblasts	Plastic	Chicken plasma	Spreading	Thom *et al.* (1979)
SV3T3	Plastic	Chicken fibroblasts	Adhesion, elongation	Yamada *et al.* (1976a)
SV1	Plastic	Chicken fibroblasts	Spreading, reduced surface protrusions	Yamada *et al.* (1976b)
NIL.HSV	Plastic	Chicken or hamster fibroblasts	Attachment, spreading, elongation, alignment, cytoskeleton	Ali *et al.* (1977)
BHK	Plastic	Calf and hamster plasma or BHK cells	Attachment, spreading	Pena and Hughes (1978)
CHO	Type I collagen	Chicken plasma or cells, human plasma or cells	Attachment	Yamada and Kennedy (1979)
BHK	Plastic	Chicken plasma or cells, human plasma or cells	Spreading	Yamada and Kennedy (1979)
SV3T3	Plastic	Chicken plasma or cells, human plasma or cells	Spreading, elongation, alignment	Yamada and Kennedy (1979)
Rat hepatocytes	Plastic	Human plasma	Attachment	Höök et *al.* (1977)
Chicken myoblasts	Gelatin	Horse serum, chicken fibroblasts	Attachment, elongation	Chiquet et *al.* (1979)
Human platelets	Collagen	Human plasma	Spreading	Hynes *et al.* (1978)
Human platelets	Collagen, fibrin	Human plasma	Spreading	Grinnell *et al.* (1979)
Rat follicular cells	Plastic	Human plasma	Cytokinesis, proliferation	Orly and Sato (1979)
Murine embryonic carcinoma	Plastic	Human plasma	Attachment, spreading, proliferation	Rizzino and Crowley (1980)

distinct from fibronectin and appear to be involved in cell adhesion have been reported recently, including a large surface glycoprotein produced by phaeochromocytoma cells (McGuire *et al.*, 1978), and laminin (see Chapter 4).

We have so far considered cell adhesion with little attention to the question of how the cells spread on the substrata. Serum fibronectin also promotes cell spreading *in vitro* (see Table II), and, as we shall see later, this effect is probably related to the effects of fibronectin on cell growth and behavior. Before turning to these other phenomena, let us review the information concerning adhesion and spreading obtained by investigation of the loss of cell surface fibronectin in oncogenic transformation (see Table II).

Once it became obvious that reduction in surface levels of fibronectin on transformation was a reasonably reproducible phenomenon (Hynes, 1976), an obvious question concerned the implications of this loss for expression of the cellular phenotype. Fibronectin purified from normal cells or from their culture medium and added to transformed cells affects a number of their properties. Cells become more firmly attached to the substratum (Yamada *et al.*, 1976a), and cells that were previously unattached adhere to the dish (Ali *et al.*, 1977). These observations are very similar to those reported earlier for serum fibronectin, and the two proteins behave similarly in these assays (Yamada and Kennedy, 1979). Normal cell fibronectin leads to increased spreading of trans-

formed cells and causes them to elongate and align with each other (Yamada et *al.*, 1976a; Ali et *al.*, 1977). The number of nuclear overlaps, an indicator of multilayering of cells, is reduced by added fibronectin (Yamada et *al.*, 1976a), and the numbers of surface ruffles and microvilli are also reduced (Yamada et *al.*, 1976b).

All of these effects are most simply interpreted in terms of a primary effect on cell–substratum adhesion (Hynes et *al.*, 1979; Yamada et *al.*, 1980). Adhesion will promote cell spreading and the increased surface area of a spread cell compared with a rounded cell can lead to a reduction in surface protrusions such as ruffles and microvilli. Multilayering arises through underlapping of cells, which is prevented when cells are firmly attached to the substratum (Bell, 1977). The effects of fibronectin on cell elongation and alignment may also be indirect, a consequence of the effects of fibronectin on adhesion, motility (Fig. 10-2; Section 3.3), and the cytoskeleton (Section 3.2).

3.2. Cytoskeletal Organization

Readdition of fibronectin to transformed cells, which lack it, also causes changes in the organization of microfilaments inside the cell (Ali et *al.*, 1977; Willingham et *al.*, 1977). Transformed cells generally have less well-ordered arrays of microfilaments. In particular, they usually lack or have reduced levels of microfilament bundles (MFB) or "stress fibers" characteristic of many *in vitro* cells (see Pollack et *al.*, 1975; Goldman et *al.*, 1977). When fibronectin is added, arrays of MFB reappear. This suggests some form of transmembrane effect of the extracellular fibronectin on the intracellular microfilaments.

The organization of actin-containing microfilaments in the basal cytoplasm of cultured corneal epithelial cells has been shown to be affected by fibronectin and other extracellular molecules (Sugrue and Hay, 1980). On lens capsule, which contains fibronectin and other matrix molecules, the basal microfila-

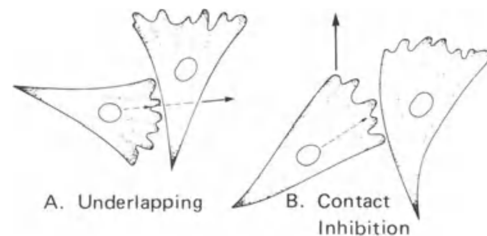

Figure 10-2. Effects of fibronection on cell overlapping and cell alignment. When a migrating cell collides with another, it has two options: either (A) it continues *beneath* the other cell, or (B) it turns away and continues migration in a different direction. What determines which happens is the degree of adhesion to the substratum of the cell that is being contacted. If the segment of cell boundary is weakly adherent, the migrating cell underlaps (case A). If the cell boundary is strongly adherent at the point of contact, then the migrating cell is inhibited in its forward migration (case B). Because fibronectin increases cell–substratum adhesion, it will increase the ratio B/A, leading to less underlapping and thus less overlapping of cells. In a culture of migrating cells, continual occurrence of event B will eventually lead to the cells becoming aligned in parallel with each other. In the figure, points of adhesion are indicated by shading.

ments are organized and the basal cell surface is smooth as *in vivo*. On Millipore filters, the microfilaments become disorganized and the basal surface blebs. However, if fibronectin or collagen in the absence of serum is added to the Millipore filter system, then the microfilaments again become organized beneath a smooth basal cell surface (see Chapter 12).

Other results suggest some relationship between fibronectin and actin-containing microfilaments. Cytochalasins B and D, which disrupt MFB, lead to rapid release of fibronectin from cell layers (Mautner and Hynes, 1977; Ali and Hynes, 1977; Kurkinen *et al.*, 1978). The fibrillar arrays of fibronectin and actin are frequently very closely related (Hynes and Destree, 1978; Fig. 10-3), and electron microscopic examination has revealed an apparent transmembrane colinearity between fibronectin-containing fibrils and MFB (Singer, 1979; Fig. 10-4).

Another intracellular cytoskeletal protein of 130,000 daltons is a constituent of attachment plaques and other sites of actin–membrane interaction (see Geiger *et al.*, 1980) and has also been shown to codistribute with fibronectin (Burridge and Feramisco, 1980; I. I. Singer, personal communication).

These results, and others that are discussed in more detail elsewhere (Hynes, 1981), are consistent with the idea of transmembrane interaction between fibronectin and actin, which could be involved in cytoskeletal organization. The molecular basis for this interaction is not yet known.

3.3. Migration

Fibronectin can promote migration *in vitro* (Ali and Hynes, 1978b; Fig. 10-5). The experiments demonstrated chemokinesis (i.e., generalized stimulation of movement) by fibronectin and did not assay directional stimulation of movement. The mechanism of this effect is unclear but most likely involves the effects of fibronectin on adhesion, spreading, and cytoskeletal organization. Fibroblast locomotion obviously involves adhesion and spreading of the leading lamella. During initial spreading of cells, fibronectin forms radially arranged fibrils, i.e., parallel to the direction(s) of spreading (Hynes and Destree, 1978). Fibronectin fibrils are also observed parallel to the long axis of the leading lamellae of fibroblasts (Hynes *et al.*, 1978), and it is reasonable to suggest that they are somehow involved in the spreading of the lamellae in the direction of migration. However, it is unclear how fibronectin could promote the polarization of cellular morphology that is involved in migration. One possibility is that an asymmetry of spreading, arising from other causes, is exaggerated by the effects of fibronectin on adhesion and spreading and that once asymmetric spreading is established it can be maintained. There is evidence that cells show some persistence in direction of migration (Gail and Boone, 1970; Albrecht-Buehler, 1977).

A priori, one might expect migration to require a certain minimal level of cell–substratum adhesion, but that too strong an adhesion would lead to inhibition of migration. Fibronectin is known to promote cell–substratum adhe-

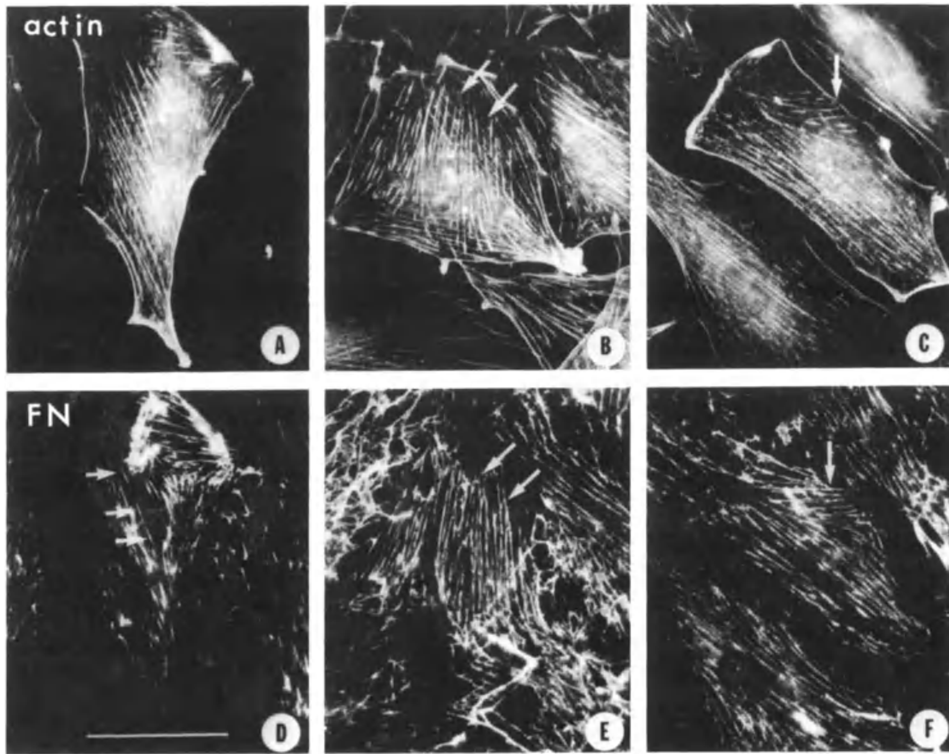

Figure 10-3. Correspondences between fibronectin (FN) and actin as seen by double-label immunofluorescence. These pairs of micrographs show actin (A, B, C) and fibronectin (D, E, F) in well-spread, nongrowing, nonmigrating NIL8 cells.

In A and D, note that in the upper right of the cell each microfilament bundle corresponds with a fibronectin fibril, and in the center of the cell many microfilament bundles lack corresponding fibronectin fibrils. Arrows in D indicate interrupted linear staining with anti-fibronectin. Also note that the cell boundary stains with anti-actin but not anti-fibronectin, whereas extracellular fibrils on the substratum and out-of-focus fibrils on top of the cell (top center, D) show the inverse staining pattern.

The other four panels show good correspondence between anti-actin and anti-fibronectin. In B and C, arrows indicate microfilament bundles that terminate before the cell edge. Corresponding arrows in E and F indicate the fibronectin fibrils continuing beyond cell boundaries.

In all panels, focus was at the bottom of the cell. Other arrays of actin microfilaments at higher planes of focus did not correspond with fibronectin staining, as seen clearly in B.

Bar = 50 μm. (From Hynes and Destree, 1978, with permission.)

sion in a dose-dependent fashion (Section 3.1). The failure to observe inhibition of migration at higher doses of fibronectin may reflect an inability to increase adhesion to a sufficient degree, e.g., because the substratum or cell surface was saturated with fibronectin.

Directional movement stimulated by fibronectin *in vitro* has been reported in the presence of a gradient of fibronectin (Gauss-Müller et *al.*, 1980). Analo-

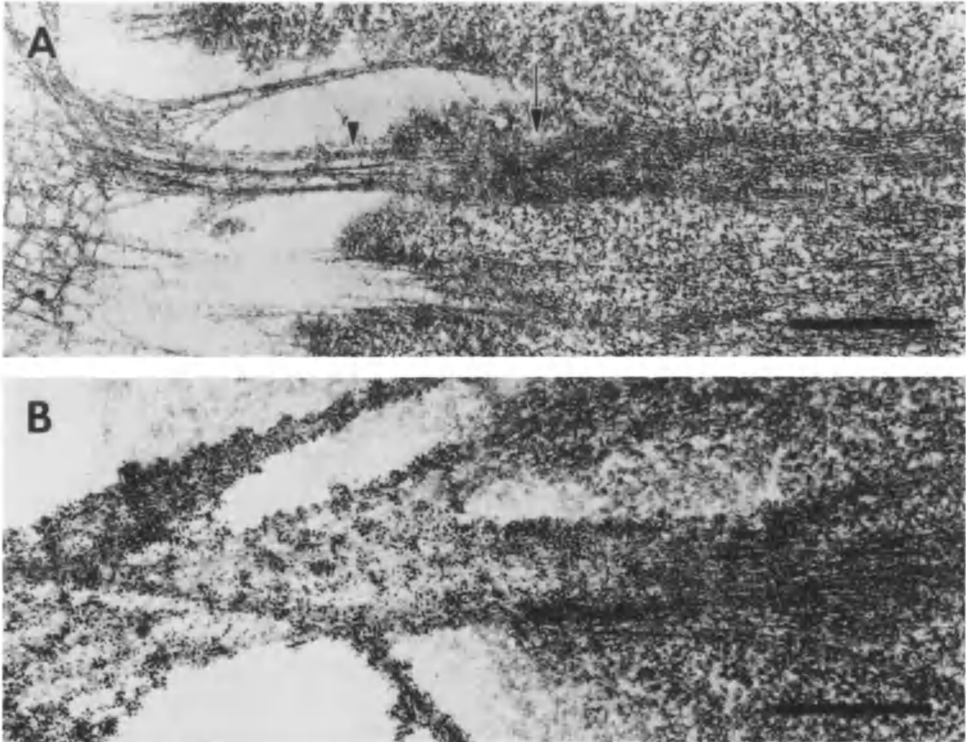

Figure 10-4. Electron microscopic visualization of the relationship between extracellular fibronectin fibrils and intracellular microfilament bundles in NIL8 hamster cells. (A) Grazing section from bottom of cell. Extracellular space is at left. Fibrils (arrowhead) approach membrane and appear colinear with microfilament bundle inside cell (arrow). (B) similar section from a culture stained for fibronectin by an indirect immunoferritin technique. Note the abundant ferritin staining of the extracellular fibrils. Bars = 0.5 μm. (Figures courtesy of Dr. Irwin I. Singer, reproduced from the 38th Annual Proceedings of the Electron Microscopy Society of America with permission.)

gies can be drawn with chemotaxis (directional movement stimulated by a gradient of a diffusible substance) or haptotaxis (directional movement stimulated by a gradient of substrate adhesiveness). We will consider these theories of directional movement in more detail in Section 5.2.3.

3.4. Phagocytosis

It has been proposed that plasma fibronectin can act as an opsonin, promoting phagocytosis by the reticuloendothelial system (Blumenstock et *al.*, 1978; Saba et *al.*, 1978a). This suggestion derives from *in vivo* evidence on clearance of particulate matter from the blood (Section 5.2.6). Because such particles are known to be ingested by Kupffer cells of the liver, a liver slice assay was developed to test for opsonic activity *in vitro*. Fibronectin promotes binding of

gelatin-coated lipid droplets or latex beads to liver slices (Blumenstock *et al.*, 1978; Molnar *et al.*, 1979). However, it is not clear whether the binding is to hepatocytes, endothelial cells, or Kupffer cells. Furthermore, in this assay system, there is no evidence for endocytosis as distinct from surface binding. Studies using pure populations of macrophages have demonstrated endocytosis of gelatin-conjugated latex by rat peritoneal exudate cells (Doran *et al.*, 1980; Gudewicz *et al.*, 1980) or cultured macrophagelike cells (Van De Water *et al.*, 1981). The binding and endocytosis are dependent on fibronectin and heparin.

So far, fibronectin-stimulated endocytosis by macrophages has been demonstrated only with gelatin-coated particles, and the role of heparin is obscure. However, earlier reports of binding of collagen and fibrin by macrophages in the presence of fibronectin suggest that the effect could be more general (Dessau *et al.*, 1978; Jilek and Hörmann, 1978). Fibronectin also binds to certain bacteria (Kuusela, 1978; Mosher and Proctor, 1980; Hynes and Destree, unpublished), raising the possibility that it could promote phagocytosis of bacteria. This has not yet been demonstrated.

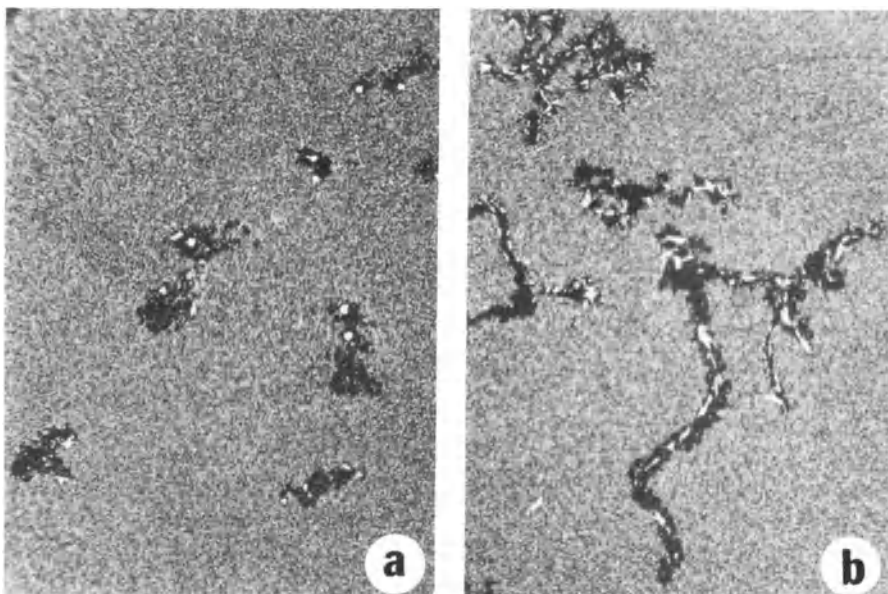

Figure 10-5. Stimulation of migration by addition of fibronectin. tsB77 rat 1 cells were plated on coverslips coated with gold particles either without (A) or with (B) the addition of fibronectin (50 μg/ml) from chick embryo fibroblasts. After culture for 24 hr, samples were fixed and viewed by dark-field microscopy. The gold particles appear as small white flecks; the cells remove the gold, leaving dark areas corresponding with their migration tracks. The cells ingest gold particles so that cells also scatter light and appear as large white specks. Fibronectin-treated cells (B) migrate over greater distances than controls (A). Fibronectin-treated cells also deposit aggregates of gold particles and fibronectin in their tracks. These show up as light-scattering material in (B) × 50. (From the work of Ali and Hynes, 1978b.)

Phagocytosis can be considered as being analogous with cell adhesion and spreading on planar substrates, the only difference being in the radius of curvature of the substratum. The attachment of particles to phagocytes, like that of cells to substrata, promotes submembranous organization of cytoskeletal elements (Reaven and Axline, 1973; Griffin et *al.*, 1976; Berlin and Oliver, 1978), which are also involved in the spreading of pseudopodia around the particle. Michl et *al.* (1979) have shown that endocytosis and spreading of macrophages involve common cell surface receptors. Therefore, it is reasonable to suggest that the involvements of fibronectin in the processes of cell adhesion and spreading and in phagocytosis reflect a common mechanism (Hynes, 1981). This idea is supported by the observation that cells that are not normally phagocytic will endocytose fibronectin-coated beads (Grinnell, 1980; Wagner and Hynes, unpublished).

3.5. Growth Control

It has been demonstrated that the degree of spreading of cells on their substratum *in vitro* can affect their rate of proliferation (Folkman and Moscona, 1978), and Rojkind et *al.* (1980), Vlodavsky et *al.* (1980), and Gospodarowicz et *al.* (1980) have shown that cell proliferation is promoted by the presence of extracellular matrix. As discussed above, fibronectin promotes cell adhesion and spreading and is a constituent of extracellular matrices. Consistent with these observations, it has been reported that serum fibronectin can also promote proliferation of some cell types. Fibronectin stimulates growth of ovarian follicular cells (Orly and Sato, 1979) and of embryonic carcinoma cells (Rizzino and Crowley, 1980). Indeed, fibronectin plus hormones can substitute for serum in supporting the growth of these cell types; in the absence of fibronectin no cell growth is observed. Similarly, certain cell types will grow in suspension with milk as a source of growth factors, but cells that do not grow in suspension will grow in milk only if it is supplemented with fibronectin, which allows them to attach and spread (Steimer and Klagsbrun, 1981).

It seems likely that further research will show this phenomenon of synergy between mitogenic hormones and cell attachment and spreading factors to be more general. A requirement for anchorage to a substratum is a common feature of normal cells that survive in culture. It appears that the requirement for anchorage and spreading and the requirement for serum factors or hormones are interrelated (Folkman and Moscona, 1978; O'Neill et *al.*, 1979; Gospodarowicz et *al.*, 1978, 1980). Transformed cells are somehow freed from this requirement for anchorage and/or serum (see Shin et *al.*, 1975).

The exact mechanisms of anchorage-mediated growth control are unclear, but Penman and co-workers (Benecke et *al.*, 1978; Farmer et *al.*, 1978; Ben Ze'ev et *al.*, 1980) have shown that cell attachment and spreading can have significant and different effects on intracellular processes such as protein synthesis and RNA metabolism *in vitro*. Culture of cells on extracellular matrices can

also greatly alter their responses to well-defined growth factors. For example, corneal epithelial cells cultured on plastic respond to FGF but not to EGF (Gospodarowicz et *al.*, 1978), whereas when cultured on collagen (Gospodarowicz et *al.*, 1978) or on fibroblast feeder layers (Sun and Green, 1977), the same cells respond to EGF as they do *in vivo*. In addition, when cultured on these matrices, corneal epithelial cells differentiate extensively; thus, the matrix is affecting the phenotype of the epithelial cells. To date, only a few attempts to substitute for the growth-promoting activity of extracellular matrices by individual purified matrix components (collagens and fibronectin) have been successful (see Chapter 12), but further work with combinations of components is obviously required. We will return to a further consideration of this question in Section 5.2.2.

3.6. Cellular Differentiation

Fibronectin affects cellular differentiation in several *in vitro* systems. As mentioned earlier (Section 2), both the myogenic and the chondrogenic lineages apparently lose fibronectin during differentiation. Cultured myoblasts have surface fibronectin; as the cultures become denser, the amount of fibrillar matrix of fibronectin increases (Hynes et *al.*, 1976; Chen, 1977; Walsh et *al.*, 1981), but as myotubes differentiate they lack fibrillar surface fibronectin (Chen, 1977; Furcht et *al.*, 1978; Walsh et *al.*, 1981) and there is a drop in amount of fibronectin at the time of fusion (Chen, 1977; Podleski et *al.*, 1979). Addition of exogenous fibronectin to rat myoblast cultures inhibits fusion, whereas antibody to fibronectin promotes fusion (Podleski et *al.*, 1979). These results suggest that fibronectin, exogenous or endogenous, can interfere with myogenesis. In contrast, Chen et *al.* (1978) have reported that an extracellular matrix containing fibronectin accelerates myoblast fusion. At present, it is not possible to reconcile this observation with the others, although the results could reflect different effects of fibronectin at different stages (see Section 5.2.2). The balance of the evidence suggests that fibronectin is normally lost at the time of fusion and that its continued presence is inhibitory to myogenesis.

Similarly, as chondrocytes differentiate *in vitro* and begin synthesis of their characteristic extracellular matrix, they lose fibronectin (Dessau et *al.*, 1978). Reduced amounts of fibronectin may be due to a reduction in capacity to bind, rather than reduction in synthesis of fibronectin (Hassell et *al.*, 1979). Inhibition of chondrocyte differentiation by culture conditions (Dessau et *al.*, 1978) or by vitamin A (Hassell et *al.*, 1979) leads to accumulation of fibronectin. Chondrogenesis can also be directly affected by exogenous fibronectin. Addition of fibronectin causes chondrocytes to flatten, reduce their synthesis of sulfated proteoglycans and type II collagen, and reacquire matrices of fibronectin and type I collagen (Pennypacker et *al.*, 1979; West et *al.*, 1979).

Therefore, as mesenchymal cells differentiate to cartilage or muscle, fibronectin synthesis and/or accumulation are decreased. In contrast, differentiation

to fibroblasts is associated with a continued high level of fibronectin. Such high levels are inhibitory to chondrogenesis and myogenesis. Fibronectin effects may be reflected in the various "dedifferentiation" phenomena that have been reported (e.g., Coon, 1966), in which chondrocytes or myoblasts appear to become flattened and "fibroblastic."

4. Fibronectin Distribution *in Vivo*

There are three major locations of fibronectin *in vivo*. Fibronectin appears as a soluble glycoprotein in bodily fluids, including plasma (300 μg/ml), cerebrospinal fluid (3 μg/ml; Kuusela et *al.*, 1978), and amniotic fluid (60–80 μg/ml; Crouch et *al.*, 1978). The cellular source of plasma fibronectin is not known, but is probably endothelial cells or hepatocytes. Endothelial cells are known to synthesize large amounts of fibronectin *in vitro* (Table I), but it is not known whether this is of the plasma type. One report of fibronectin synthesis by hepatocytes (Voss et *al.*, 1979) has not yet been confirmed. The source of cerebrospinal fluid is similarly unclear. Amniotic fluid fibronectin is larger than plasma or most cellular fibronectins and, as we have seen, is synthesized by cultured amniotic epithelial cells (Table I).

The second major location of fibronectin is in basement membranes. Fibronectin first appears at the blastocyst stage in mouse development in the inner cell mass and in Reichert's membrane between the ectoderm and the primitive endoderm (Zetter and Martin, 1978; Wartiovaara et *al.*, 1979). Teratocarcinoma cell aggregates developing *in vitro* are analogues of inner cell masses, and fibronectin is synthesized *in vitro* by lines of endodermal cells derived from teratocarcinoma cells (Table I). Fibronectin is also found in the area of mesoderm formation, separating the mesoderm from both the ectoderm and the endoderm (Wartiovaara et *al.*, 1979). As development proceeds, fibronectin is commonly found at such interfaces between different cell layers.

Although there are some discrepancies in the literature, most basement membranes appear positive for fibronectin by immunofluorescence (see Linder *et al.*, 1975, 1978; Schachner *et al.*, 1978; Matsuda et *al.*, 1978; Stenman and Vaheri, 1978; Quaroni et *al.*, 1978; Thesleff *et al.*, 1979; Couchman et *al.*, 1979; Kurkinen et *al.*, 1979; Krieg et *al.*, 1979; Wartiovaara et *al.*, 1979, 1980; Alitalo *et al.*, 1980b; Courtoy et *al.*, 1980; Mayer *et al.*, 1981). In the next section, some specific locations will be considered in the context of functions of fibronectin *in vivo*, but it is worth pointing out a few generalities. The basement membranes beneath endothelial cells of most or all blood vessels contain fibronectin. Most epithelial cells, including those of the small and large intestines, stomach, bronchi, lung, kidney, and many glands, are attached to basement membranes that contain fibronectin and that separate the epithelial layer from underlying mesoderm. Muscle cells, both striated and smooth, are surrounded by fibronectin.

The third location in which fibronectin is found is in loose stromal connective tissues throughout the body and in the capsules and sheaths of muscles,

nerves, cartilage, spleen, lymph nodes, blood vessels, etc. (Linder *et al.*, 1975, 1978; Stenman and Vaheri, 1978; Dessau *et al.*, 1978; Couchman *et al.*, 1979). Here, it may be associated with collagen fibrils.

The sources of the basement membrane and connective tissue fibronectins are in most cases not known, nor are their exact molecular natures. Most of the data come from immunofluorescence and a small amount of immunoelectron microscopy (Schachner *et al.*, 1978; Couchman *et al.*, 1979; Courtoy *et al.*, 1980; Mayer *et al.*, 1981). From this description, it can be seen that fibronectins are widely distributed in the body. However, apart from their locations, we know little about them. Speculations about the functions of fibronectin in these various locations *in vivo* are necessarily based largely on information obtained with *in vitro* systems (Sections 2 and 3). Section 5 will attempt to synthesize such speculations with a view to suggesting testable hypotheses of the functions of fibronectins *in vivo*.

5. Possible Functions of Fibronectin *in Vivo*

5.1. Fibronectin as a Molecule with Multiple Binding Sites

Structural analyses of fibronectin in parallel with studies of its binding affinities *in vitro* have lead to the current picture of fibronectin as a series of domains with different binding sites strung together in a flexible and asymmetric molecule (see Chapter 4 and reviews by Mosher, 1980; Yamada *et al.*, 1980, Hynes, 1981). For purposes of this discussion, Fig. 10-6 shows the arrangement of several of the known binding sites. Other important binding sites that are not yet accurately positioned on the molecule are those for heparin and hyaluronic acid and for gangliosides, and other binding activities of less obvious significance (actin, DNA) are also known (see Chapter 4).

Given fibronectin's known structure, it is easy to see how it might function as an adhesive ligand between different extracellular matrix molecules and between cells and matrices (Fig. 10-7). Fibronectin is known to form ternary aggregates with fibrin and heparin and with collagen and heparin (Chapter 4). This suggests that fibronectin might be involved in the structural organization of extracellular matrices including basement membranes, acting for instance as a cross-linking agent between collagens, heparan-containing proteoglycans, and hyaluronic acid (Fig. 10-7A). Fibronectin has high-affinity binding sites for each of these macromolecules as well as the potential for covalent bonding to collagen (demonstrated *in vitro*) or to proteoglycans (hypothetical disulfide bonding). In culture, fibronectin has been shown to codistribute with collagen and with sulfated proteoglycans (Section 2), and all these macromolecules can be found together in various extracellular matrices (e.g., kidney glomerular basement membrane; see Chapter 11). An example of such a codistribution *in vivo* is shown in Fig. 10-8 (see also Fig. 4-1). Future studies will investigate more precisely the nature of the supramolecular order derived from interactions between the constituent macromolecules.

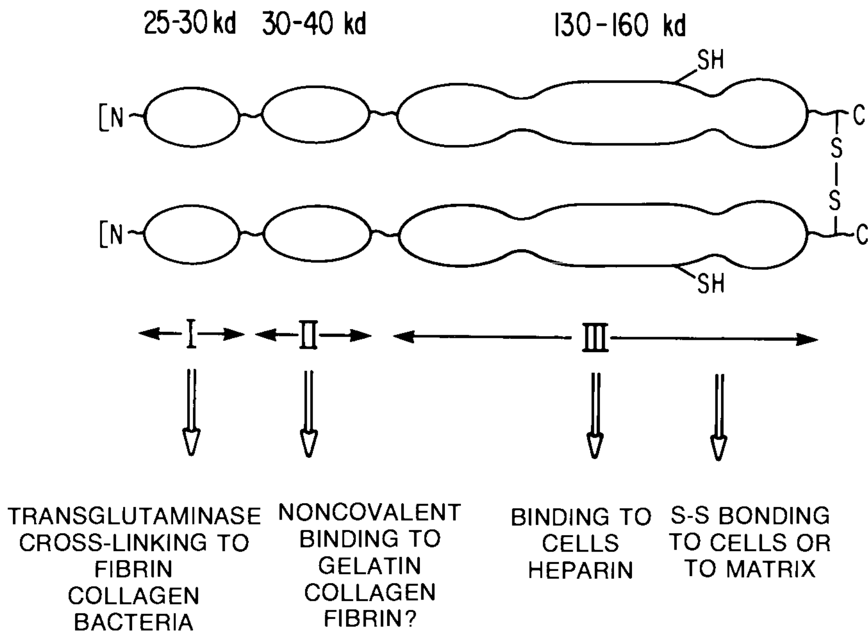

Figure 10-6. Structure of fibronectin showing functional domains. The intact molecule is elongated but two globular domains (I and II) can be derived from it by proteolysis. The evidence for the different binding activities is reviewed in Chapter 4. Binding of domain II to fibrin is inferred only from competition of fibrin versus gelatin binding. The size of the larger piece derived from proteolysis (III, 130–160 kd) differs among different reports. The exact locations of cell- and heparin-binding sites within domain III are not known.

There are also other, minor constituents of extracellular matrix. One is the acetylcholinesterase of muscle, which is present in the basal lamina in the synaptic cleft of neuromuscular junctions (McMahan *et al.*, 1978). As mentioned earlier (Section 4), fibronectin is a constituent of the basement membrane around muscle fibers, although it is not known whether it is present within the synaptic cleft. Acetylcholinesterase occurs in several forms, one of which is asymmetric and has a collagenlike tail (Bon *et al.*, 1979). The affinity of fibronectin for collagen raises the possibility that acetylcholinesterase might be bound to the basement membrane via fibronectin. Consistent with this idea, it has recently been shown that fibronectin will bind *in vitro* to the asymmetric (but not to the globular) acetylcholinesterase and can be cross-linked to it by plasma transglutaminase (Emmerling *et al.*, 1981). While these results do not prove that the same thing happens *in vivo*, they raise the possibility.

The presence of cell-binding sites in fibronectin suggests that it could act *in vivo* as a ligand between cells and extracellular matrices (Fig. 10-7B). It clearly can *in vitro*, as shown by the various cell-binding assays (Section 3.1). The little that is known about the arrangement of fibronectin at the boundaries

between cells and matrices is consistent with its serving such a role *in vivo*. As discussed in Section 4, fibronectin is seen in many or most basement membranes at the resolution of the light microscope. In the few cases where the arrangement of fibronectin and other constituents has been studied at the electron microscopic level, fibronectin has been found not only in the internal, but also in the external laminae rarae of the basal lamina, i.e., adjacent to the cells (Couchman *et al.*, 1979; Courtoy *et al.*, 1980; Mayer *et al.*, 1981). Perhaps the clearest case is the glomerular basement membrane of the kidney, which lies between epithelial cells (podocytes) and endothelial cells. Both laminae rarae stain for fibronectin, and heparan sulfate is also found along the surfaces of the glomerular basement membrane, whereas collagen type IV appears to be present in the central lamina densa (see Chapter 11). These patterns are consistent with a molecular arrangement such as that shown in Fig. 10-7B, although they by no means prove that it occurs. It seems likely that such arrangements involving fibronectin (or other cell-attachment molecules such as laminin) as ligand(s) between cells and various extracellular matrices will turn out to be very important in tissue organization, both static and dynamic.

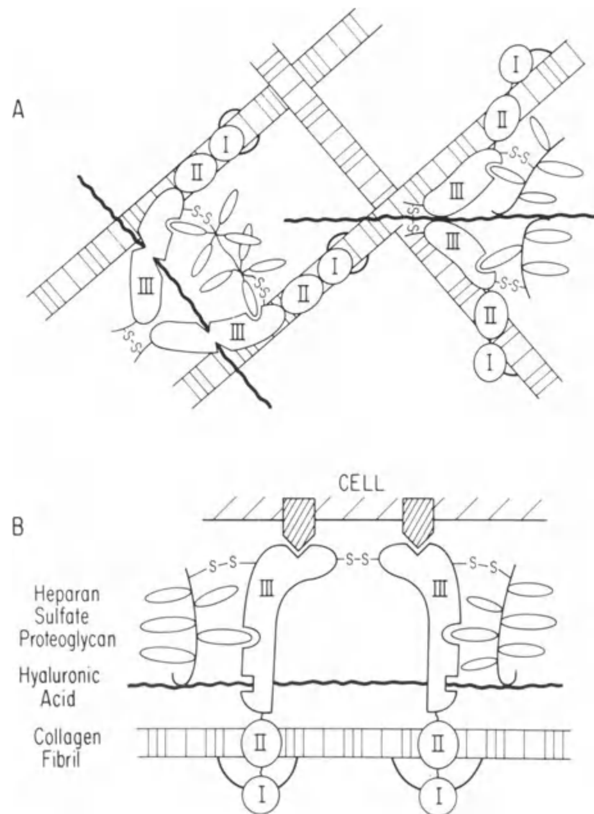

Figure 10-7. Schematic diagrams of possible ligand functions of fibronectin. The diagrams are based on the structure of fibronectin shown in Figure 10-6. The binding sites for heparan sulfate, hyaluronic acid, and cell receptor have deliberately been placed in arbitrary positions in the different diagrams because their exact locations are not known.

(A) Two possible interactions of fibronectin with collagen fibrils, heparan sulfate, proteoglycans, and hyaluronic acid. In each case, domain II binds noncovalently to the collagen and domain I is cross-linked via a transglutaminase-induced cross-link. Domain III binds both glycosaminoglycans noncovalently and is depicted bonding by disulfide to the polypeptide of the proteoglycans. The two arrangements could correspond to parallel and orthogonal collagen fibrils.

(B) Possible function of fibronectin as a cell–matrix ligand. Interactions with matrix components as in (A) plus interaction with a cell-surface-binding site are postulated. Such an arrangement would allow fibronectin to bind the cell to the matrix.

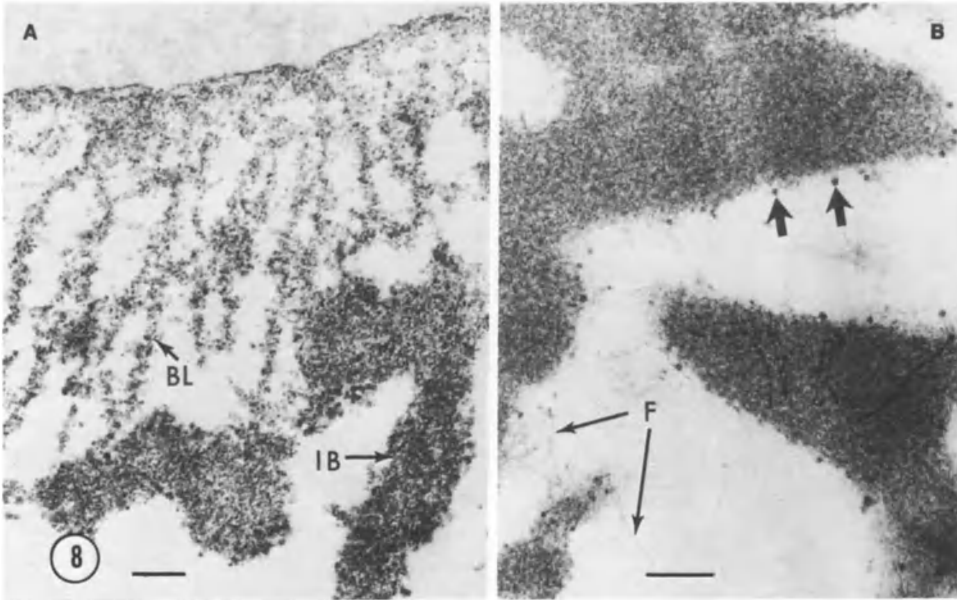

Figure 10-8. Codistribution of fibronectin and glycosaminoglycans in the area vasculosa of stage 12 chicken embryos. (A) Immunoperoxidase labeling of fibronectin in the basal lamina (BL) and interstitial bodies (IB). Bar = 165 nm. (B) Ruthenium red staining of proteoglycan granules (arrows). Data are from Mayer *et al.* (1981). F, small fibrils. Bar = 200 nm.

Let us briefly consider the formal possibilities for ligand-mediated interactions. In the static case, such as a typical epithelial cell on a basement membrane, there are three basic requirements.

1. The cell must have a *surface receptor* for the ligand.
2. There must be a *source of ligand*. This source can be local, either the cell itself *(cis)* or the cells across the basement membrane *(trans)*; or it can be a distant source such as plasma fibronectin *(exogenous)*. All that is necessary is that the supply be sufficient to replace ligand lost by turnover.
3. The *other matrix constituents* must be present at a sufficient and stable level. Again local or exogenous supplies are conceivable.

So long as the status quo with respect to these requirements is maintained, the situation will be stable. Obviously, if the cell loses its receptor, or if the ligand or one of the other constituents becomes limiting, then the situation will change: the interaction of the cell with the matrix and its metabolism (see Section 5.2) will be altered. In the extreme case, the cell will detach from the matrix, but more subtle intermediate changes are possible by changing the levels or nature of the ligands. It is also possible to conceive of spatial inhomogeneities in the matrix and ligands or in the responses of cells to them. Leaving aside for the present the question of how such inhomogeneities arise, one can

see how they might produce differential cellular behavior. Spatial inhomogeneities, either alone or in concert with temporal changes, can also lead to translocations of cells (see Section 5.2.4).

This formal discussion of possible models for cell–matrix interactions mediated by fibronectin or similar molecules is intended as a framework for consideration of possible functional roles of fibronectin in the following sections. These sections will deal with fibronectin, but many of the same ideas may be applicable to similar molecules.

5.2. Possible Effects of Fibronectin on Cellular Behavior

5.2.1. Adhesion and Morphology

As we know that fibronectin promotes cell–substratum adhesion *in vitro* (Section 3.1) and is present in many basement membranes *in vivo*, it is logical to suggest that it performs a similar function *in vivo*, although there is little direct evidence for this. Quaroni *et al.* (1978) point out that the amounts of fibronectin detected by immunofluorescence in the basement membrane of the small intestine decrease toward the tip of the villus, where in fact the cells detach. Because most epithelial layers are stable, one normally expects to see little change. However, during development one might predict alterations in adhesiveness of the cells to the underlying matrix correlating with alterations in amounts of fibronectin present. If one could observe such correlations it would add weight to the argument. The studies to date on fibronectin during the development of specific epithelia have all been by indirect immunolocalization methods (Wartiovaara *et al.*, 1979; Kurkinen *et al.*, 1979; Thesleff *et al.*, 1979), which provide only nonquantitative information, and we are also hampered by lack of direct information concerning adhesiveness of the cells to the basement membrane.

Another, possibly related, effect that basement membranes or extracellular matrix are thought to have on cells is in the organization of intracellular cytoskeletal elements. As discussed in Section 3.2, fibronectin has such effects *in vitro*. Cytoskeletal organization *in vivo* is known to change during the development of many epithelial tissues (see Chapter 12). Does local fibronectin distribution or amount alter at the same times? We do not know yet, but this is an answerable question. Some observations are available for one developing system that has been much studied over the years, the avian embryonic cornea. The cornea develops in response to inductive stimuli from the lens. Induction can be triggered by various extracellular matrices (see Chapter 12). Kurkinen *et al.* (1979) have described the changing distribution of fibronectin in the eye. Sugrue and Hay (1980) have shown that fibronectin affects the arrangement of microfilaments in the basal regions of corneal epithelial cells explanted onto various substrata (Section 3.2). More studies of this type will be necessary to investigate the effects of various matrix constituents, singly and in combination, on the adhesion and morphology of epithelial cells.

5.2.2. Growth and Differentiation

In Section 3.5, we discussed the *in vitro* effects of fibronectin on growth of a few cell types and considered the possibility that this might be related to the ability of fibronectin to promote adhesion and spreading on the substratum. Similarly, basement membranes may have effects on basal epithelial cell proliferation that could be related to their effects on adhesion and morphology discussed in the previous section. It seems likely that further analysis of this possibility will also require more *in vitro* work on the effects of various extracellular matrix constituents on proliferation of cells. Early work along these lines includes the work of Folkman and colleagues on the effects of cell shape on growth, of Penman and colleagues on the effects of adhesion and spreading on intracellular metabolism, and of Reid and Gospodarowicz and colleagues on the effects of extracellular matrices on cell behavior (Section 3.5). While none of these studies directly analyzes the *in vivo* effects of a relevant basement membrane or matrix on proliferation, they may all have a bearing on this question that is difficult to analyze *in situ*.

Also possibly related to the questions of adhesion and spreading are the effects of basement membranes or other extracellular matrices on cellular differentiation. In Section 3.6, we discussed several systems whose differentiation *in vitro* is affected by fibronectin. In both chondrogenesis and myogenesis, fibronectin is found associated with the cells early in the developmental sequence, but later on endogenous fibronectin is present in reduced amounts and exogenous fibronectin is inhibitory to further development. In the case of chondrocytes at least (Section 3.6), altered response of cells to an extracellular matrix component seems to be effected by a change in cellular receptor rather than by a change in amount of the ligand itself (see Section 5.1). In contrast with this model, Newman and Frisch (1979) have presented a system that postulates that the patterns of formation of chondrogenic promordia during limb development *in vivo* could be governed by the concentration of a diffusible matrix constituent such as fibronectin. They suggest that local concentrations might promote cell–cell aggregation leading to chondrogenic nodules. In theory, one could combine the two hypotheses in sequence: a first stage of differentiation where fibronectin promotes cell–cell aggregation, followed by a change in the cells such that they no longer bind fibronectin and are able to proceed with chondrogenic differentiation.

An *in vivo* system where further analysis of these ideas may be possible is the formation of cartilage and bone in reponse to implanted demineralized bone matrix. Mesenchymal cells invade the matrix, differentiate into chondroblasts, and produce cartilage matrix. Following vascularization, chondrolysis, bone formation, and hematopoiesis ensue. This sequence is induced by the implanted matrix. Early in the sequence, plasma fibronectin binds to the matrix and accumulates during mesenchymal cell proliferation (Weiss and Reddi, 1980). At a later stage when chondrogenesis is underway, fibronectin is less conspicuous. These results are discussed in more detail in Chapter 12.

Obviously, to proceed further in understanding the role that matrix molecules play in affecting cellular differentiation, it will be necessary to learn more

both about the *in vitro* effects of constituents such as fibronectin on differentiation and about their temporal and spatial distributions in developing systems such as limb buds and epithelial glands.

5.2.3. Migration

As reviewed in Section 3.3, fibronectin promotes cellular migration *in vitro* (Fig. 10-5; Ali and Hynes, 1978b). Could it be involved in promoting or directing cellular migration *in vivo*? Before considering the evidence that exists for an involvement of fibronectin migration *in vivo*, it is worth considering the requirements for an involvement of a matrix component in translocation of cells. As with simple adhesion of cells to a basement membrane (see Section 5.1), the cells must have a receptor for the matrix component. However, in order for translocation to occur, there must be a differential distribution of the matrix component, either spatial or temporal, or both. Several possible mechanisms can be envisioned.

In the simplest model, there is an arrangement of matrix components, say fibronectin, along the track of cellular migration, but there is no gradient of concentration. If the cells start at one position, have an affinity for fibronectin, and respond to it by increased migratory activity, then they will migrate along the track. Preferential adhesion to the fibronectin will discourage the cells from wandering off the track into surrounding tissues. Because fibronectin forms fibrils that could be arranged along a given axis, they could also contribute "contact guidance," a phenomenon whereby asymmetries in the substratum cause cells to align and migrate parallel with them (Weiss, 1961).

This sort of simple track model may be sufficient to explain certain cell migration systems, such as the neural crest, where cells start in one location and migrate along well-defined paths. One might ask how it can be arranged in such a model that the cells do not move backwards along the same path. Two simple possibilities are (1) that cells show persistence of directional movement and rarely double back on their tracks (see Gail and Boone, 1970; Albrecht-Buehler, 1977), or (2) that population pressure of cells following behind, through contact inhibition of movement, prevents cells from turning back. However, if these possibilities prove insufficient to explain unidirectionality of movement, one could postulate modifications of the model to provide directionality. Obvious possibilities are a vectorial component in the track, or a temporal variation in concentration of the migration-inducing component. A vectorial component could arise from a gradient in concentration of fibronectin or of another constituent. Cells would move up a gradient of a positive effector such as fibronectin (positive haptotaxis; see Carter, 1967) and down a gradient of a negative effector. Vectors could theoretically also be derived from assymmetric molecules. Collagen fibrils have an intrinsic polarity, as may fibronectin (see Fig. 10-6). Such intrinsic vectors in the track could in principle be sensed by the cells. Temporal changes in the track could also be postulated: for example, it could be laid down in sequence ahead of the cells; the cells would then continually be advancing onto a newly laid track. To prevent backtracking, the

track could be removed behind the cells, either by cells of the surrounding tissues or by the migrating cells themselves, which could destroy the track as they advanced.

A final possibility, which differs somewhat from those discussed above, is that cells might generate their own fibronectin and lay their own tracks as they proceed. However, for such a migration system to produce directional movement, it is necessary that there be a prior track of fibronectin-binding sites in the matrix. Formally, this model does not differ from the others. The difference is only that the cell brings both receptor and ligand, and the track consists only of the other matrix components to which the ligand binds rather than including the ligand as well. That is, the source of ligand is "cis" rather than "trans" or exogenous (see Section 5.1).

Having considered the possible properties of the matrix tracks that cells might follow, it is necessary to ask what determines when cells start and when and where they stop migrating. Starting could be triggered by the appearance of the track or by a change in the cells, which either (1) could become freed from their prior attachments, thus allowing them to respond to the track; (2) could acquire a cellular receptor for the stimulatory ligand only when they commence migration; or (3) both. Stopping could also be signaled by the track's end, by loss of the cells' ability to respond to the track, or by the cells' strong adhesion to the target area.

Thus, it can be seen that a molecule such as fibronectin, which may be involved in promoting cellular migration *in vivo*, can be expected to be present when and where migration occurs, but need not be there only at the time of migration and need not be present in a gradient of concentration. With this in mind, let us now consider the data available for the presence of fibronectin in association with cellular migration *in vivo*. Other matrix molecules that may affect migration are discussed in Chapters 9 and 12.

During gastrulation in chicken embryos, fibronectin can be detected on the ventral surface of the ectoderm by immunofluorescence (Fig. 10-9; Critchley *et al.*, 1979). The distribution correlates in a striking fashion with areas of cell migration: (1) the streaks in the area pellucida lie in the area and direction of mesenchymal migration from the primitive streak; (2) the network around the blastoderm margin is in a region of centrifugal expansion of the blastoderm across the vitelline membrane; and (3) a strong band of fibrils in the germinal crescent on the border between area opaca and area pellucida is an area of both active mesodermal cell migration and primordial germ cell migration.

All three regions conform to our discussion of tracks. In particular, the fibrillar band in the germinal crescent region appears immediately prior to Hamburger–Hamilton stage 5, when mesodermal cells enter this region. Because mesodermal and primordial germ cells proceed in opposite directions along this fibrous band, then either the track has no polarity and directionality arises from other sources, or the two cell types are responding to different vectors or respond differently to a single vector. Finally, the fibrillar band becomes disorganized after the entry of the mesodermal cells.

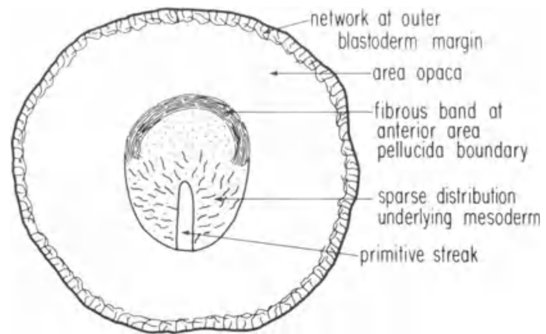

Figure 10-9. Locations of fibronectin on the ventral surface of the blastoderm of the chicken gastrula. Mesodermal cells migrate out from the primitive streak, across the ectodermal surface in the area pellucida, and around the fibrous band at the anterior edge of the area pellucida. Primordial germ cells also migrate along this fibrous band. The blastoderm margin is expanding centrifugally. Fibronectin is found in all these areas of cell migration. (From Critchley *et al.*, 1979.)

At a slightly later stage (2 days, stage 12, 16 somites), fibronectin is found in several areas of active cell migration (Mayer *et al.*, 1981). Of particular interest is the presence of fibronectin in interstitial bodies, extracellular matrix bodies that are characteristic of embryos. These interestitial bodies also contain proteoglycans (Fig. 10-8; Mayer *et al.*, 1981). Interstitial bodies containing fibronectin are found in the neural crest pathways, lateral to the neural tube and dorsal to the somites, and neural crest cells make contact with these bodies (Mayer *et al.*, 1981). There is currently no evidence for variations in concentration of fibronectin along the pathway prior to migration of the crest cells; cells implanted in the path at an early stage will migrate in either direction. Thus, we might propose that the track has no vectorial component but merely promotes or guides migration.

The area vasculosa at the same stage is another region of active cell migration. This region is the descendant of the blastoderm margin of the gastrula where both cell migration and fibronetin are observed (Fig. 10-10). At stage 12, the area vasculosa comprises a circumferential blood vessel (sinus) whose radius increases with time. The sinus is preceded by mesenchymal cells that migrate on the basal lamina of the epiblast (Fig. 10-10, insert). In the region of mesenchymal cell migration, the basal lamina is highly convoluted and enriched in interstitial bodies (Fig. 10-10). Both these extracellular matrix materials stain strongly for fibronectin (Fig. 10-8; Mayer *et al.*, 1981). In this case, therefore, there appears to be a temporal/spatial variation in a fibronectin-containing extracellular matrix, apparently involved in cell migration.

At a still later stage of development, fibronectin is found in the extracellular matrices of the eye in two areas of cellular migration (Kurkinen *et al.*, 1979). Two successive cell migrations occur in the development of the cornea. The first migration wave occurs at stages 22–25, when endothelial cells migrate along the posterior surface of the primary corneal stroma (see Chapter 12). This surface of the stroma, but not the rest, stains for fibronectin before the endothelial cells enter (see Fig. 10-11). These observations are consistent with the layer of fibronectin-positive matrix acting as a track for migration. At stage 28–30, mesenchymal cells invade the primary stroma, which, until this stage,

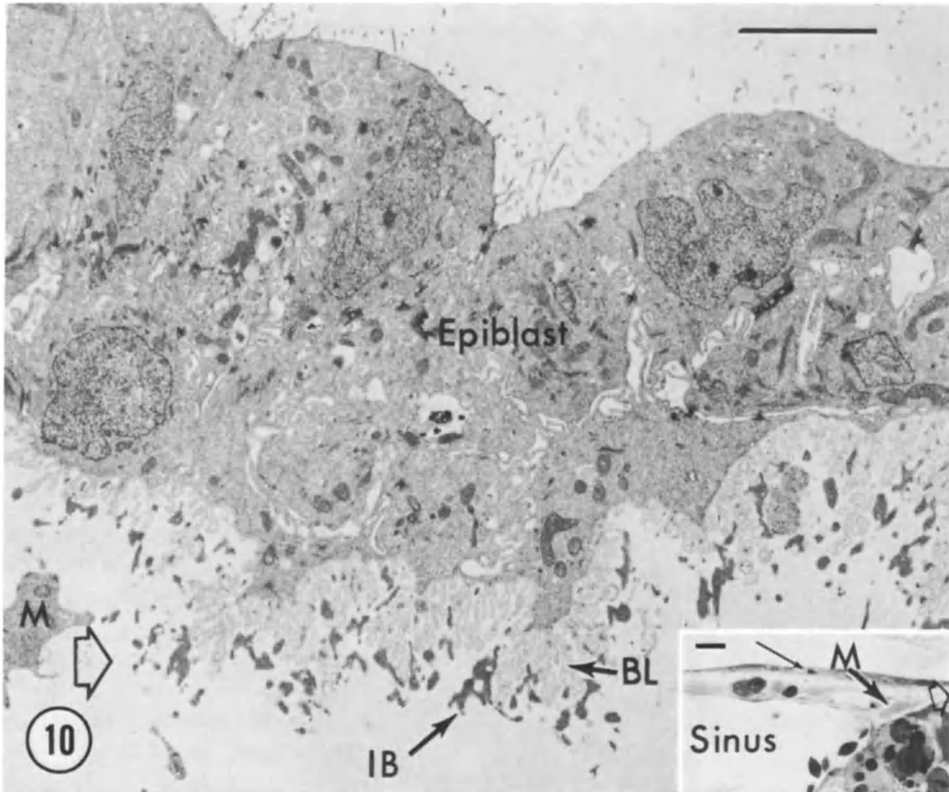

Figure 10-10. Area vasculosa of 2-day-old chicken embryo. Inset shows low-power light micrograph of terminal sinus expanding (open arrowhead) between the epiblast (thin arrow) and the yolky endoderm. Mesenchymal cells (M) precede the sinus. Bar = 10 μm.

The main micrograph is an electron micrograph of epiblast from a position such as that marked by the open arrowhead in the inset. The endoderm has been removed. Note the convoluted basal lamina (BL) and many heavily stained interstitial bodies (IB). Bar = 4 μm. A similar section stained for fibronectin is shown in Fig. 10-8A. (Data from Mayer *et al.*, 1981.)

is not rich in fibronectin. After the mesenchymal cells enter, the stroma becomes fibronectin-rich. This could be an example of cells bringing their own fibronectin with them and migrating along a matrix track of fibronectin-binding sites (Fig. 10-11).

In amphibian embryos, some information is available concerning two migrating cell types, neural crest cells and primordial germ cells. Neural crest cells appear to migrate on an extracellular matrix, the fibrils of which lie parallel to the direction of cell migration (Löfberg *et al.*, 1980). The biochemical nature of the fibrils is unknown, but preliminary results indicate the presence of fibronectin (Wylie, unpublished data) and glycosaminoglycans (Löfberg *et al.*, 1980) in the neural crest pathway of amphibians.

In the case of primordial germ cell migration, one area over which the cells of Xenopus embryos migrate *in vivo* is the gut mesentery. Primordial germ cells can also be explanted onto monolayer cultures of adult mesentery cells. Under these conditions, the primordial germ cells migrate over the surface of the mesentery cells and are aligned parallel to the actin bundles inside the cells (Wylie *et al.*, 1979). Biochemical and immunofluorescence studies of this system show that the cultured mesentery cells do produce fibronectin and lay down fibrils that also appear to align with the intracellular actin fibers (Heasman *et al.*, unpublished data). There results are consistent with the hypothesis that the primordial germ cells migrate along fibronectin fibrils laid down by the mesentery cells.

Fibronectin is reported (on the basis of immunofluorescence data) to be present in sea urchin blastulae at cell–cell boundaries (Spiegel *et al.*, 1980). Primary mesenchymal cells in this embryo migrate on the inner surface of the ectoderm by means of filopodia, which might attach to the matrix in the blastocoele via fibronectin.

It is important to note that, for all the systems discussed here, the only evidence for a functional role of fibronectin in the various cell migrations is circumstantial; fibronectin is present at the right time and place but has not been shown to promote migration. However, the observations do conform with the predictions of the models for matrix involvement in migration discussed earlier. Further analysis will require intervention experiments to test these hypotheses directly.

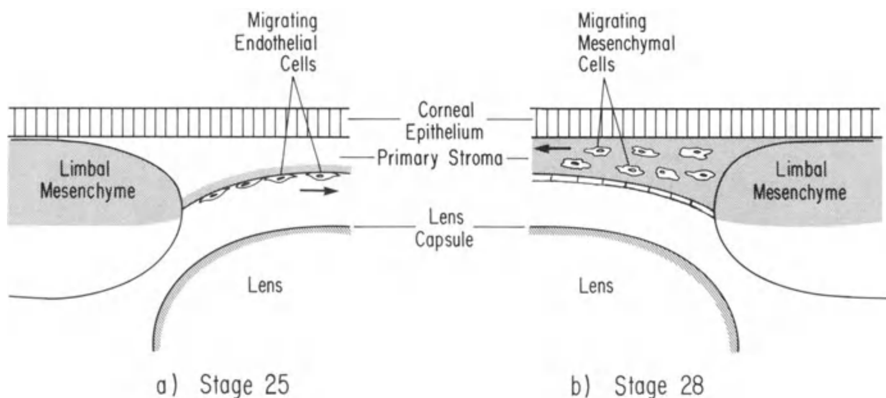

Figure 10-11. Distribution of fibronectin (shaded area) in the cornea during cell migration. (a) Stage 25. Endothelial cells migrate on the posterior surface of the primary stroma. This is the only part of the primary stroma positive for fibronectin, but limbal mesenchyme is also positive. (b) Stage 28. Endothelial layer is now complete. Mesenchymal cells migrate in from the limbal mesenchyme into the primary stroma, which becomes fibronectin-positive. The difference between the possible roles of fibronectin for the two migrating cell types might be viewed, according to the discussion of Sections 5.1 and 5.2.3, as follows. For the endothelial cells, the source of fibronectin in the track is "trans" or "exogenous," whereas for the mesenchymal cells, the source is "cis," i.e., produced by the cells themselves. (Figure is based on the immunofluorescence data of Kurkinen *et al.*, 1979.)

5.2.4. Malignancy

One of the prime initial reasons for interest in fibronectin was the observation that transformed cells *in vitro* lack or have greatly reduced amounts of surface fibronectin (see reviews by Hynes, 1976, 1979; Yamada and Olden, 1978; Vaheri and Mosher, 1978; Hynes *et al.*, 1979). Subsequent studies showed that loss of surface fibronectin generally correlates with acquisition of tumorigenic potential (see Chen *et al.*, 1976, 1980; Smith *et al.*, 1979; Asch *et al.*, 1981) although some exceptions were reported (Der and Stanbridge, 1978; Kahn and Shin, 1979). In particular, loss of surface fibronectin *in vitro* seems to correlate best with acquisition of metastatic potential (Smith *et al.*, 1979; Chen *et al.*, 1980; Asch *et al.*, 1981). Given the known effects of fibronectin *in vitro* (Section 3), and its likely effects on cells *in vivo* (Section 5), it is of interest to consider how altered levels of cell-associated fibronectin might affect malignant cell behavior *in vivo*.

A primary question concerns the reason for the observed lower levels of fibronectin. Biosynthesis of fibronectin by transformed cells is lower than that by normal cells, but the reduction in rate of synthesis is generally insufficient to account for the reduction in surface levels. Increased turnover rates and decreased ability of transformed cells to bind fibronectin (Hynes *et al.*, 1978; Wagner *et al.*, 1981) also contribute to the reduced surface levels, and some transformed cells continue to synthesize and secrete fibronectin at normal rates (Vaheri and Ruoslahti, 1975). The causes of the increased turnover and reduced binding are not yet understood but could include loss of receptors; production of degradative enzymes affecting either the receptor, the fibronectin, or another constituent (see Quigley, 1979); or alterations in interactions with the cytoskeleton (see Section 3.2 and Hynes, 1981). These mechanisms of loss are likely to have different consequences for cellular behavior in response to exogenous fibronectin. In cases where there is a major effect on the ability of the cells to bind fibronectin, addition of exogenous fibronectin will be unlikely to make much difference, whereas cells whose major alteration is in fibronectin synthesis will likely respond to supplementation with exogenous fibronectin, as has been reported for several cell types (Sections 3.1 and 3.2).

If we now turn to consider the consequences *in vivo*, it is clear that they will also differ depending both on the alteration in the transformed, or tumor, cell and on the situation in which the cell finds itself. Let us consider first the case of reduced biosynthesis. In most situations, a tumor cell is surrounded, at least initially, by normal cells, so reduced production of fibronectin by a few tumor cells will have little effect on the ambient level of fibronectin in the matrices around the cells. Major changes would only occur after the tumor mass had become significant. The exact point at which the depression becomes significant depends on the other available sources of fibronectin. However, in this situation it seems unlikely that reduction in fibronectin levels could be of much importance during early development of the tumor.

In contrast with this case, alterations in the ability of a cell to bind fibronectin would have significant consequences at an early stage. In the preceding sections we considered the possible effects of matrix, and in particular of

fibronectin, on cell adhesion to basement membranes, cell proliferation, differentiation, and migration. Insofar as these phenomena are affected by the interactions of the cell via the action of fibronectin as a ligand, an alteration in the cells' ability to bind fibronectin (i.e., altered or lost receptor) might be expected to cause major alterations in cellular behavior. The same would be true if the tumor cell produced an enzyme such as a protease that could destroy the receptor, the ligand, or other components of the matrix. This would produce local disruption of the cell–matrix interaction and consequent interference with the response of the cell to the influence of the matrix.

Hence, it is difficult to make generalizations about the expected alterations in behavior of malignant cells because (1) the alterations are likely to differ for different situations and (2) we are still at an early stage of understanding the effects of matrices and fibronectin on normal cells *in vivo*. Little is to be gained from detailed speculation, but let us consider a simple example. Take the hypothetical case of an epithelial cell that normally sits on a basement membrane containing fibronectin produced in part by the cell itself (cis) and in part by mesodermal cells across the basement membrane (trans). Assume the cell becomes transformed and loses its ability to bind to fibronectin, or alternatively, begins to secrete a protease that degrades fibronectin. The transformed cell will no longer attach firmly to the matrix of the basement membrane. It will probably retract, and round up, and could proliferate in an uncontrolled fashion because its growth responses are altered by its lack of attachment to the matrix (see Sections 3.5 and 5.2.2). The cell's ability to take on its characteristic differentiated phenotype would most likely also be altered. These are the characteristics of hyperplasia. In the case we are considering, these effects would all be less severe if the lesion were simply in the cis biosynthesis of fibronectin. However, in either case, subsequent effects could arise from complete detachment of cells from the primary tumor because of their reduced interactions with the matrix. If such detached cells were capable of migration, release from normal attachments to their neighbors or to local basement membranes might be sufficient to initiate cell migration (see Section 5.2.3). Erickson et *al.* (1980) have shown that some tumor cells respond to the neural crest migration pathway, so once a malignant cell is released it may have the potential to migrate over large distances. Ability to penetrate matrices by degradation or simply by inability to be retained by binding to the matrix could also contribute to invasion and metastasis.

The phenomena involved in malignancy are obviously complex, and further studies are necessary for a better understanding of these phenomena and of the normal interaction of cells with matrices.

5.2.5. Hemostasis and Thrombosis

A specific case of cell interaction with extracellular matrix that is of considerable importance is the interaction of the blood platelet with the basement membrane and connective tissue that are exposed when the endothelial layer lining blood vessels is injured. Platelets bind to the matrix, spread over it, and

undergo radical shape changes involving cytoskeletal reorganization. The altered platelets release a variety of soluble components, some of which lead to aggregation of further platelets onto the initially bound platelets, forming a "platelet plug." The clotting cascade is triggered, leading to formation of fibrin and its deposition on and around the platelets (Fig. 10-12).

What role(s) might fibronectin play in this process? Several of the cellular events that make up the sequence are ones in which fibronectin has been implicated in other cell systems. These include cell–matrix adhesion, cell spreading, cytoskeletal organization, and cell–cell adhesion. As it is known that fibronectin binds to fibrin and becomes cross-linked to it by factor XIIIa transglutaminase (Mosher, 1976), it seems likely that this interaction plays some role (see Fig. 10-12). There are several possible sources of fibronectin, both exogenous and endogenous to the platelets: it is present in large amounts in the endothelial basement membrane (Section 4) and in plasma. Evidence for surface fibronectin on platelets is controversial, but small amounts have been detected inside platelets, possibly in α granules (see Section 2.1).

A number of observations are consistent with an involvement of fibronectin at several stages of the process depicted in Fig. 10-12. Endothelial cells, both *in vivo* and *in vitro*, are nonthrombogenic (i.e., do not interact with platelets). In both situations, their dorsal or luminal surfaces are free of fibronectin (Birdwell *et al.*, 1978; Vlodavsky *et al.*, 1980). However, if the monolayer of cells is injured in either case, matrix containing fibronectin is exposed and is thrombogenic. Furthermore, culture variants of endothelial cells that do have a fibronectin-containing extracellular matrix on their dorsal surfaces are thrombogenic (Zetter *et al.*, 1978; Vlodavsky *et al.*, 1980). Basement membrane fibronectin would be an example of a trans source of ligand for platelet adhesion. Of course, the matrices, *in vivo* and *in vitro*, contain other constituents and it is not clear that fibronectin is the active ingredient.

Some experiments with simpler model systems do, however, support the idea that exogenous fibronectin might play a functional role. Platelets in whole plasma will bind to purified collagen sheets. They either spread or aggregate depending on the conditions. Such platelets become surrounded by fibrillar arrays that stain for fibronectin (Hynes *et al.*, 1978). If, instead, the platelets are allowed to interact with the collagen in fibronectin-depleted plasma, very little fibronectin appears around the adherent platelets and platelet spreading is inhibited (Hynes *et al.*, 1978; Grinnell *et al.*, 1980). These results suggest that exogenous plasma fibronectin can act under some conditions to promote platelet spreading on collagen matrices. However, endogenous (cis) platelet fibronectin could contribute to some of the steps, for platelets activated by thrombin release several intracellular products, including fibronectin (Plow *et al.*, 1979; Ginsberg *et al.*, 1980).

At present it is unclear which, if any, of the fibronectins–plasma, platelet, or basement membrane—contribute to the various steps of platelet adhesion, response, and aggregation, but one observation does suggest a role *in vivo*. In a family with a form of Ehlers–Danlos syndrome, a disease associated among other things with bleeding problems, there is some evidence for defective

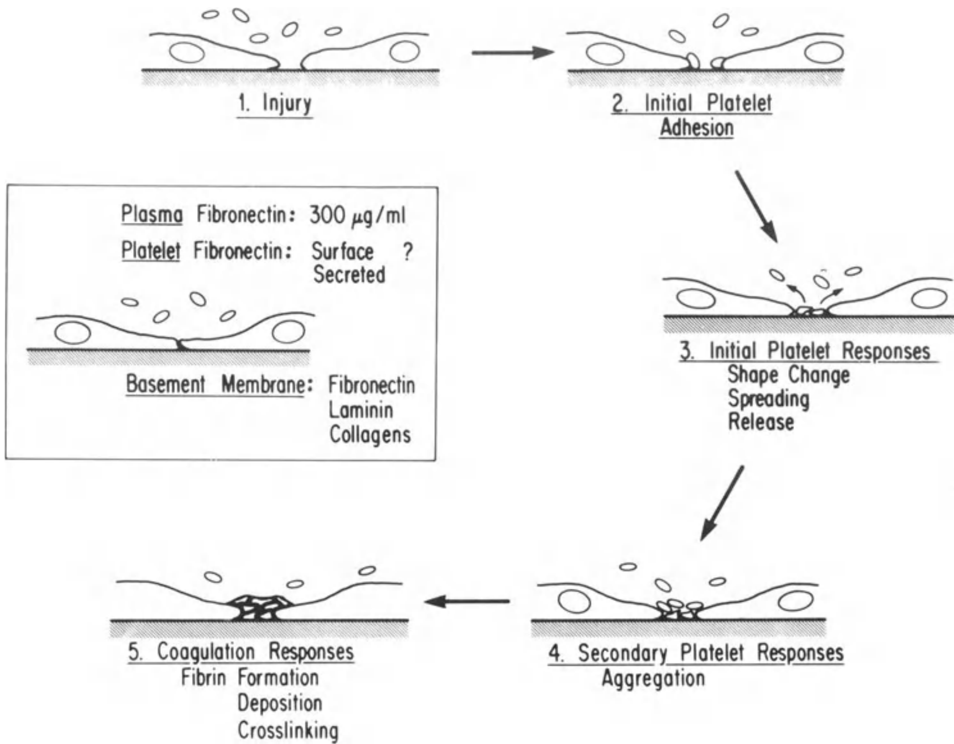

Figure 10-12. Schematic representation of the interaction of platelets with a damaged blood vessel wall. Possible sources of fibronectin are indicated. After injury of the capillary wall exposing the basement membrane (1), fibronectin might be involved in initial platelet adhesion to the basement membrane (2), platelet spreading and shape change (3) (which involve intracellular organization of actin microfilaments), platelet aggregation (4), or binding to fibrin and cross-linking by factor XIIIa transglutaminase (5). Analogous functions for fibronectin have been demonstrated in other systems.

fibronectin (Arneson *et al.*, 1980). Affected patients bruise and bleed easily, and in an *in vitro* assay their platelets fail to aggregate. If normal plasma fibronectin is added to the aggregation assay, the platelets do aggregate.

If indeed plasma fibronectin plays a role in hemostasis and thrombosis, this would provide an explanation for its presence in large amounts. Plasma fibronectin might also provide a source of fibronectin to bind to extracellular matrices in the body or act as an opsonin for the reticuloendothelial system. In the next section we will briefly consider this last possibility.

5.2.6. Phagocytosis

Opsonins are molecules that bind to particulate matter and render it palatable to phagocytic cells. This is a ligand function analogous with the cell–ligand–matrix interactions discussed in previous sections. The suggestion that

fibronectin acts as an opsonin was initially made by Saba and co-workers on the basis of *in vivo* studies of clearance of particulate matter from the blood (Saba *et al.*, 1978a; Blumenstock *et al.*, 1978; Saba and Jaffe, 1980). Levels of an $\alpha 2$ glycoprotein now known to be identical with plasma fibronectin generally correlate with the clearance rate of various gelatin-coated particles. *In vitro* studies have lent some support to the idea that fibronectin can act as an opsonin (Section 3.4).

Because fibronectin binds to collagen and fibrin, it is possible that as an opsonin it functions in clearance from the blood of debris derived from breakdown of extracellular matrices or fibrin clots. The ability of fibronectin to bind to actin and DNA could play a similar role in clearance of cellular debris. Preliminary indications that fibronectin binds to the collagenlike tail of the C1q component of complement (Isliker *et al.*, 1981) could also have a bearing. After complement-mediated cytolysis, C1q remains bound to the dead cells in addition to the C3b component of complement and immunoglobulins. C3 and the Fc region of immunoglobulins both act as opsonins for phagocytosis (Silverstein *et al.*, 1977), and perhaps a complex of fibronectin with C1q does also. Finally, fibronectin binds to various bacteria (Section 3.4), and some preliminary data show that treatment with cryoprecipitate of plasma, which contains fibronectin, can improve the condition of patients suffering from bacterial septicemia (Saba *et al.*, 1978b; Scovill *et al.*, 1978).

6. Concluding Remarks

It is clear from the chapters in this book that extracellular matrices play important roles in the behavior of cells. Extracellular matrices have been implicated in adhesion, morphology, proliferation, differentiation, and migration of normal cells and are probably involved in several aspects of the behavior of malignant cells.

Fibronectin is an extracellular matrix protein about which we know a fair amount, both in terms of structure and with respect to its effects on cellular behavior. While fibronectin is clearly not responsible for all the properties of extracellular matrices, it must be considered a candidate for involvement in each of them. This chapter has attempted to review the data on this subject critically and to discuss the ways in which fibronectin might be involved, with a view to identifying and clarifying the unanswered questions. As more information becomes available on other extracellular matrix glycoproteins, similar analyses will need to be applied to them. At present, fibronectin is the best characterized and provides a "handle" that should allow investigations both of functional aspects and of other matrix components. Only time and more work will determine which functions are attributable to which molecules.

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Chapter 11

The Glomerular Basement Membrane

A Selective Macromolecular Filter

MARILYN GIST FARQUHAR

1. Introduction

The basement membrane of the glomerular capillaries of the mammalian kidney has been a favorite object for studies on the structure, function, and composition of basement membranes because of its important biological role in glomerular filtration of macromolecules, and because of the relative ease with which it can be isolated for study, free from contamination by other extracellular matrix components. This chapter will review what is known about the characteristics of the glomerular basement membrane (hereafter abbreviated as GBM) and will compare wherever possible the available data with those on basement membranes from other sources.

1.1. What Are Basement Membranes and Where Are They Found?

To begin with, one should define the term *basement membrane*. Basement membranes are continuous sheets of extracellular matrix material composed of collagenous and noncollagenous glycoproteins and are found wherever cells (other than connective tissue cells) meet extracellular matrix. In effect, they are the natural substrates on which all cells (except connective tissue cells) grow. They closely adjoin and are the products of the overlying cell layers (e.g., endothelium, epithelium, or smooth muscle cells).

Prior to the use of the electron microscope in biological research, the term *basement membrane* was understood to refer to a PAS-staining layer (found in the glomerulus, under epithelia in the skin and internal organs, and around muscle fibers) that was visible in the light microscope. Since the introduction of the electron microscope, this term, which is often used interchangeably with *basal lamina* or *basement lamina*, is generally understood to refer to the moderately electron-dense layer found wherever cells (other than connective tissue

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cells or blood cells) meet extracellular matrix. In essence, basement membranes serve to delimit the domain of connective tissue and provide a barrier between it and the domain of non-connective-tissue elements. This means that basement membranes (or basal laminae) are found in all of the following locations: at the dermal–epidermal junction of the skin; at the base of all lumen-lining epithelia throughout the digestive, respiratory, reproductive, and urinary tracts; underlying endothelia of capillaries and venules; around Schwann cells, adipocytes, skeletal and cardiac muscle cells; and at the base of parenchymatous cells of exocrine (pancreas, salivary glands) and endocrine (pituitary, thyroid, adrenal) glands wherever they face perivascular connective tissue.

In all the locations mentioned, the basement membrane consists of a lamina densa, 20–50 nm in thickness, which runs parallel to the basal cell membranes of the cell layer in question (epithelium, endothelium, etc.) and is separated from the latter by a lighter, approximately 10-nm layer—referred to as the lamina lucida or lamina rara. Thus, the basement membrane usually faces cell membranes on one surface and extracellular matrix components on the other; however, there are a few locations where basement membranes face cell layers on both surfaces. The best known example occurs in the renal glomerulus of mammals where the GBM faces vascular endothelium on one surface and epithelium on the other. The fact that the GBM does not face extracellular matrix makes it a particularly favorable object for biochemical analyses of basement membranes, as it eliminates the problems introduced by contamination of basement membrane fractions with interstitial collagens and other matrix components, a situation that is unavoidable with basement membranes derived from most other sources.

1.2. What Is the Structure and Function of the Kidney Glomerulus?

In order to understand the functional role of the GBM, some knowledge of glomerular organization and physiology is needed. Therefore, anticipating that not all readers will have this background, a brief overview will be given here and a more detailed discussion of glomerular physiology appears in a later section (see also Farquhar *et al.*, 1981).

The function of the renal glomerulus is to filter the blood, producing a protein-free filtrate of the blood plasma (the glomerular filtrate, the first step in urine formation), while retaining the cellular elements of the blood and the plasma proteins in the circulation. Each glomerulus consists of a tuft of anastomosing capillaries (Fig. 11-1). It is at the level of these capillaries that filtration takes place. Physiologic studies (Brenner *et al.*, 1978) indicate that the glomerular filter has both size-selective and charge-selective properties, that is, there is increasing restriction to passage of macromolecules across these capillaries with increasing size and increasing net negative charge of the molecules. To carry out this unique filtration function, the structure of glomerular capillaries is highly specialized. To begin with, they are divided into peripheral regions (facing Bowman's capsule), which are believed to constitute the main filtration

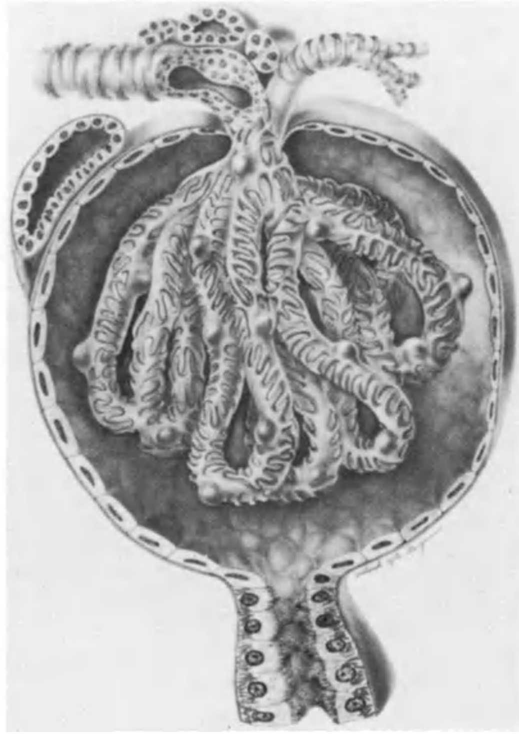


Figure 11-1. Schematic representation of the renal glomerulus, a tuft of capillaries surrounded by a capsule (Bowman's capsule). The latter consists of an inner, squamous epithelial layer surrounded by a basement-membrane-like layer of extracellular matrix material. The blood enters the glomerulus through the afferent arteriole (top), which immediately branches into the capillary network. The glomerular filtrate is forced out of the capillaries by the hydrostatic pressure of the blood,and collected in the capsular space between the capillaries and Bowman's capsule. From here it is funneled into the renal tubule (below) where readsorption of most of the fluid and salts takes place. (From Bloom and Fawcett, 1975, *A Textbook of Histology*, 10th ed.)

surface, and axial regions buried deeper in the glomerular tuft (Figs. 11-2 and 11-3). The glomerular capillary wall is considered to be constructed of four components—three cell types, endothelial, epithelial,* and mesangial cells, and the extracellular basement membrane (Figs. 11-2 and 11-3).

Filtration is assumed to occur mainly along peripheral regions where the walls of these capillaries are very thin.† Here the wall consists of the GBM, an

* By convention, the epithelium, because of its direct attachment to the GBM, is considered part of the wall of these specialized capillaries.

† Some filtrate can also be assumed to percolate through the mesangial regions to reach the GBM and eventually the urinary spaces (see Michael *et al.*, 1980, for a review of this topic). The relative amount of the total filtrate that takes this route is unknown. It is assumed to be minor under normal conditions and increased under pathologic conditions when the filtration surface at the periphery of these capillaries is modified (e.g., by a reduction in the number and collective area of the filtration slits) (Seiler et *al.*, 1980).

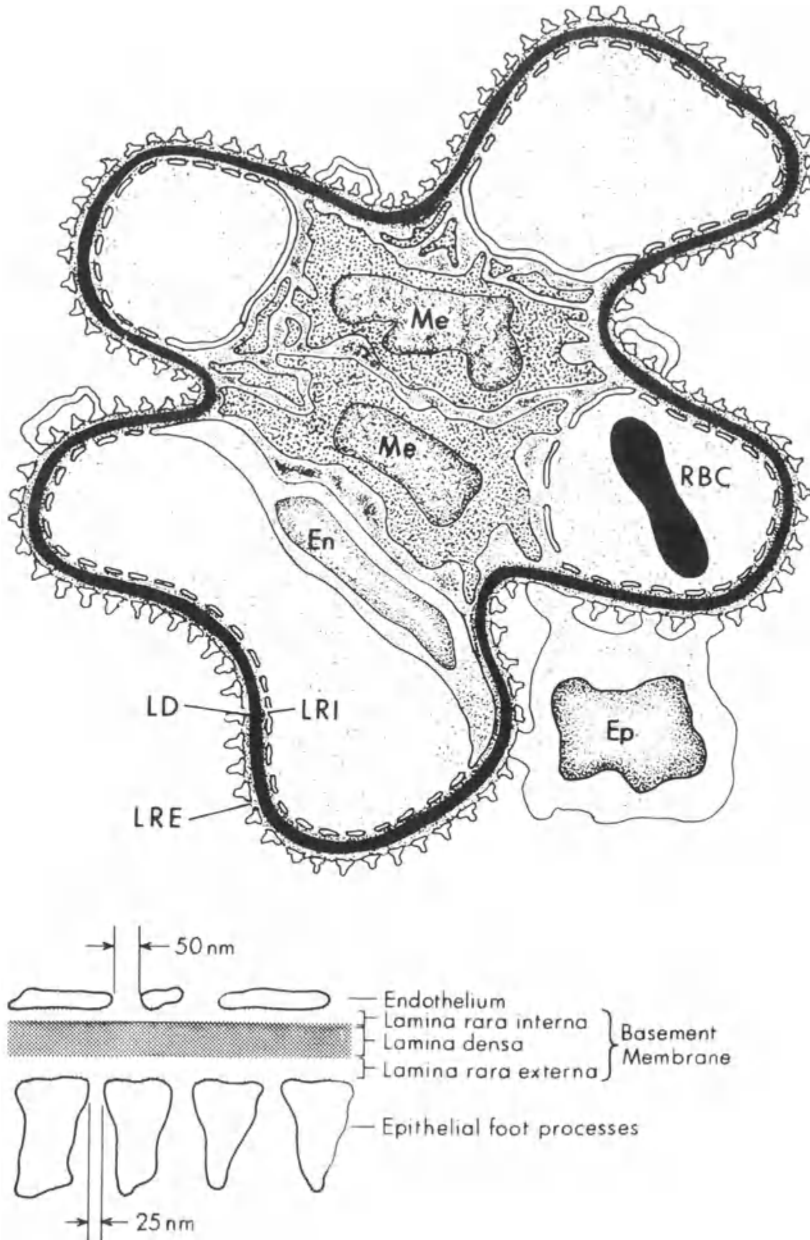


Figure 11-2. Diagram of a glomerular lobule showing the peripheral regions where the walls are very thin and the central or axial regions where the cell bodies of the endothelial (En) and mesangial (Me) cells are concentrated. It is assumed that normally filtration occurs mainly along the peripheral regions of these capillaries. The extracellular spaces between adjoining mesangial cells, between endothelial and mesangial cells, and between mesangial cells and the GBM contain a basement membrane-like material known as the mesangial matrix. It has a lower density and is looser in texture than the GBM, and it often contains fibrillar elements. (Redrawn after Latta *et al.*, 1960.)

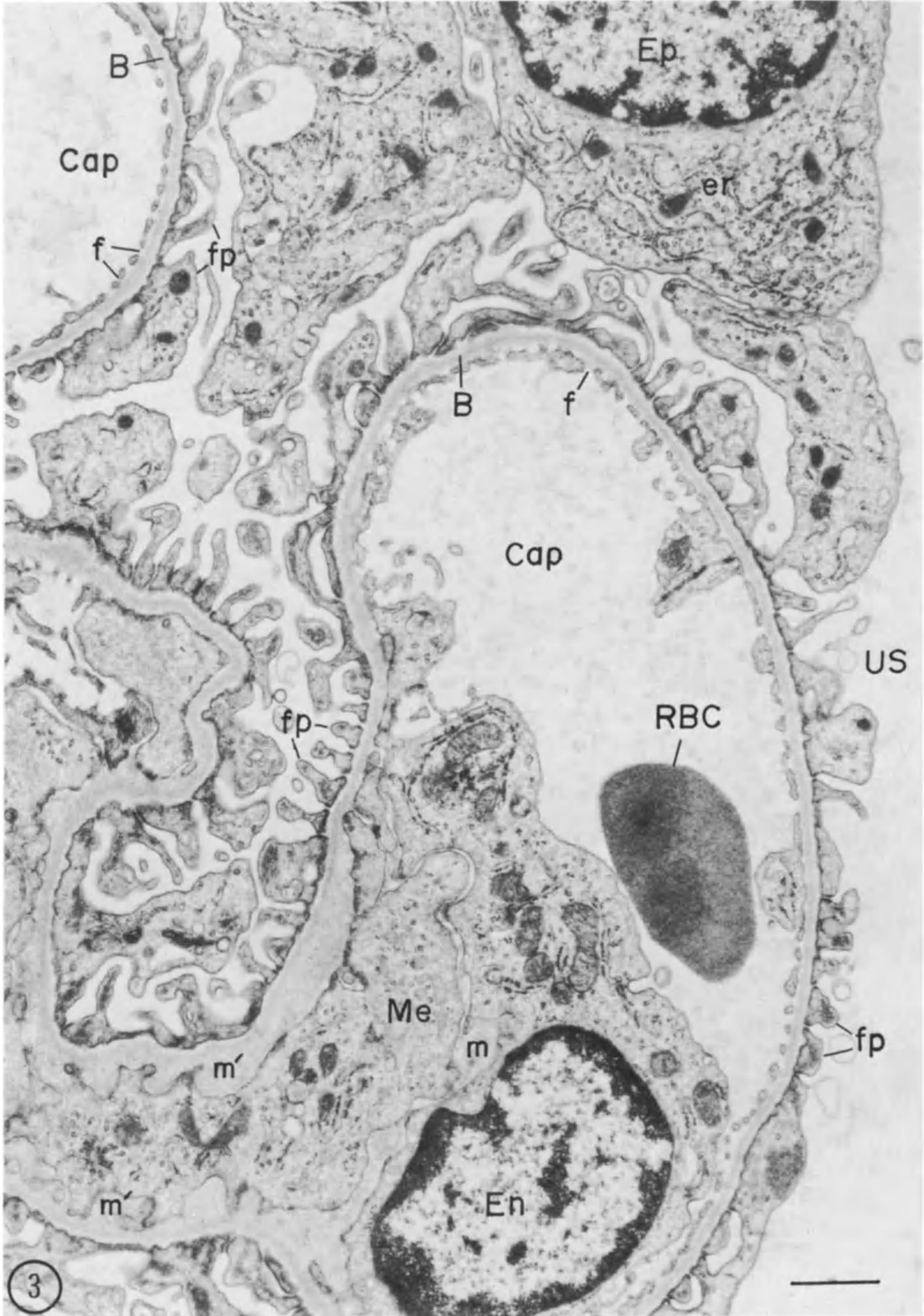
attenuated endothelial layer found along its luminal surface, and the characteristic hoof-shaped, interdigitating foot processes of the epithelium that lie along its abluminal surface facing the urinary spaces (Figs. 11-4 and 11-5). Because the endothelial layer is interrupted by porous openings or fenestrae and the epithelium is interrupted by the slits between the foot processes, the GBM is the only continuous layer among these elements and is, therefore, on morphological grounds, the logical candidate for the structure that represents the molecular filter. Permeability studies carried out with electron-dense tracers have verified that such is indeed the case (Farquhar, 1975, 1978, 1980; Venkatachalam and Rennke, 1978, 1980).

The glomerular capillaries are unique in many respects:

1. Their basement membranes are thicker and more substantial than basement membranes in most other locations. This reflects the fact that they are formed during development by fusion of the endothelial and epithelial basement membranes.
2. The GBM faces cell layers (the endothelium and epithelium) on both surfaces, whereas, as already mentioned, in most other situations, the basement membrane normally faces a cell layer on one side and extracellular matrix on the other.*
3. The endothelial fenestrae are open, that is, they lack the usual diaphragms that are invariably present in other fenestrated capillaries (refer to Fig. 11-39). As a consequence of this arrangement, the GBM is directly exposed to the blood plasma on its endothelial surface.
4. The epithelial cells (or podocytes) with their elaborate interdigitating foot processes, which form the filtration slits, are unique to these vessels.† The integrity of the foot processes and slit arrangement is dependent on the presence of an unusually thick, sialic acid-rich, cell surface coat (Fig. 11-4), which can be stained with cationic stains (Fig. 11-5).
5. The mesangial cells, likewise, are specialized cellular elements seen only in association with glomerular capillaries. They resemble in some respects the pericytes (modified smooth muscle cells) in other capillaries, but differ in being located on the luminal (rather than on the adventitial) side of the capillary and in being concentrated in the axial regions.
6. The spaces between adjoining mesangial cells, between mesangial cells and endothelial cells, and between mesangial cells and the GBM are filled with a special matrix component known as the mesangial matrix (Fig. 11-3), which differs in its morphology and composition from the GBM.

* In most areas, therefore, the lamina rara "interna" occupies the side of the basement membrane facing ECM. In the glomerulus, the lamina rara interna faces endothelium (see pp. 2–4 for further discussion of this terminology problem).

† By convention, the epithelium, because of its direct attachment to the GBM, is considered part of the wall of these specialized capillaries.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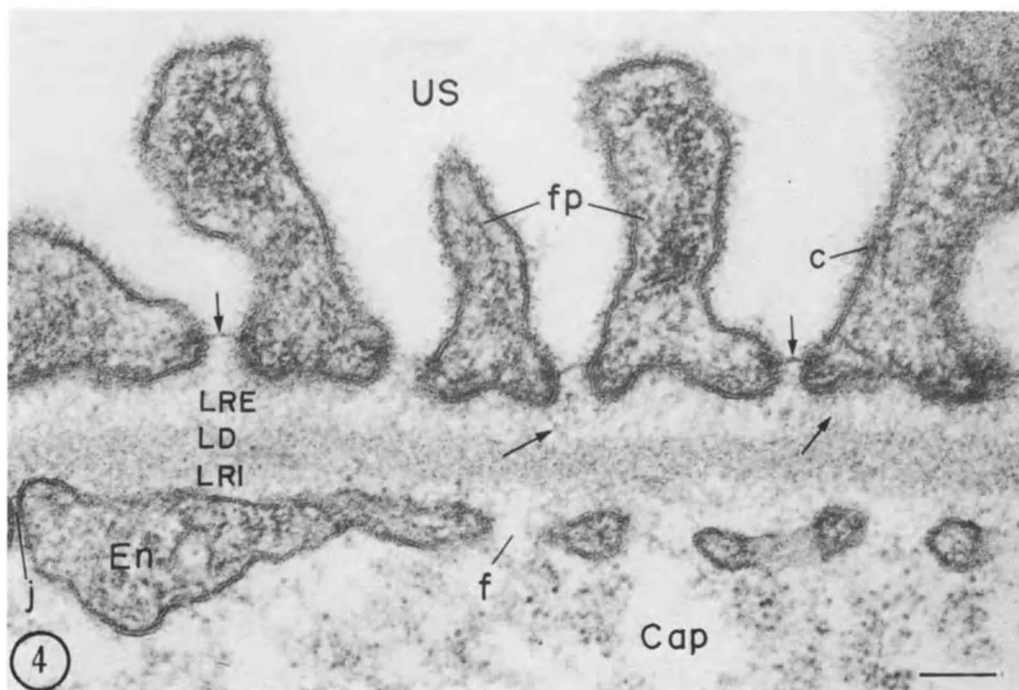


Figure 11-4. Portion of a rat glomerulus showing a peripheral region of a capillary (Cap) where filtration is assumed to take place. The filtration surface consists of the attenuated endothelium (En) with its fenestrae (f), the GBM, and the epithelial foot processes (fp). The latter are separated by ~ 25-nm spaces known as the filtration slits, which are bridged at their base by the so-called slit membranes (short arrows). A thick cell coat (c) is visible on the cell membrane of the foot processes above the level of the slit membranes. Note that the endothelial fenestrae are open, that is, they lack the diaphragms that are present in most other fenestrated capillaries (see Fig. 11-39) so that at their level the GBM is directly exposed to the blood plasma. The GBM consists of three layers: a central dense layer, the lamina densa (LD), and two adjoining layers of lower density, the lamina rara interna (LRI) and externa (LRE), adjoining the endothelium and epithelium, respectively. The lamina densa is composed of a fine (~ 3 nm) filamentous meshwork, and wispy filaments are seen extending from the lamina densa to the membranes of the foot processes (long arrows) and endothelium on either side. The glomerular filtrate passes through the endothelial fenestrae, permeates the GBM, and passes through the filtration slits to reach the urinary spaces (US), which are in continuity with Bowman's space. j, junction between two endothelial cells. × 100,000, bar = 0.1 μm. (From Farquhar and Kanwar, 1980.)

← **Figure 11-3.** Low-power electron micrograph of a portion of a renal glomerulus from a rat kidney showing portions of two glomerular capillaries (Cap), one of which contains a red blood cell (RBC). In the peripheral regions of these capillaries the walls are very thin and consist only of an attenuated layer of endothelial cytoplasm interrupted by fenestrae (f), the GBM (B), and the foot processes (fp) of the epithelium (Ep). Part of a mesangial cell (Me) and the cell body of an endothelial cell (En) are present in the axial regions (bottom). Mesangial matrix is found in the spaces between endothelial and mesangial cells (m) and between the GBM and the pointed processes of the mesangial cells (m'). The cell body of an epithelial cell whose cytoplasm is filled with abundant rough endoplasmic reticulum (er) is present (top). US, urinary spaces. × 13,000, bar = 1 μm.

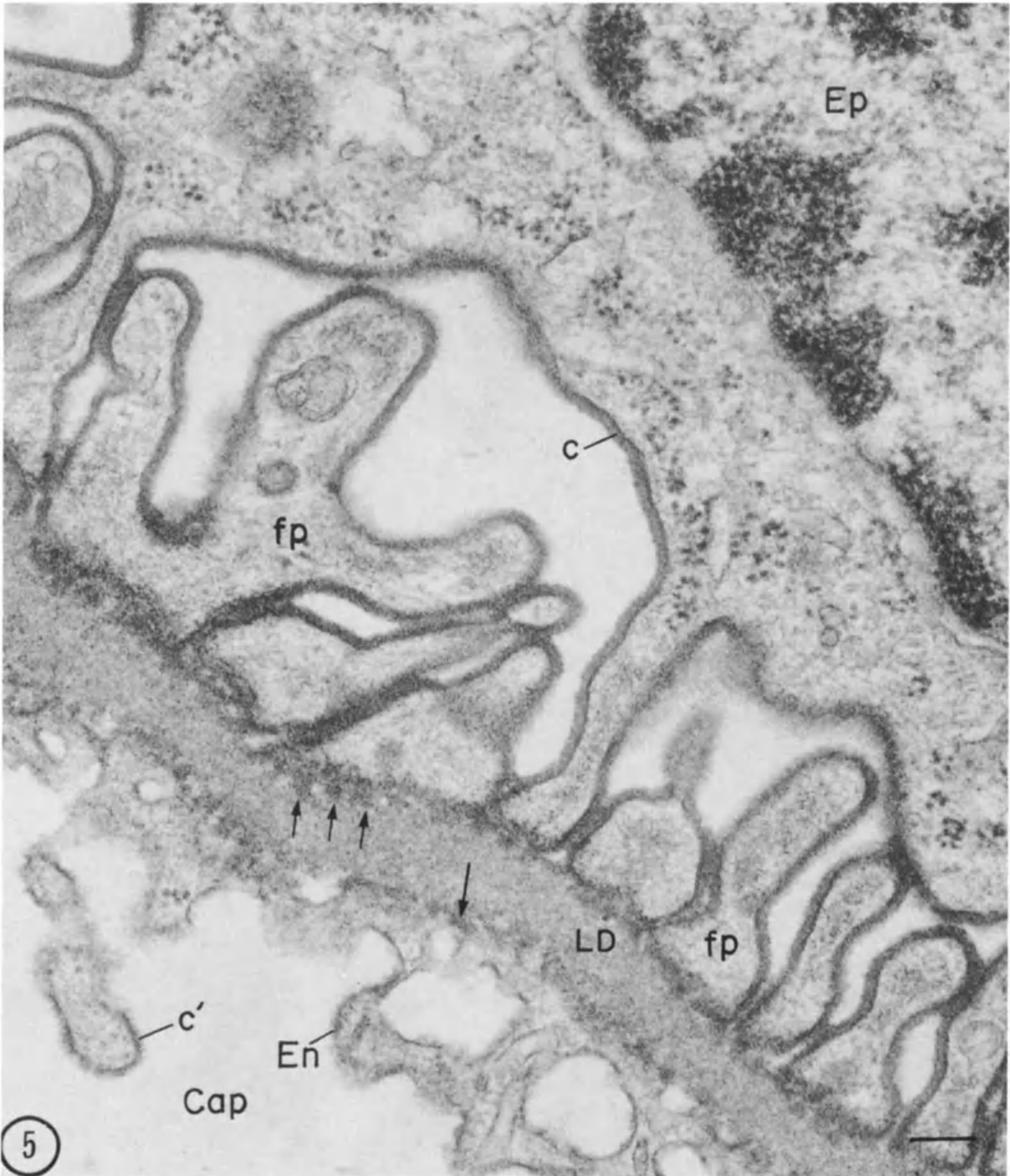


Figure 11-5. Field from the glomerulus of a normal rat kidney perfused with lysozyme. Lysozyme, which is a small cationic protein (pI = 11.0), binds to negatively charged groups and acts as a stain for anionic substances. Here it binds in a thick layer to the conspicuous sialic acid-rich, polyanionic cell surface coat (c) on the epithelial cell body (Ep) and its foot processes (fp). A similar but thinner cell coat is seen on the endothelium (c'). Lysozyme also binds to the anionic sites in the laminae rarae interna (↓) and externa (↑) of the GBM, thereby rendering them more dense than the lamina densa (LD). × 85,000, bar = 0.1 μm. (From Caulfield and Farquhar, 1978.)

2. Structural Organization of the GBM and Mesangial Matrix

The extracellular matrix of the glomerulus consists of the GBM and the mesangial matrix. Our main attention in this chapter will be focused on the GBM because of its primary role in glomerular filtration; however, as the mesangial matrix is often altered in disease states, information on its structure and composition is of considerable importance, and thus the mesangial matrix will be compared and contrasted to the GBM wherever possible.

The GBM is organized into three layers of different structural and chemical composition: a dense middle layer, the lamina densa, and two adjoining layers of lower electron density, known as the lamina rara interna and externa, which are located between the lamina densa and the membranes of the adjoining endothelium and epithelium, respectively (Fig. 11-4). The lamina densa is composed of a tightly woven meshwork of fine filaments, each of which is 3 nm in diameter. The laminae rarae, as their name implies, have a lighter background density, but they are by no means empty spaces or retraction artifacts as once believed.

Several distinct types of fibrous elements can be recognized in the GBM (Farquhar, 1978): (1) the 3-nm filaments that comprise the lamina densa (Fig. 11-4); (2) large fibrils (10-nm diameter) located in the lamina rara interna (Fig. 11-6) and in the mesangial matrix; and (3) filaments of variable size seen in both of the laminae rarae extending between and apparently connecting the lamina densa and the adjoining cell membranes (Fig. 11-4).

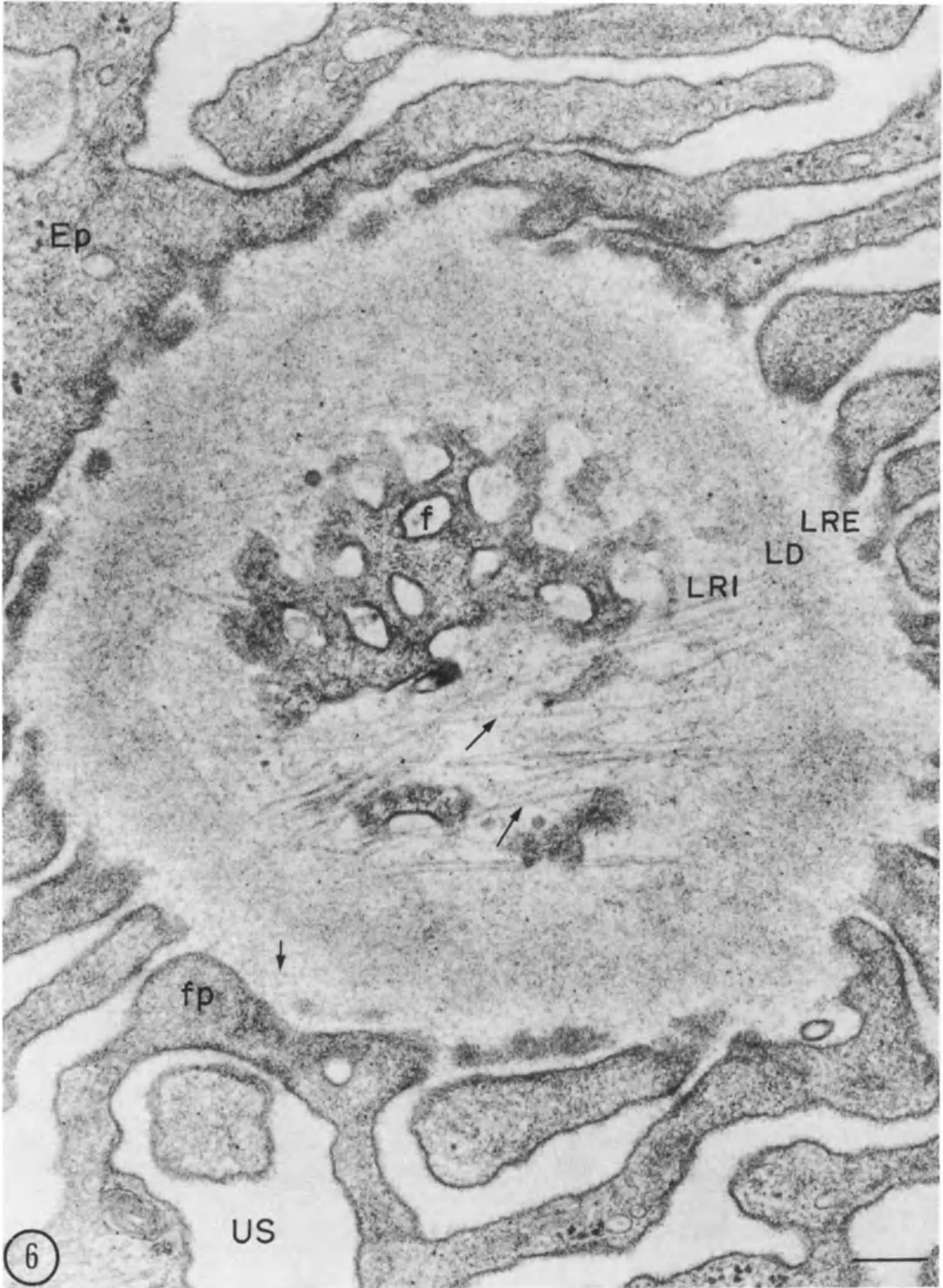
When sections are stained with cationic stains, such as ruthenium red or Alcian blue, additional structures can be demonstrated. After ruthenium red staining, a network of granules that have been identified as proteoglycan granules (Kanwar and Farquhar, 1979a,b) can be detected in the laminae rarae (Figs. 11-7 and 11-8). They are arranged in a quasi-regular, latticelike array (Figs. 11-7 and 11-9), much as in the corneal basal lamina (Trelstad et *al*., 1974). Another class of fine (2-nm diameter) filaments can be seen connecting the proteoglycan granules to each other, to the lamina densa, and to the adjoining cell layers (Fig. 11-7).

From recent immunocytochemical studies (summarized below), it is clear that the lamina densa and laminae rarae differ in composition. Ideally, one would like to identify precisely, by immunocytochemical staining, the chemical nature of each of the individual classes of microfibrils found in the GBM; however, due to the technical difficulties involved and the present state-of-the-art in applying immunocytochemistry at the electron microscopic level, this goal has not yet been achieved.

3. Chemical Composition of the GBM

3.1. Background Information

The inventory of the GBM components, like that of other basement membranes, is now known to include collagenous proteins, e.g., type IV collagen,


Ep
f
LRE
LD
LRI
fp
US
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(Kefalides, 1973, 1978, 1979), an unknown number of glycoproteins (Spiro, 1972, 1978) of which the best characterized is laminin (Timpl *et al.*, 1979a; Rohde *et al.*, 1979), and proteoglycans (Kanwar and Farquhar, 1979b,c; Kanwar *et al.*, 1981).

Information on the composition of the GBM has come from three main approaches: preparation and biochemical analysis of purified GBM fractions; immunocytochemistry carried out at the light or electron microscopic level; and cytochemical studies employing selective extraction *in situ*. The collective findings from all these approaches are summarized below.

Until only a few years ago, the bulk of the available information on the composition of the GBM was derived from biochemical studies on purified GBM fractions, studies in which Kefalides (1973, 1978, 1979) and Spiro (1972, 1978) and their collaborators have pioneered. This approach was made possible by the introduction in the early 1950s of a technique for the preparation of GBM fractions (Krakower and Greenspon, 1951). This involves isolation (by collection on a graded series of sieves) of glomeruli free from contamination by other kidney structures, especially the kidney tubules with their basement membranes and associated interstitial collagen and other matrix elements. Purified GBM fractions were then prepared therefrom by sonication, a procedure that undoubtedly resulted in removing (all or in part) both the laminae rarae and the mesangial matrix and thereby in concentrating the lamina densa. As 15% of the glomeruli retain their capsules during the glomerular isolation procedure, the main contaminant of such fractions is the extracellular matrix layer of Bowman's capsule.

Characterization of GBM components has been complicated by the fact that the GBM is extensively cross-linked: by disulfide bonds; by collagen-type cross-links (hydroxylysine); and by other unknown interactions, so that it is quite insoluble. Therefore, harsh procedures (such as pepsin digestion, or treatment with SDS after reduction and alkylation) must be used to solubilize it, and a wide variety of heterogeneous fragments have resulted. An additional complication is that proteolysis is believed to occur both *in vivo* and *in vitro*, a factor that further increases the heterogeneity of the fragments. Nevertheless, it has been clear for some time that the GBM contains a mixture of collagenous and noncollagenous glycoproteins with hydroxylysine-linked and asparagine-linked polysaccharide units, respectively (Spiro, 1972, 1978; Kefalides, 1973, 1978, 1979). It was not clear, however, until rather recently whether the two

← **Figure 11-6.** Glomerular capillary cut in grazing section, showing the layers of the capillary wall partially *en face*. The endothelial fenestrae (f) appear as irregularly circular, open portholes, and the three layers of the GBM—the lamina densa (LD), the lamina rara interna (LRI), and the lamina rara externa (LRE)—are cut broadly. Two types of fibrils are visible in the GBM: (1) a fine (~ 3 nm) fibrillar meshwork, which makes up the lamina densa and extends across the lamina rara externa to the epithelial cell membrane (short arrow), and (2) large (10 nm) double-walled (tubular) fibrils (long arrows), which are located between the endothelium and the GBM. × 56,000, bar = 0.2 μm. (From Farquhar and Kanwar, 1982.)

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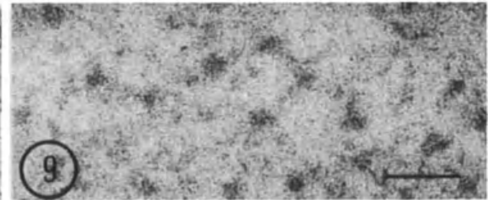
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types of carbohydrate units were on different molecules or whether they could occur on the same peptide chain. It is now clear that they can occur on the same glycopeptide molecule (Levine and Spiro, 1979).

The majority of the biochemical studies on the GBM have concentrated on an analysis of the collagenous components because, owing to their distinctive composition, they are easier to study. Characterization of the glycoproteins present has been very slow, and, as of now, no single glycoprotein has been purified from the GBM. This is because, in contrast to collagen, proteoglycans, and elastin, glycoproteins do not contain distinctive constituents that can be used in their identification. This, together with the fact that asparagine-linked heteropolysaccharides occur together with hydroxylysine disaccharide units as part of the procollagen chain (Levine and Sprio, 1979), plus the aforementioned highly cross-linked nature of the GBM has hindered isolation of intact glycoproteins from the GBM. To date, documentation of the presence of specific glycoproteins in the GBM has come from immunocytochemistry in which cross-reactivity of the GBM to glycoproteins isolated from other sources (primarily, a basement membrane-secreting tumor) has been demonstrated (see below).

3.2. Proteoglycans

Documentation of the presence of proteoglycans in the GBM has been obtained only recently (Kanwar and Farquhar, 1979a–c). Although proteoglycans had been shown to be present in basement membranes derived from other sources such as lens capsule (Spiro, 1972) and embryonic basement membranes (see Hay, 1981), their presence in the GBM had been denied based on the failure to detect uronic acid (the usual test for the presence of glycosaminoglycans) in isolated solubilized GBM (Spiro, 1972; Kefalides, 1973, 1979). The presence of proteoglycans in the GBM was first suspected on morphologic grounds when it was shown that anionic sites can be stained in the laminae rarae by a variety of cationic stains or probe molecules, i.e., lysozyme (Fig. 11-5), ruthenium red (Figs. 11-7 to 11-9), cationized ferritin (Figs. 11-10 and 11-11). Their identity was confirmed by cytochemical studies employing extraction *in situ* (Kanwar and Farquhar, 1979b). The approach used was to perfuse specific enzymes into the kidney (after flushing the kidney with a buffer) followed by perfusion with aldehyde fixative containing ruthenium red. The dye-stained network was no

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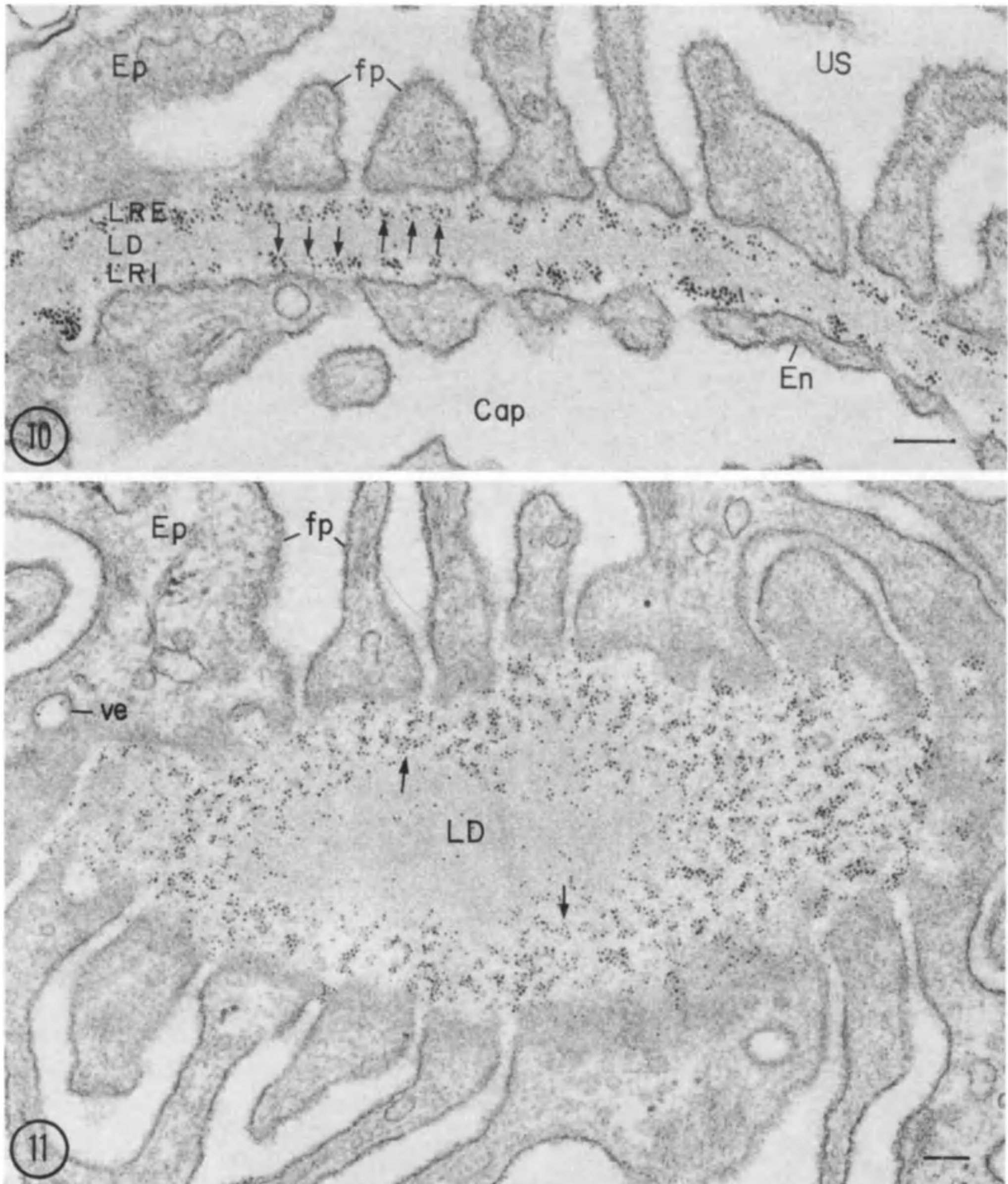
← **Figures 11-7 to 11-9.** Portions of glomerular capillaries stained with ruthenium red. A network of polygonal, proteoglycan granules can be seen in the laminae rarae interna (↓) and externa (↑) of the GBM, which is cut in grazing section in Fig. 11-7 and in cross section in Fig. 11-8. Fig. 11-9 is an enlargement of part of the lamina rara externa in Figure 11-7 showing the quasi-regular, lattice-like arrangement of the particles, which are located at regular (60 nm) intervals. Fine (2 nm) filaments connect the particles to one another and to the cell membranes of the epithelium and endothelium (En). Figure 11-7, × 104,000; Fig. 11-8, × 80,000; Fig. 11-9, × 208,000. Bars = 0.1 μm. (From Kanwar and Farquhar, 1979a.)



Figures 11-10 and 11-11. Portions of a glomerulus of a rat given cationized ferritin (CF) (pI = 7.3–7.5) *in vivo* by intravenous injection, to label anionic sites in the GBM. CF binds to anionic sites in the laminae rarae interna (LRI) and externa (LRE) of the GBM. Figure 11-10 shows that CF is distributed in discrete clusters located at regular ~ 60-nm intervals (arrows). Figure 11-11, which is from a grazing section of the same glomerulus as that in Fig. 11-10, illustrates the reticular pattern of CF binding in the lamina rara externa (arrows). Note that CF binds only to the GBM and that no binding to the endothelium (En) or epithelium (Ep) is seen. ve, Vesicle. Figure 11-10, × 80,000; Fig. 11-11, × 60,000. Bars = 0.1 μm. (From Kanwar and Farquhar, 1979a.)

longer demonstrable after perfusion with Pronase, heparitinase,* (Fig. 11-13), or nitrous acid,† but was unaffected by perfusion with neuraminidase, chondroitinase ABC (Fig. 11-12), or hyaluronidases. Similar findings were obtained when isolated GBM were incubated with the same enzymes followed by incubation in cationized ferritin to label the anionic sites: The sites were unaffected by incubation with chondroitinase ABC (Fig. 11-14) or neuraminidase (Fig. 11-15), but were no longer demonstrable after incubation with heparitinase* (Fig. 11-16). Thus, the cytochemical results clearly demonstrated that the particles were composed largely of proteoglycans rich in heparan sulfate.

Subsequently, biochemical studies were performed in which the glycosaminoglycan (GAG) moieties were extracted from isolated GBM prepared by the method of Meezan *et al.* (1978), which involves detergent treatment rather than sonication of glomeruli, and the extracted GAG were identified by cellulose acetate electrophoresis. The results (Kanwar and Farquhar, 1979c) confirmed that heparan sulfate is the major GAG present; in addition, a small amount of hyaluronic acid was detected. The compositional analysis was further confirmed by isolation of GAG after radiolabeling *in vivo*, and, with the increased sensitivity provided by this approach, a small amount of chondroitin sulfate (i.e., chondroitin 4- or 6-sulfate or keratan sulfate) was also detected (Lemkin and Farquhar, 1981). Quite recently, the intact proteoglycans were extracted from isolated GBM after radiolabeling kidneys with [^{35}S]sulfate by perfusion *in vitro* (Kanwar *et al.*, 1981). The results indicate that there are two types of proteoglycans with different densities but similar molecular weights (~130,000). The predominant proteoglycan is heparan sulfate, which comprises ~85% of the total proteoglycans extracted; chondroitin sulfate proteoglycan accounted for the remaining 15% (See also Linker *et al.*, 1981; Parthasarathy and Spiro, 1981).

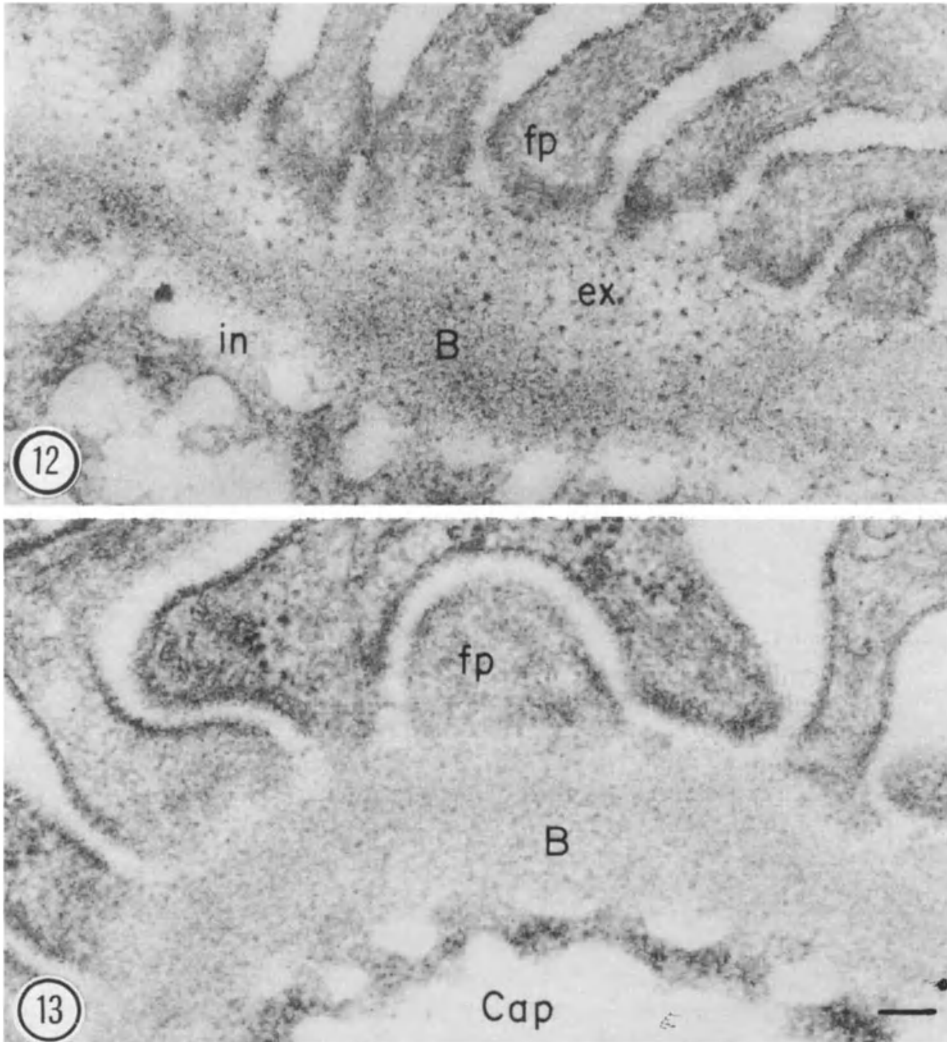
An additional finding was that ~45% of the total GBM proteoglycans could not be extracted by the usual procedures used for proteoglycans, involving use of 4 M guanidine chloride (Chapter 2); they were apparently covalently bound to other GBM constituents. Comparative analyses revealed no differences in the GAG chains released from the extractable and nonextractable proteoglycans. It is of interest to note that a heparan sulfate proteoglycan has also been isolated from the matrix produced by a basement membrane-secreting tumor known as the EHS sarcoma, and antibodies prepared against these proteoglycans were shown to cross-react with the GBM as well as with basement membranes derived from several other sources (Hassell *et al.*, 1980).

3.3. Collagenous Components

It has been known for some time that the GBM contains collagenous proteins, but the basement membrane collagens are by no means as well character-

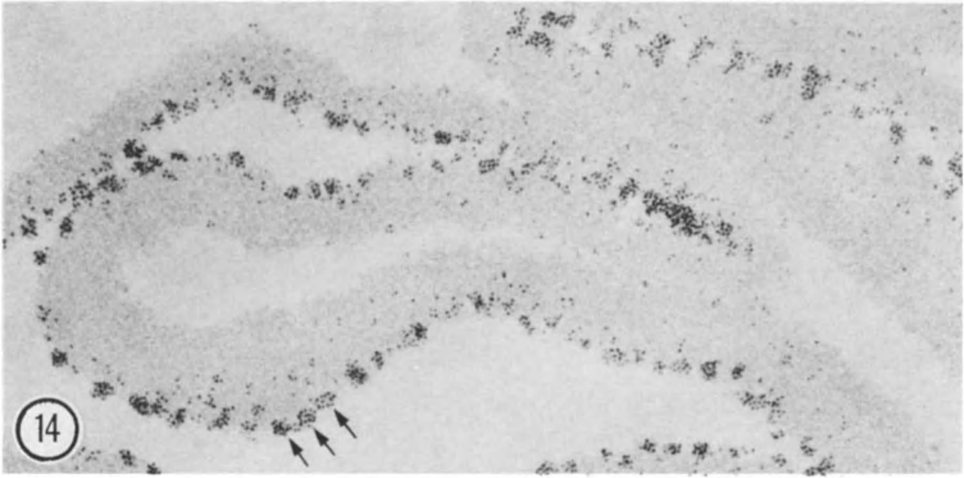
* Purified heparitinase acts only on heparan sulfate and not on heparin or other GAG (Linker and Hovingh, 1972).

† Under the conditions utilized, nitrous acid specifically degrades heparan sulfate and heparin but not other GAG.



Figures 11-12 and 11-13. Figure 11-12 is a small field from a glomerular capillary of a kidney perfused with chondroitinase ABC, and Fig. 11-13 is from a kidney perfused with purified heparitinase prior to staining with ruthenium red. Note that the particles in the laminae rarae interna (in) and externa (ex) were *not* removed by chondroitinase treatment, but they were removed by digestion with heparitinase. The specific removal of these sites by heparitinase indicates that they consist of heparan sulfate because the enzyme specifically digests only this GAG. Both × 70,000, bar = 0.1 μm. (From Kanwar and Farquhar, 1979b.)

Figures 11-14 to 11-16. Loops of isolated GBM subjected to treatment with specific enzymes followed by incubation with cationized ferritin (CF) to label the GBM anionic sites. CF binding (arrows) is not affected by treatment with chondroitinase ABC (Fig. 11-14) or neuraminidase (Fig. 11-15), but is abolished by heparitinase (Fig. 11-16). CF molecules bind only to the outer or exposed side of the GBM loops because the latter consist of intact, closed tubes, and the tracer does not have access to the inner or unexposed side of the GBM. All × 70,000, bar = 0.1 μm. (From Kanwar and Farquhar, 1979b.) ⟶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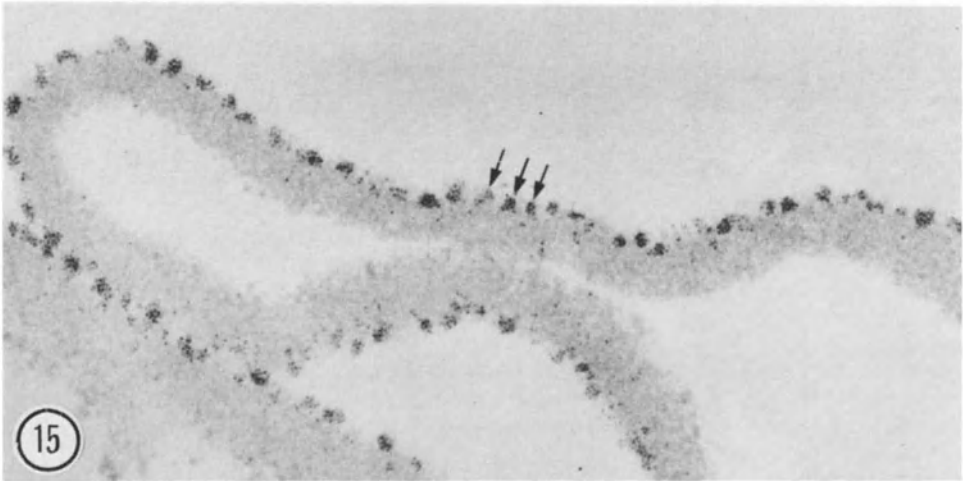
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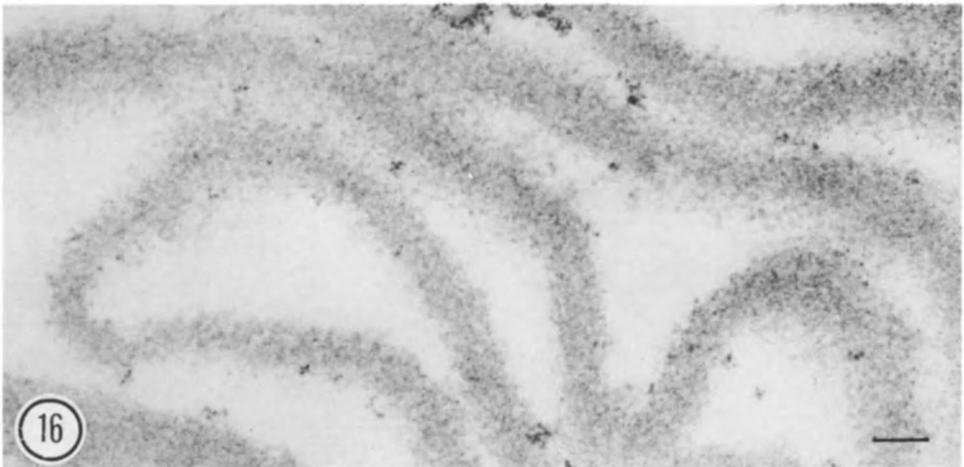
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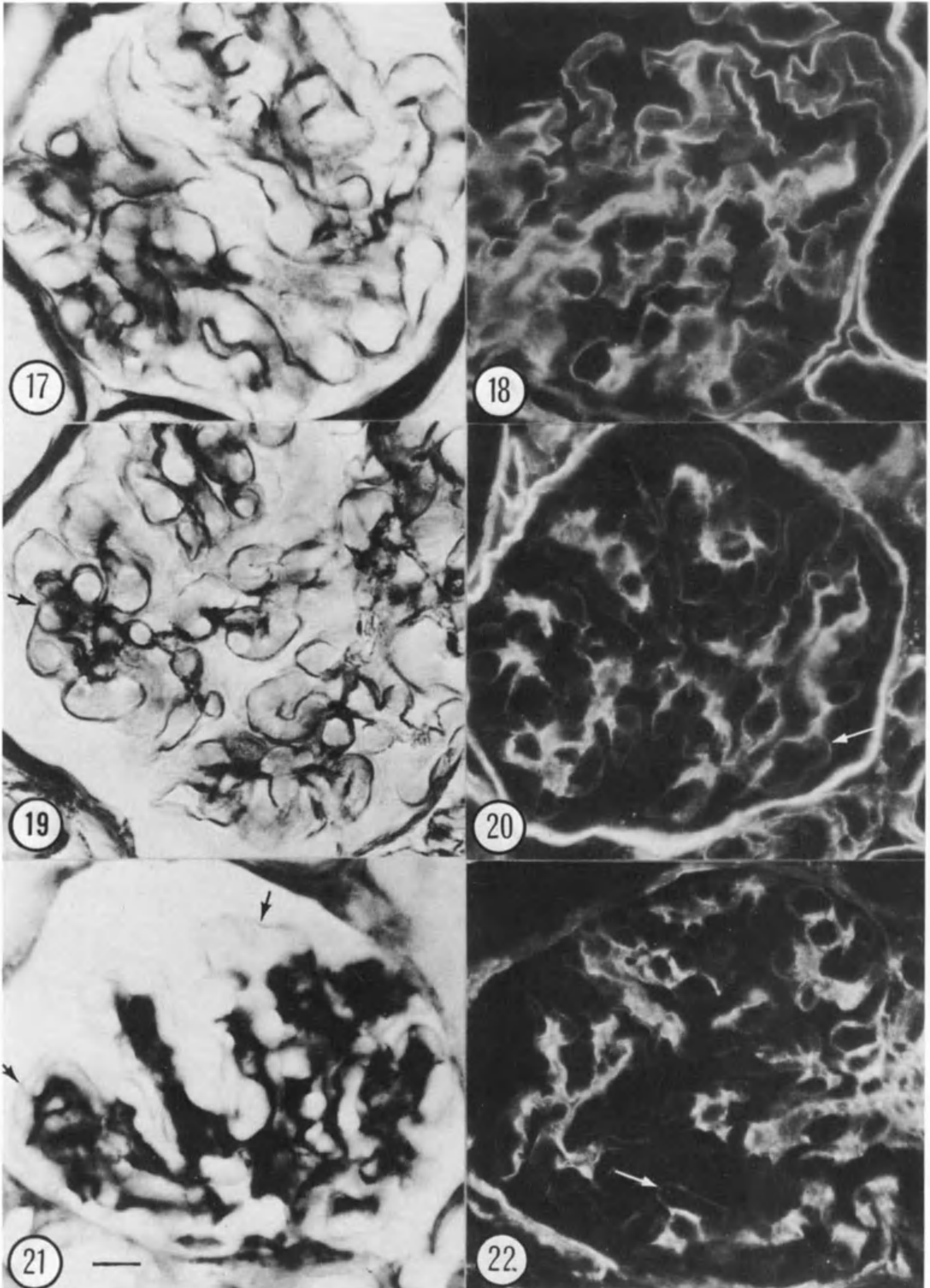
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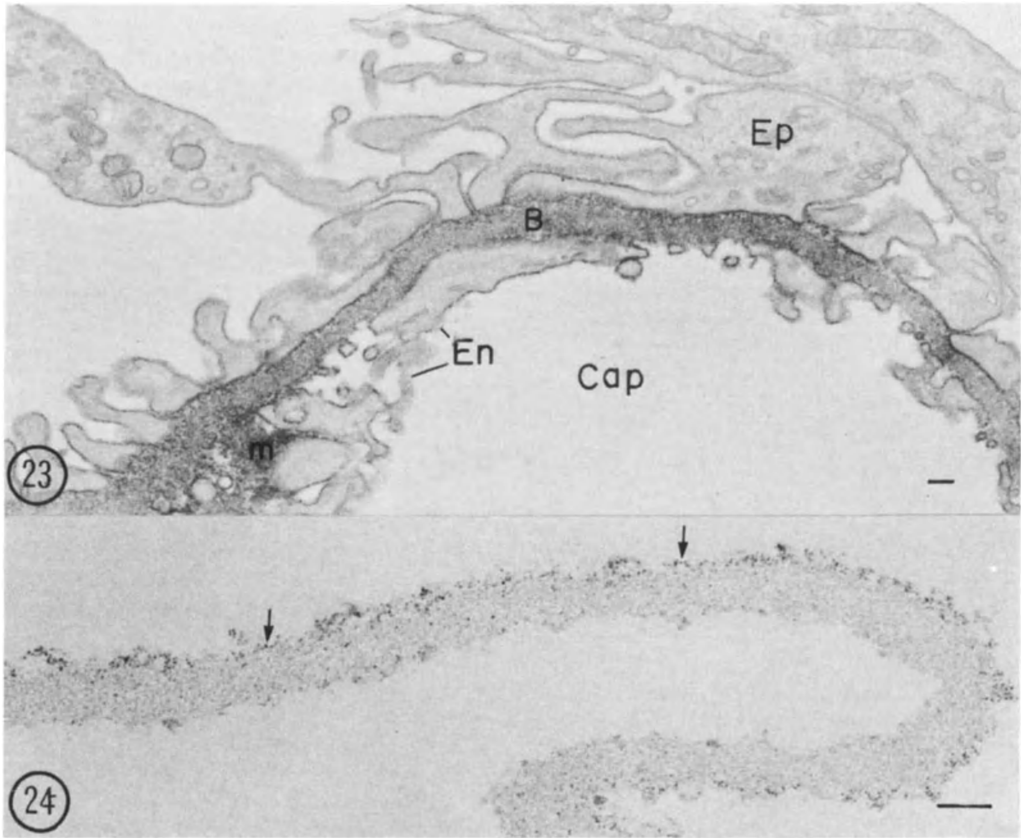
ized as interstitial collagens, and there is still disagreement as to how many different types there are, i.e., type IV collagen, type V collagen, 7 S collagen, etc. (see Chapter 1 and review by Heathcote and Grant, 1981). However, most workers agree that the GBM contains type IV collagen, and clearly the GBM as well as the mesangial matrix react with antibodies prepared against type IV collagen (Timpl et *al.*, 1978; Scheinman et *al.*, 1980a; Roll et *al.*, 1980; see Figs. 11-19 and 11-20). Moreover, immunocytochemical studies carried out at the electron microscopic level have localized type IV collagen to the lamina densa of the GBM (Roll et *al.*, 1980; Courtoy et *al.*, 1981; see Figs. 11-23 and 11-24).

3.4. Laminin

Laminin is a high-molecular-weight (800,000–1,000,000) sialoglycoprotein with a high concentration of carbohydrate (15%) that was originally purified by Timpl et *al.* (1979b) from the EHS sarcoma (Chapter 4). Based on immunocytochemical staining, it appears to be a ubiquitous basement membrane component, for it has been demonstrated in virtually every basement membrane examined including the GBM. The functions of laminin are not yet established, but there is good evidence for its involvement in the attachment of cells, especially of epithelial cells, to their substrate (Chapter 4).

Laminin was localized to the GBM, mesangium, and Bowman's capsule by immunofluorescence in the original studies of Timpl and his co-workers (Rohde et *al.*, 1979) and by Scheinman et *al.* (1980b) (see Figs. 11-17 and 11-18). Subsequently, it was localized at the ultrastructural level within the GBM and mesangial matrix (Madri et *al.*, 1980; Courtoy et *al.*, 1981) and in the basement membrane at the base of the epidermis of the skin (Foidart et *al.*, 1980a). In all these studies, it was noted that the distribution of laminin within the basement membrane layers was not uniform; it was concentrated in the laminae rarae and absent or present at low concentration in the lamina densa (Figs. 11-25 and 11-26). In the glomerulus, one study (Roll et *al.*, 1980) described laminin only in the lamina rara interna, whereas in the other (Courtoy et *al.*, 1981), laminin was found in both laminae rarae (interna and externa). Differences in results might be attributable to the different techniques used in the preparation and incubation of tissues for immunocytochemistry. Immersion fixation in glutaral-

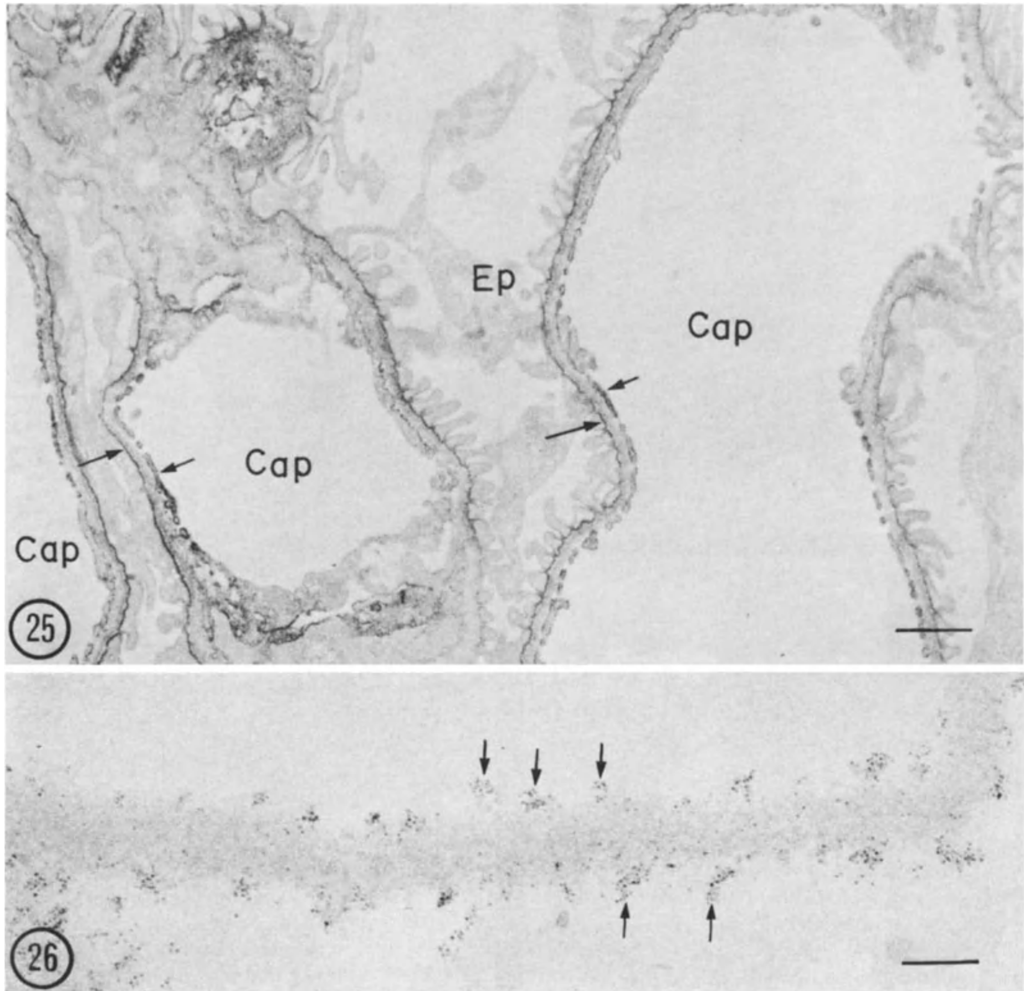
← **Figures 11-17 to 11-22.** Distribution of laminin, type IV collagen, and fibronectin detected by immunofluorescence in unfixed cryostat sections (Figs. 11-18, 11-20, and 11-22) or by immunoperoxidase in sections of aldehyde-fixed kidneys (Figs. 11-17, 11-19, and 11-21). Staining for all three proteins is seen in Bowman's capsule, in the GBM, and in the mesangial matrix. The relative distribution of the staining for the three proteins varies: staining for laminin is more intense in the GBM at the periphery (Figs. 11-17 and 11-18); staining for collagen type IV is prominent in both the mesangium and the GBM (arrows, Figs. 11-19 and 11-20); staining for fibronectin is more intense in the mesangium and only faint staining is seen in the GBM (arrows, Figs. 11-21 and 11-22). Figure 11-17, × 600; Figs. 11-18 to 11-22, × 800. Bar = 10 μm. (From Courtoy et *al.*, 1981.)



Figures 11-23 and 11-24. Localization of type IV collagen to the lamina densa of the GBM by an indirect immunoperoxidase technique *in situ* (Fig. 11-23) and by an indirect immunoferritin technique applied to isolated (unfixed) GBM (Fig. 11-24). Peroxidase reaction product is seen throughout the lamina densa of the GBM (B) and mesangial matrix (m). Ferritin particles form a closely packed layer along the outer (exposed) surface of the lamina densa (arrows) in the isolated GBM.
Figure 11-23 is from a ~ 10 μm cryostat section taken from a kidney that was lightly fixed (by perfusion with aldehyde), incubated with affinity-purified antibodies to type IV collagen (prepared by R. Timpl from monospecific antisera of rabbits immunized against collagen type IV purified from the EHS sarcoma), followed by incubation in Fab fragments of sheep anti-rabbit IgG conjugated to peroxidase. Figure 11-24 is from isolated (unfixed) GBM incubated in the same anti-type IV collagen antibody, followed by incubation in sheep anti-rabbit IgG conjugated to ferritin. Figure 11-23, × 30,000; Fig. 11-24, × 70,000. (Bars = 0.1 μm. (From Courtoy et *al.*, 1981.)

dehyde followed by incubation of frozen thin sections of kidney with an immunoferritin technique was used in the former study, whereas brief perfusion fixation followed by incubation of cryostat sections of kidney (Fig. 11-25) or isolated unfixed GBM (Fig. 11-26) with immunoperoxidase and immunoferritin conjugates, respectively, was used in the latter study.

The localization of laminin to the laminae rarae is of considerable interest because it places this glycoprotein in the proper location to play a role in the



Figures 11-25 and 11-26. Localization of laminin to the laminae rarae interna and externa of the GBM by an immunoperoxidase technique *in situ* (Fig. 11-25) and an immunoferritin technique on isolated GBM (Fig. 11-26). Figure 11-25 shows three capillary loops (Cap) with peroxidase reaction product. The latter is seen as two thin dense lines, one deposited in the lamina rara externa (long arrows) at the base of the foot processes and the other deposited in the lamina rara interna (short arrows) between the endothelium and the GBM. Figure 11-26 shows clusters of ferritin molecules adhering in patches ~ 120 nm apart on both sides of a segment of isolated GBM. In this case the GBM loop was opened during the isolation procedure so that the ferritin conjugates had access to both sides of the GBM. Note that in contrast to the situation with anti-type IV collagen antibody (Fig. 11-24), the ferritin is deposited at a distance of 20–50 nm from the lamina densa, i.e., in a location corresponding to the residual laminae rarae. Specimen preparation as in Figs. 11-23 and 11-24, except that incubation was in affinity-purified antibodies to laminin (prepared by R. Timpl from rabbits immunized against laminin purified from the EHS sarcoma). Figure 11-25, × 10,000; Fig. 11-26, × 100,000. Bars = 0.1 μm. (From Courtoy *et al.*, 1981.)

attachment of the endothelium and the epithelium to the GBM. The attachment of endothelial and epithelial cells to the GBM is quite strong and not easily disturbed. It is unaffected by removal of divalent cations or treatment with strongly acidic or basic buffers, but it has been shown to be dependent on the presence of sialic acid (presumably in sialoproteins or sialoglycolipids), as perfusion of glomeruli with neuraminidase (Figs. 11-27 and 11-28) causes detachment of both endothelial and epithelial cells from the GBM (Kanwar and Farquhar, 1980). Thus, the location of laminin in one or probably both laminae rarae, plus the fact that removal of sialic acid promotes cell detachment, makes laminin a prime candidate for playing a role in the attachment of endothelial and epithelial cells to the GBM.

3.5. Fibronectin

Fibronectin is a widespread matrix glycoprotein that is found associated with collagen fibrils, with nonstriated matrix fibrils, with interstitial bodies in developing tissues, and with basement membranes (Chapters 4 and 10). There has been some debate, however, as to whether it is a ubiquitous component of basement membranes.

In the case of the GBM, there have been a number of studies in which antibodies raised against tissue or plasma fibronectin were localized in the glomerulus by immunofluorescence, and several immunocytochemical studies have also been carried out at the electron microscopic level. There is general agreement among all these studies that fibronectin is present in high concentration in the mesangial matrix (Figs. 11-21, 11-22, 11-29, and 11-31); however, there is disagreement concerning whether or not it is present in the GBM itself. Several studies reported finding staining for fibronectin in the peripheral regions of the capillaries by immunofluorescence (Figs. 11-21), where it is assumed to be associated with the GBM (Pettersson and Colvin, 1978; Oberley et *al.*, 1979; Scheinman et *al.*, 1980b; Courtoy et *al.*, 1981), whereas several others reported that it was absent from this location (see Madri et *al.*, 1980). In one of the two ultrastructural studies carried out so far (Courtoy et *al.*, 1980, 1981), fibronectin was detected in the GBM, where it was localized to the laminae rarae (Figs. 11-29 and 11-30), but in the other study (Madri et *al.*, 1980) it was reported to be absent from the GBM. Once again, differences in the techniques used and differences in the species studied (rat, mouse, and human) may serve

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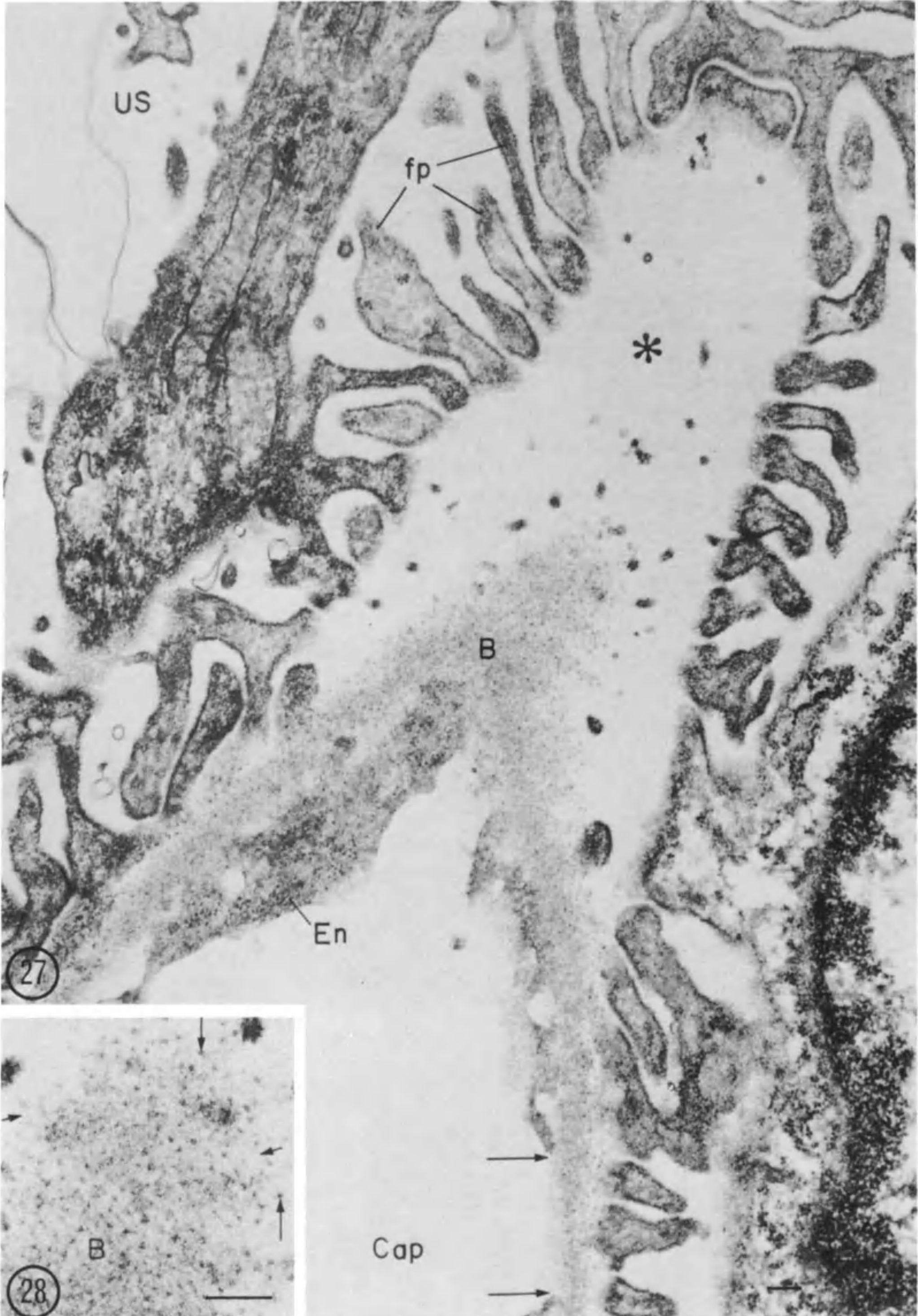
Figures 11-27 and 11-28. Field from a glomerular capillary perfused with neuraminidase. The ⟶ epithelial foot processes (fp) have been partially detached from the GBM (B) creating a large space (asterisk) between the two. Smaller areas of endothelial detachment are also present (between arrows). Figure 11-28 shows that neither the ruthenium red-stained proteoglycan particles (long arrows) nor the 3-nm connecting filaments (short arrows) have been affected by the enzyme treatment. Figure 11-27, × 25,000; fig. 11-28, × 50,000. Bars = 0.2 μm. (From Kanwar and Farquhar, 1980.)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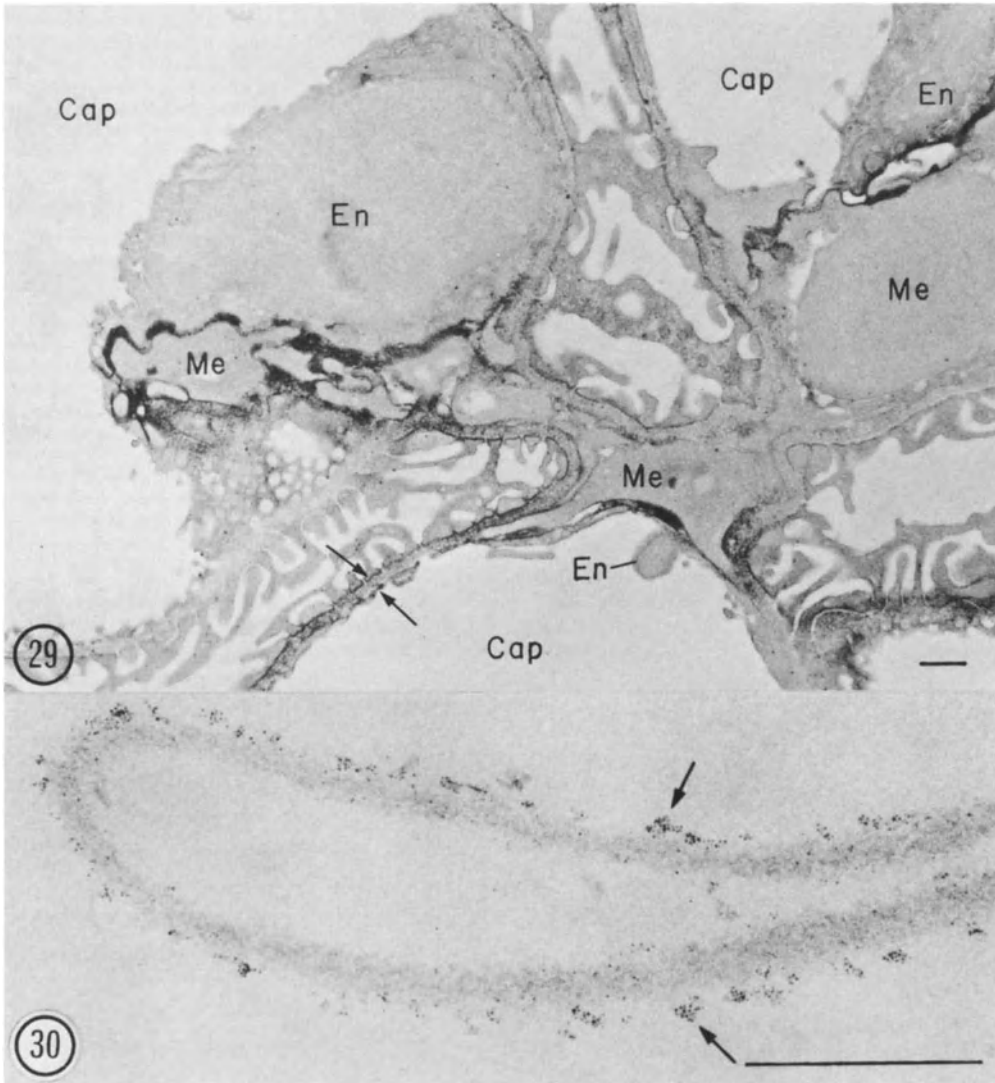


Figures 11-29 and 11-30. Localizatin of fibronectin in the glomerulus by the immunoperoxidase technique in situ (Fig. 11-29) and immunoferritin technique on isolated GBM (Fig. 11-30). In Figure 11-29, peroxidase reaction product is concentrated in the mesangial matrix between the endothelial (En) and the mesangial (Me) cells. It is also present in the laminae rarae in some places (arrows). In Figure 11-30, ferritin molecules are seen distributed in patches or clusters along the residual lamina rara externa (arrows) of this closed GBM loop. As in the case of laminin (Fig. 11-26), the clusters are located in the residual laminae rarae at a distance > 20 nm from the lamina densa and at least 100 nm from each other. Specimen preparation as in Figs. 11-23 and 11-24, except that localization was done with antisera to fibronectin (prepared by R. Hynes from rabbits immunized against fibronectin isolated from NIL8 hamster cells). Figure 11-29, $\times$ 12,500; Fig. 11-30, $\times$ 65,000. Bars = 0.5 μm. (From Courtoy et *al.*, 1980.)

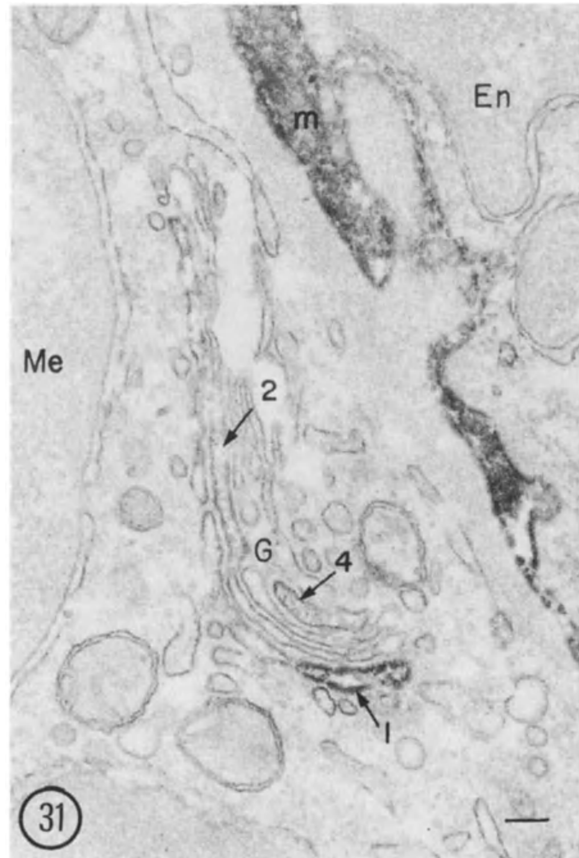


Figure 11-31. Immunoperoxidase, showing localization of fibronectin intracellularly within several of the stacked Golgi (G) cisternae (1, 2, and 4) of a mesangial cell (Me), as well as extracellularly in the mesangial matrix (m) at the mesangial–endothelial (En) interface. × 55,000, bar = 0.1 μm. (From Courtoy *et al.*, 1980.)

to explain the discrepant findings. However, the fact that four different groups of investigators have detected fibronectin in the peripheral GBM by immunofluorescence, plus the fact that ultrastructurally it was localized to the laminae rarae by three different immunocytochemical approaches (immunoperoxidase and immunoferritin on lightly fixed cryostat sections and immunoferritin on unfixed, isolated GBM), makes it likely that fibronectin is present not only in the mesangium, but also, albeit in lower concentration (Figs. 11-21 and 11-22), in the GBM itself. Like laminin, its location in the laminae rarae would place this protein (which is well known to function in the attachment of many other cells to their substrates) in a favorable position to perform a function in the attachment of the endothelial and epithelial cells to the GBM. It is also of interest that within the mesangial matrix, fibronectin is especially concentrated between mesangial cells and endothelial cells (Fig. 11-29), which led us to suggest that it may play a role in the attachment of these cells to one another or to the mesangial matrix (Courtoy *et al.*, 1980).

3.6. Other Components

Apart from collagen type IV, proteoglycans, laminin, and fibronectin, attempts to characterize other components of the GBM have made little headway. A number of other proteins have been reported to show cross-reactivity (by immunofluorescence) to the GBM. The list of such proteins that have been purified (to a variable extent), used for the production of specific antibodies,and shown to immunostain the GBM includes the microfibrillar protein of elastic tissue (Kewley *et al.*, 1977), 7 S collagen (Risteli *et al.*, 1980), type V collagen (Roll *et al.*, 1980), Goodpasture's antigen (Wick and Timpl, 1980), and, most recently, a sulfated glycoprotein called entactin (Carlin *et al.*, 1981; Bender *et al.*, 1981). Clearly, more work is needed before the significance of these findings will be understood. There is also a long history of immunohistochemical localization of glomerular antigens, especially nephritogenic antigens, carried out on poorly or incompletely characterized antigens or antibodies, consideration of which is beyond the scope of this chapter (see Kefalides, 1979, for a discussion of this problem).

3.7. Comments on Techniques for Isolating the GBM

As already mentioned, the adjacent endothelial and epithelial cell layers are tightly attached to the GBM *in situ*; therefore, unless care is taken to remove them, bits of cell membrane and associated cytoplasm will regularly contaminate any GBM fractions obtained. To date, two main methods have been used for denuding the GBM and ridding it of adherent cell debris: (1) sonication with or without subsequent TCA treatment (Krakower and Greenspon, 1978) and (2) detergent treatment (Meezan *et al.*, 1978). Most of the early work on characterization of GBM components was done using sonicated GBM, which, as mentioned earlier, undoubtedly removes part or all (depending on the extent of sonication) of the laminae rarae and mesangial matrix, and yields mainly the lamina densa. GBM prepared by detergent treatment according to the technique of Meezan *et al.* (1978) yields intact, unbroken loops of GBM (see Figs. 11-14 to 11-16) with the mesangial matrix apparently intact, and with all the components identified so far as belonging to the laminae rarae (heparan sulfate proteoglycan, laminin, fibronectin) still present (Figs. 11-14, 11-26, and 11-30).

The main compositional difference detected so far between sonicated and detergent-treated GBM preparations is the presence of proteoglycans in GBM fractions prepared by the detergent method of Meezan and co-workers.The reason that uronic acid (indicative of the presence of GAG) was not detected by Kefalides (1973) and Spiro (1972) and their collaborators in their early studies was undoubtedly due in part to the fact that the membranes they analyzed had been subjected to extensive sonication, and sonication has been shown to disrupt the proteoglycan network in the laminae rarae and mesangial matrix (Kanwar and Farquhar, 1979a). It remains to be seen if other components are preferentially extracted or lost by one or the other treatment. In choosing which method to use, the detergent method is clearly the one of choice if one wants

to isolate the entire glomerular extracellular matrix, but one must take great pains to rid these membranes of debris trapped on the inside of the intact GBM loops. However, if one wants to study the lamina densa, sonication (or sonication coupled with TCA treatment) is the method of choice.

3.8. Summary of Nature and Location of GBM Components

The number of well-established glomerular matrix components is very few and includes only four: type IV collagen, proteoglycans, laminin, and fibronectin. The presence of type IV collagen and proteoglycans in GBM has been established by extraction and biochemical characterization as well as by localization *in situ* (by immunocytochemistry or by selective extraction). Type IV collagen is concentrated in the lamina densa and proteoglycans in the laminae rarae of the GBM. Both are present in the mesangial matrix. The demonstration of the presence of laminin in the glomerulus rests entirely on the immunoreactivity of glomeruli (by immunocytochemistry) to anti-laminin antibodies prepared against laminin derived from the EHS sarcoma. Laminin has been localized to the laminae rarae (both laminae rarae in one study and only the interna in another) and the mesangial matrix. The demonstration of the presence of fibronectin in the glomerulus also rests entirely on immunocytochemical results. There is general agreement that fibronectin is concentrated in the mesangial matrix. Past studies disagree concerning whether or not fibronectin is localized in the GBM, but our recent immunocytochemical studies at the ultrastructural level place it in the laminae rarae. Evidence for all other components or antigens suspected or claimed to be present in the GBM is tenuous, and documentation of their presence in the GBM requires further work and clarification.

4. What Do We Know about the Biosynthesis of GBM Components?

Studies on the biosynthesis of the GBM have had two main aims: (1) determination of the glomerular cell types that manufacture the GBM or its individual components, and (2) characterization of the steps involved in the biosynthesis and intracellular processing of each of the proteins made and incorporated into the GBM, especially the collagenous peptides, and comparison of the results with the steps worked out for interstitial collagens (Chapter 6).

4.1. Which Glomerular Cells Make GBM Components?

In principle, all three cell types, endothelial, epithelial, and mesangial cells, should be capable of making basement membrane components, as it has been shown that endothelial, epithelial, and smooth muscle cells derived from

other sources are capable of making basement membrane collagen, fibronectin, and proteoglycans in culture (see review by Hay, 1981). It was already noted here and reviewed in detail elsewhere (Reeves et *al.*, 1980) that during glomerular development, both the epithelial cells and the presumptive endothelial cells produce a basement membrane, and the two basement membranes eventually merge to form the mature GBM.*

The epithelial cell was pinpointed as the main site of GBM synthesis many years ago when material resembling the lamina densa was found inside the rough endoplasmic reticulum of epithelial cells and demonstrated to react with antibodies prepared against whole GBM (Andres et *al.*, 1962). In addition, the fact that when GBM was labeled with silver (administered in the drinking water), metallic silver was preferentially deposited along the epithelial side and gradually displaced toward the luminal side, suggested that the GBM was synthesized by the epithelial cells and removed by the endothelial and/or mesangial cells (Walker, 1973). Autoradiographic studies in which [^{3}H]proline or [^{3}H]leucine was administered *in vivo* also suggested that the epithelial cells were the primary source of the GBM (Romen et *al.*, 1976).

More recently, several workers have succeeded in culturing cells of glomerular origin and in establishing apparently homogeneous epithelial and mesangial cell lines. So far, no endothelial cell lines have been established (the glomerular endothelial cells have proved to be difficult to propagate in culture). The information obtained from biosynthetic studies on cultures of these homogeneous cell lines is as follows: (1) Both epithelial and mesangial cells have the capacity to synthesize a collagen with similar antigenic determinants to type IVcollagen (MW 140,000–170,000) (Scheinman and Fish, 1978; Killen and Striker, 1979; Foidart et *al.*, 1980b). (2) Mesangial cells, like their smooth muscle cell cousins, also produce interstitial collagens, types I, II, and V (Striker et *al.*, 1980), a finding that explains the occasional presence of collagen fibrils in the mesangium. (3) Both epithelial and mesangial cells produce proteoglycans, but, interestingly enough, the types of proteoglycans produced differ. Epithelial cells synthesize predominantly heparan sulfate proteoglycans, and mesangial cells produce predominantly chondroitin sulfate proteoglycans (Farin et *al.*, 1980). (4) Both epithelial cells and mesangial cells have been shown to be capable of synthesizing fibronectin in culture (Killen and Striker, 1979; Foidart et *al.*, 1980b). As fibronectin has been localized to the Golgi complex (see Fig. 11-31) of mesangial cells *in situ* (Courtoy et *al.*, 1980), it might be anticipated that these cells would also be capable of producing fibronectin in culture.

The study of epithelial and mesangial cell lines in culture has been very informative with respect to indicating some of the biosynthetic capabilities of these two cell types. However, information on the specific capabilities of these cell types *in situ* is virtually nonexistent, and little or nothing is known at

* It is of interest that Huang (1979) has introduced a method that he believes distinguishes between the epithelial and the endothelial–mesangial basement membranes *in situ* based on their differences in density after guanidine treatment.

present concerning the relative contributions of each of the glomerular cell types to the synthesis of the GBM under normal or pathologic conditions.

4.2. Synthesis of Collagenous Peptides

Studies on the steps involved in the biosynthesis of collagenous peptides (investigated by radiolabeling with radioactive proline *in vivo*, by incubation of isolated glomeruli *in vitro*, or, more recently, by incubation of glomerular cells in culture) have yielded the following information of interest: Label is incorporated into a high-molecular-weight precursor (180,000–195,000) that is directly incorporated unchanged (i.e., without proteolytic processing) into the GBM matrix (Kefalides, 1979; Spiro, 1978; Healthcote and Grant, 1981). In time, the collagenous peptides are assembled into higher-molecular-weight aggregates, which appears to involve hydroxylysine-derived cross-links. The turnover of the collagenous proteins in the GBM is very slow (Price and Spiro, 1977). It is on the order of weeks or months rather than hours. The intracellular pathway for biosynthesis of GBM type IV collagen has not yet been worked out in the glomerulus, but based on analogies with information obtained on other systems (e.g., Reichert's membrane, lens capsule), it is assumed to be similar to that for other collagens (see Kefalides, 1979; Chapter 6).

4.3. Synthesis of GAG

Biosynthesis of GAG and their incorporation into the GBM has been demonstrated *in vivo* by radiolabeling with [^{35}S]sulfate and [^{3}H]glucosamine (Lemkin and Farquhar, 1981) and *in vitro* by radiolabeling perfused kidneys with [^{35}S]sulfate (Kanwar *et al.*, 1981). In both cases, heparan sulfate was the major GAG synthesized, but small amounts of hyaluronic acid and chondroitin sulfate were also detected *in vivo*, as was chondroitin sulfate *in vitro*. No data are as yet available on the turnover of glomerular proteoglycans or GAG; one study on whole kidney cortex (Barry and Bowness, 1975) has indicated that the half-life of renal GAG is in the same range (3–5 days) as that of GAG derived from other sources. This implies that the turnover of the GAG present in the GBM is much more rapid than that of the collagenous components of the GBM. The intracellular events in proteoglycan synthesis have not yet been studied in the glomerulus, but probably are similar to those that take place in other cells (Chapter 5).

4.4. Synthesis of Fibronectin and Laminin

The synthesis of fibronectin has been extensively studied in numerous cell types (Chapters 4 and 10), but it has not yet been investigated in the glomerulus. Information on the biosynthesis of laminin is quite recent and so far is limited

to studies on amniotic epithelial cells, embryonic carcinoma cells, and a human tumor cell line in culture (Chapter 4); nothing is known at the moment about its biosynthesis in the glomerulus. However, both these glycoproteins are assumed to be *N*-glycosylated with asparagine-linked oligosaccharide moieties containing terminal sialic acid residues. The pathways for biosynthesis of such glycoproteins have been well established (Chapter 4).

5. What Do We Know about the Function of the GBM as a Filter?

5.1. Clearance Studies

As mentioned at the outset, the glomerulus serves to filter the blood plasma. Clearance studies done by physiologists in the 1940s and 1950s using various probe molecules (proteins and dextrans) established that the glomerular capillary wall as a whole behaves like a sieve in that the passage of macromolecules is increasingly restricted as the molecular weight and effective diameter increase up to the size of albumin (MW 70,000). Albumin is the major plasma protein and must be retained in the blood, and normally only traces are filtered. Based on such studies, it was postulated that the walls of glomerular capillaries, as well as other capillaries, contain pores, ~9 nm in diameter. Recent work has further established the importance of charge as a factor in glomerular filtration (Brenner *et al.*, 1978; Bohrer *et al.*, 1978; Venkatachalam and Rennke, 1978, 1980). It was found that negatively charged molecules are filtered in much smaller amounts than neutral molecules of the same size (Fig. 11-32). Thus, the physiologic findings have established that the glomerulus has both size-selective and charge-selective properties, and they imply that the glomerular capillary wall is negatively charged.

5.2. Studies Using Electron-Dense Tracers

Early work by cell biologists and electron microscopists in this field was preoccupied with efforts to determine where the hypothetical pores postulated by the physiologists were located, that is, to determine which glomerular layer represents the critical barrier serving to retain albumin in the circulation. Many studies were done in which electron-dense tracers were used to identify the barrier, and conflicting results were obtained. As a result, a debate arose concerning whether the GBM or the epithelial slits were the main barrier that serves to retain plasma proteins (reviewed by Farquhar, 1975, 1980). At present, however, everyone who has worked on this problem is in agreement (1) that the GBM is the main filter serving to retain albumin and other plasma proteins in the circulation, and (2) that the epithelial slits do not serve in this capacity (Farquhar, 1975, 1978, 1980; Rennke et *al.*, 1975; Ryan and Karnovsky, 1976; Ryan et *al.*, 1976; Laliberté et *al.*, 1978; Venkatachalam and Rennke, 1978, 1980;

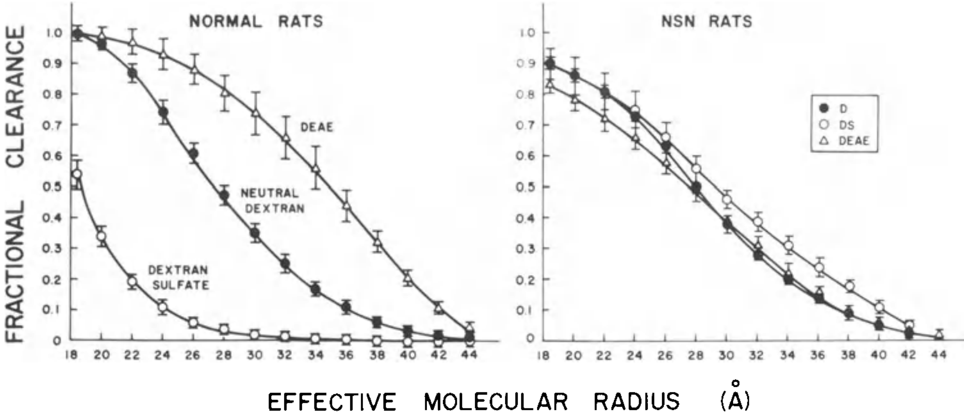


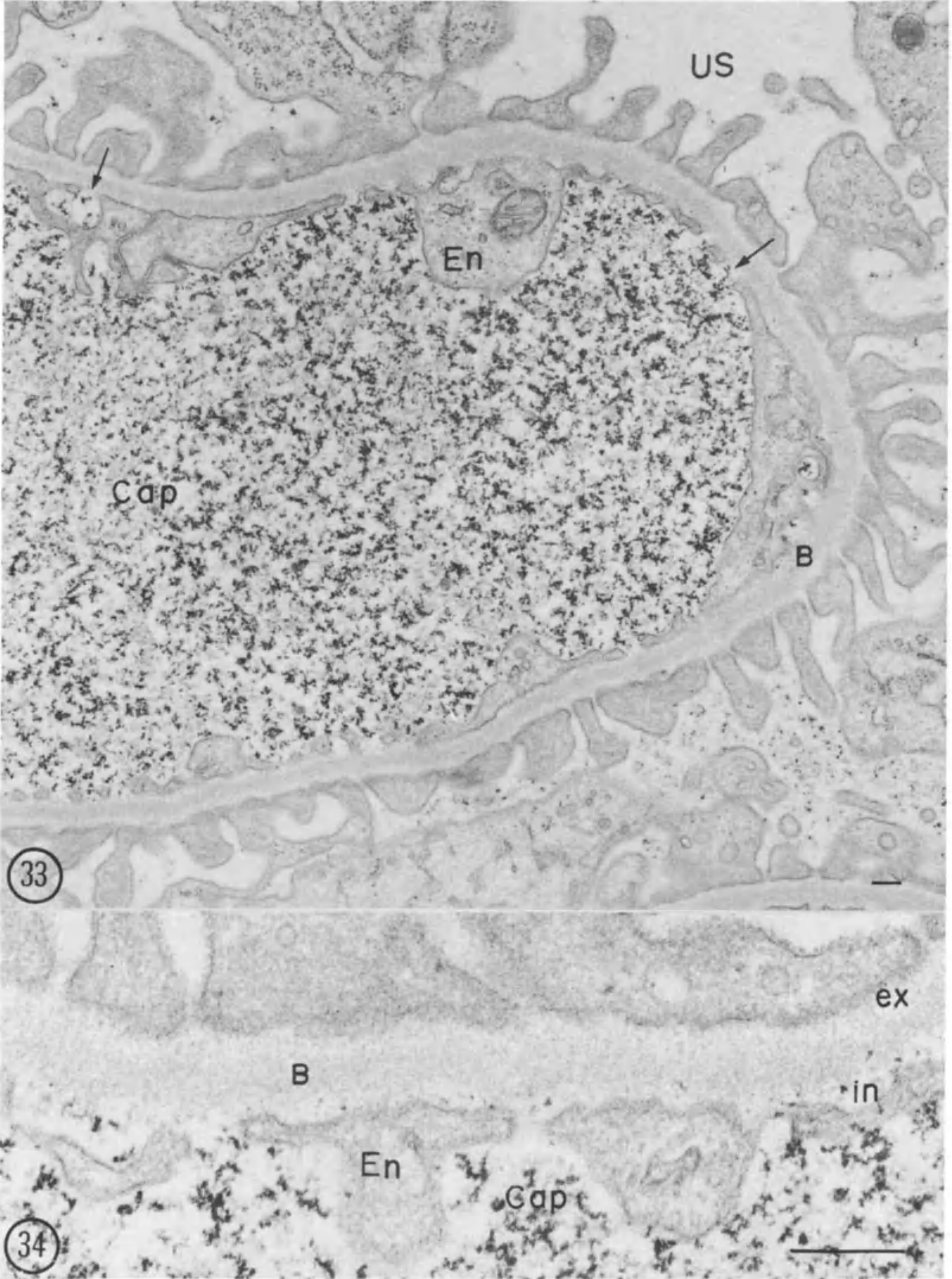
Figure 11-32. Clearance curves showing the size- and charge-selective properties of the normal glomerulus (left) and the loss of charge discrimination that occurs in some glomerular diseases associated with proteinuria (right). Size discrimination is manifest by the fact that there is increasing restriction to glomerular filtration with increasing effective molecular radius of the molecules, regardless of their charge. Charge discrimination is manifest by the fact that in normal rats, the clearance of negatively charged dextran sulfate is retarded, and that of positively charged DEAE dextran is increased over that of neutral dextran of a comparable size.

In rats with nephrotoxic serum nephritis (NSN), an experimentally induced glomerular disease in which there is proteinuria, the fractional clearance of all three forms of dextran are similar, indicating a loss of charge discrimination in this disease. (From Bohrer et *al.*, 1978.)

Farquhar and Kanwar, 1982). This conclusion was reached as a result of studies with a variety of anionic (native ferritin, catalase, endogenous albumin, or IgG) and neutral (dextrans) tracers (see Figs. 11-33 to 11-35), all of which indicate that the tracer molecules do not penetrate beyond the lamina rara interna of the GBM, and thereby identify the GBM as the main barrier preventing the filtration of anionic or neutral macromolecules larger than 70,000 molecular weight. The cell layers are assumed to function to synthesize and maintain the GBM and to modulate its activities by controlling access to it and exit from it (Farquhar, 1978). Much of the confusion that existed earlier was created by the use of cationic proteins (myeloperoxidase and some isozymes of horseradish peroxidase), which are not satisfactory as tracers because they bind to negatively charged glomerular components, especially to the epithelial cell surface coat and the laminae rarae of the GBM (see Fig. 11-5).

5.3. Nature and Location of Anionic Sites in Glomeruli

With the question of the location of the main filter resolved, attention in this field shifted to attempting to determine which glomerular component(s) was responsible for establishing the charge-barrier function of the glomerulus.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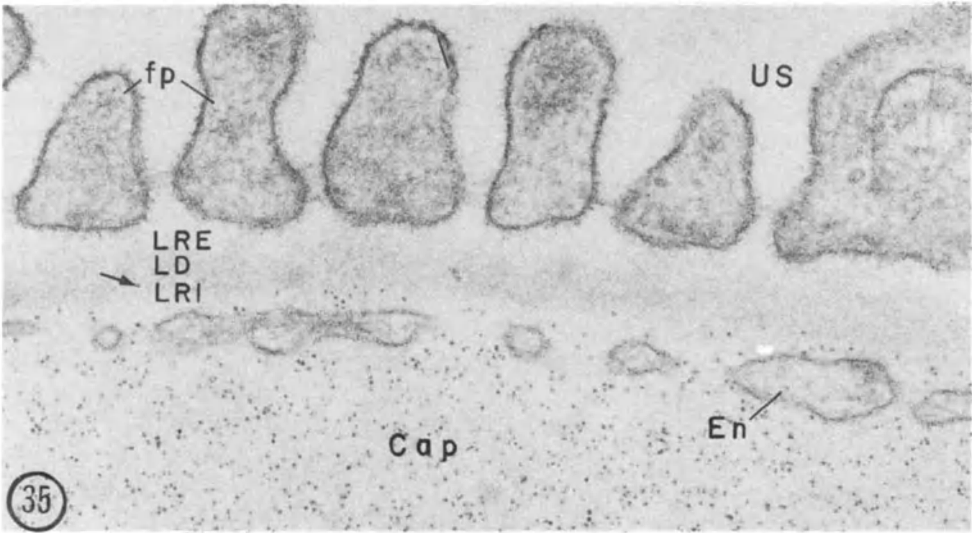
This is a matter of considerable importance because loss of the charge-barrier function has been found in several experimental glomerular diseases (Fig. 11-32), used as models for human diseases, which are characterized by leakage of plasma proteins (primarily albumin) into the urine (Brenner *et al.*, 1978).

Although cationic probe molecules are not useful as tracers, they have proved to be useful as stains for the demonstration of anionic sites. Using such cationic probes, negatively charged groups have been shown to be present in all layers of the glomerular capillary wall, i.e., in the GBM and on the surfaces of the endothelial and epithelial cells. The unusually thick, sialic acid-rich, cell surface coat of the epithelium, the so-called epithelial polyanion, captured the attention of a number of workers (Jones, 1969; Michael *et al.*, 1970; Mohos and Skoza, 1970; Latta *et al.*, 1975). This cell coat, which has not yet been characterized beyond the fact that it is rich in sialic acid, is on the wrong side of the GBM to provide it with its charge-selective properties. However, it has been shown to be essential for the maintenance of the normal foot process and slit arrangement, as neutralization of the coat by perfusion with polycations disrupts the foot processes and reperfusion with polyanions restores them to their normal state (Seiler *et al.*, 1977).

More recently, with the realization that the GBM constitutes the permability barrier for macromolecules, attention has focused on attempting to characterize charged groups in the GBM. That the GBM is a negatively charged structure was first demonstrated by the studies of Rennke *et al.* (1975) with differently charged ferritins. Their studies showed that, whereas native anionic (pI = 4.8) ferritin fails to penetrate beyond the lamina rara interna, cationized ferritins with increasing isoelectric points penetrate the GBM to a greater and greater extent. Subsequently, various cationic probes (lysozyme, cationized ferritin, ruthenium red) were used as stains to detect anionic sites in the GBM, and to determine where the greatest concentration of negatively charged groups occurred (Caulfield and Farquhar, 1976; Kanwar and Farquhar, 1979a). In the course of these studies, the network of proteoglycan granules concentrated in the laminae rarae was discovered (Figs. 11-7 to 11-9). Cationized ferritin with an isoelectric point of 7.3–7.5 proved to be a very useful probe for this purpose, as it binds to sites in the laminae rarae but not to the lamina densa or cell surface (Figs. 11-10 and 11-11), thereby demonstrating that the sites with the highest charge density or concentration of negatively charged groups in the glomerulus correspond to the sites where the heparan sulfate proteoglycans are located in the laminae rarae.

← **Figures 11-33 and 11-34.** Portions of a glomerulus from a normal rat sacrificed 3½ hr after the injection of dextran (MW 125,000). The capillary lumina (Cap) are filled with dense dextran particles. They appear to penetrate the fenestrae of the endothelium (En) (arrows) and can be seen in the lamina rara interna (in) of the GBM. No dextran is seen in the lamina densa (B), lamina rara externa (ex), or epithelial slits. Figure 11-33, × 22,000; Fig. 11-34, × 87,000. Bars = 0.2 μm. (Figure 11-33 is from Farquhar, 1980, and Figure 11-34 is from Caulfield and Farquhar, 1974.)



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Cap
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5.4. Role of GAG in the Permeability of the GBM to Macromolecules

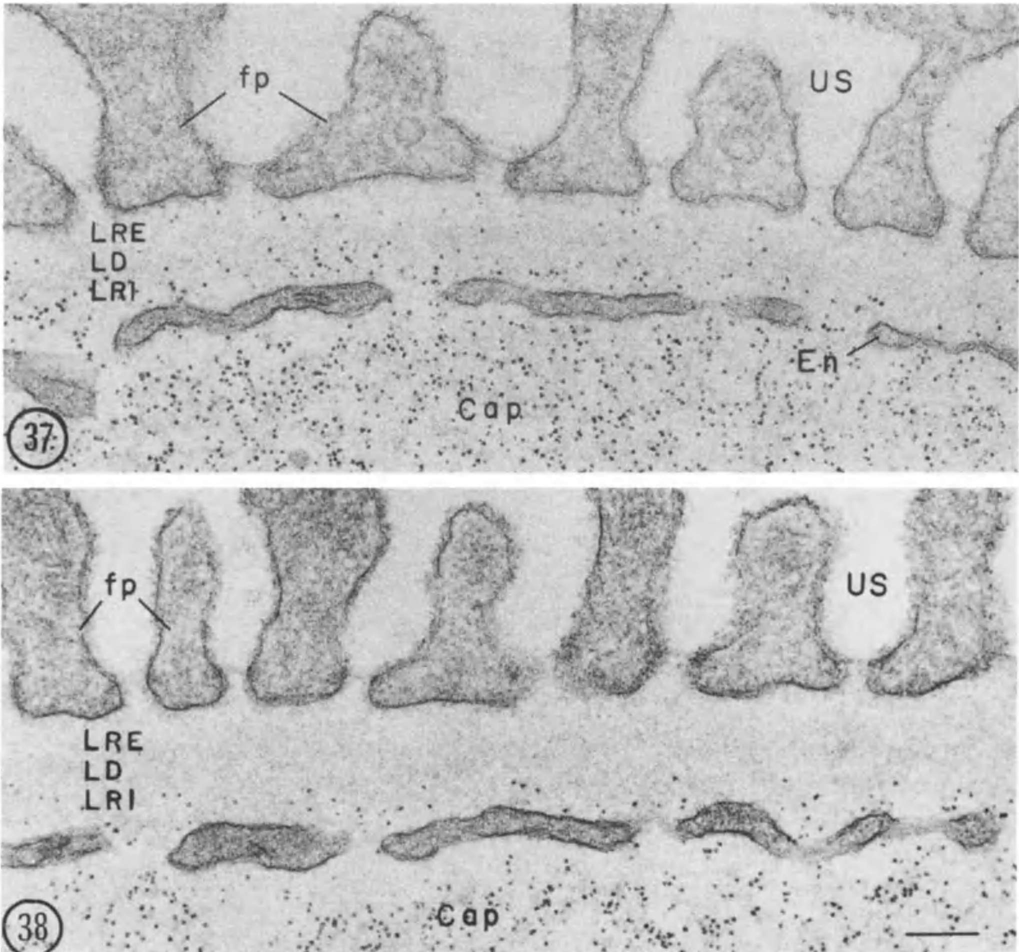
As the GAG moieties of proteoglycans are among the most highly negatively charged molecules known, their presence in the GBM makes them automatic candidates for components that might play a role in establishing or in maintaining the charge-barrier properties of the GBM. Besides their charge properties, GAG have been shown to exert a steric exclusion effect on proteins in solution (Comper and Laurent, 1978; Lindahl and Höök, 1978). Therefore, it could logically be assumed that GAG might be one of the GBM components responsible for endowing the GBM with its properties as a selective filter. Direct evidence for this assumption was provided by the demonstration that there is increased leakage of anionic ferritin (Kanwar *et al.*, 1980) and [^{125}I]-BSA (Rosenzweig *et al.*, 1981) into the urinary spaces after removal of GAG from the GBM by perfusion of kidneys with GAG-degrading enzymes *in situ*. Removal of all GAG including heparan sulfate by digestion with heparinase* led to increased leakage of both ferritin (MW 480,000) and BSA (MW 70,000) into the urinary spaces (Figs. 11-36 and 11-37); removal of hyaluronic acid alone by digestion with *Streptomyces* hyaluronidase, or removal of most GAG (hyaluronic acid, chondroitin 4- and 6-sulfate, and keratan sulfate) but not heparan sulfate by digestion with chondroitinase ABC, led to increased leakage of BSA (Rosenzweig *et al.*, 1981) but not of ferritin (Fig. 11-38). These findings suggest that both heparan sulfate and hyaluronic acid may play a role in endowing the GBM with its properties as a selective filter.

Are alterations in GAG responsible for the loss of charge selectivity found in some glomerular diseases? At present this question cannot be answered with certainty owing to lack of sufficient data. However, it is of interest that reduced binding of cationic probes to anionic sites in the laminae rarae has been reported in experimental animals with glomerular diseases associated with proteinuria (Caulfield and Farquhar, 1978; Kelley and Cavallo, 1980; Farquhar and Kanwar, 1982).

It is also worth noting that the role of GAG in modifying basement membrane permeability is probably not limited to the GBM because similar proteoglycan networks are widely, probably universally, distributed in all renal basement membranes (including those of the renal tubule, Bowman's capsule, and

* Partially purified heparinase [as opposed to purified heparitinase (see footnote on p. 349)] acts on all known GAG except keratan sulfate (Linker and Hovingh, 1972).

← **Figures 11-35 and 11-36.** Figure 11-35 shows a portion of a glomerular capillary from a control kidney perfused with native ferritin (10 mg/ml). Most of the ferritin molecules are restricted to the capillary lumen (Cap) or to the lamina rara interna (LRI) of the GBM. Occasionally, molecules are seen in the lamina densa (LD) (arrow), but they are rarely seen in the lamina rara externa (LRE) or in the urinary spaces (US). Figure 11-36 shows a similar field from a kidney perfused with heparinase* prior to perfusion with native ferritin. Numerous ferritin molecules have penetrated the lamina densa (arrows) and are present in the urinary spaces in large numbers. Both × 100,000, bar = 0.1 μm. (From Kanwar *et al.*, 1980.)

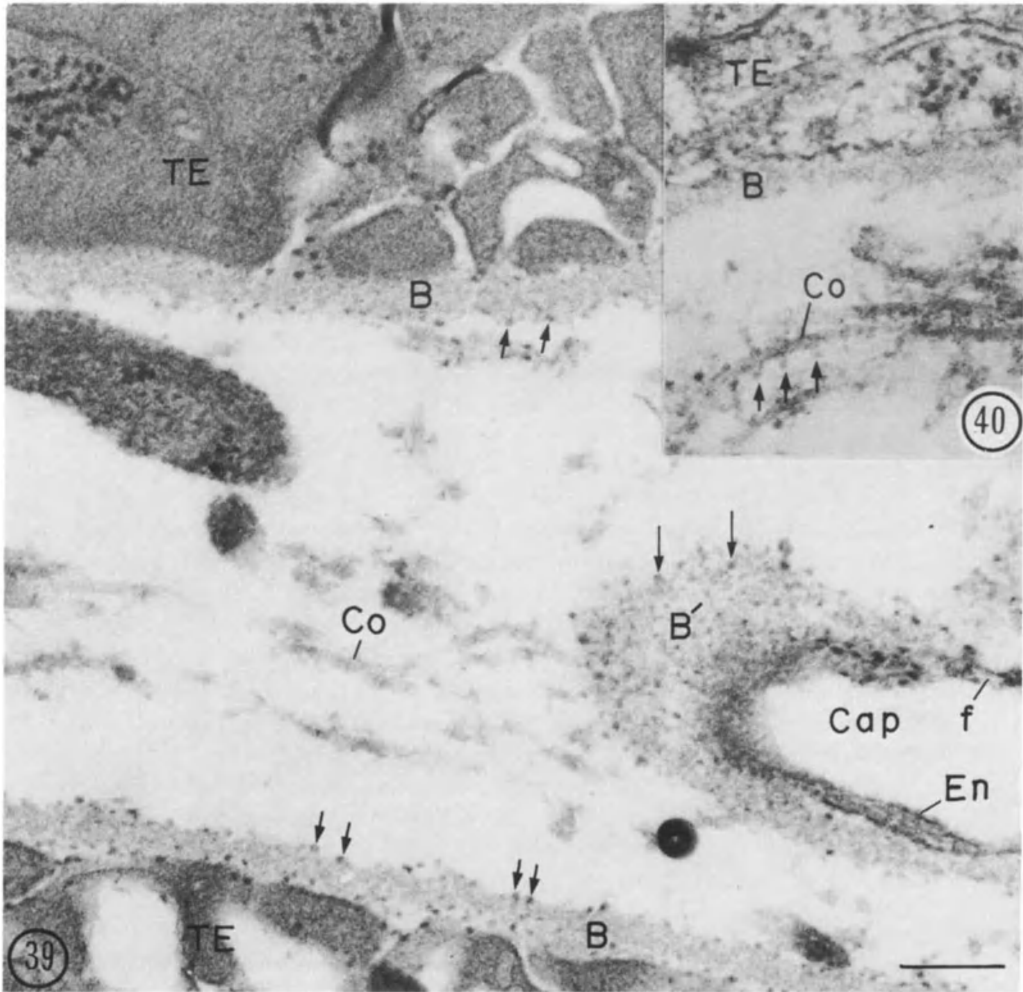


Figures 11-37 and 11-38. Portions of glomerular capillaries from kidneys digested with heparinase (Fig. 11-37) or chondroitinase ABC (Fig. 11-38), followed by perfusion with native ferritin. After heparinase treatment, increased amounts of ferritin have penetrated the GBM and are seen in the lamina densa (LD) and lamina rara externa (LRE), whereas after chondroitinase ABC, as in controls (Fig. 11-35), little or no ferritin is seen beyond the lamina rara interna (LRI). Both × 100,000, bar = 0.1 μm. (From Kanwar et *al.*, 1980.)

the arteriolar or peritubular capillary endothelium; (see Figs. 11-39 and 11-40) and in basement membranes in many other locations as well (see Trelstad *et al.*, 1974; Hay, 1981).

5.5. What Are the Permeability Properties of the Lamina Densa?

Information on the permeability properties of the collagenous lamina densa has come from experiments utilizing differently charged ferritins



Figures 11-39 and 11-40. Peritubular region of the cortex from a kidney stained with ruthenium red, demonstrating the wide distribution of heparan sulfate-rich proteoglycan granules in renal basement membranes. Figure 11-39 shows the basement membranes (B) at the base of two tubule epithelial cells (TE) and the basement membrane (B′) of a peritubular capillary (Cap), which is cut in grazing section. All of these basement membranes contain proteoglycan granules that are stained with ruthenium red (arrows). Figure 11-40 is a similar field from a kidney perfused with heparitinase prior to staining, demonstrating that the particles in the tubular basement membranes (B) as well as those in the endothelial basement membranes (not shown) are removed by this treatment. The proteoglycans associated with the collagen fibrils (Co) in the interstitia (arrows) are unaffected by this enzyme but are removed by chondroitinase ABC. En, endothelium; f, endothelial fenestra with its diaphragm. Both × 70,000, bar = 0.2 μm. (From Farquhar and Kanwar, 1982.)

(Rennke *et al.*, 1975; Kanwar and Farquhar, 1979a). As already mentioned, native anionic ferritin does not normally penetrate to any great extent beyond the lamina rara interna, but cationized ferritin can penetrate the lamina densa to reach and bind to the lamina rara externa. These findings indicate that the porosity of the lamina rara interna and lamina densa is equal to or greater than the size of ferritin (~12 nm), but normally ferritin and other anionic proteins are prevented from entering the GBM by charge repulsion. If the net charge of the ferritin is reduced, however, it can penetrate the GBM.

6. Concluding Remarks

Figure 11-41 summarizes in diagrammatic form what is known about the structure and composition of the GBM. The GBM consists of three layers of different composition, the lamina rara interna, the lamina densa, and the lamina rara externa. The lamina densa is a compact, filamentous layer in which type IV collagen has been localized by immunocytochemistry. The laminae rarae are the sites of localization of a network of heparan sulfate proteoglycans and of two attachment proteins, laminin and probably also fibronectin. The localization of proteoglycans has been established by special staining (e.g., ruthenium red) and enzyme digestion procedures, and the localization of laminin and fibronectin has been demonstrated by immunocytochemistry. It should be noted that only those constituents of the GBM that have been localized are shown in Figure 11-41, and it is most likely that the inventory is incomplete. A number of other components have been found in fractions of isolated GBM (chondroitin sulfate proteoglycans, hyaluronic acid) or have been identified as GBM components by immunostaining (microfibrillar protein of elastin, type V collagen, 7 S collagen), but their location is not yet established.

Several models have been proposed for the molecular organization of the GBM (Spiro, 1978; Kefalidès, 1979; Venkatachalam and Rennke, 1980; Schurer

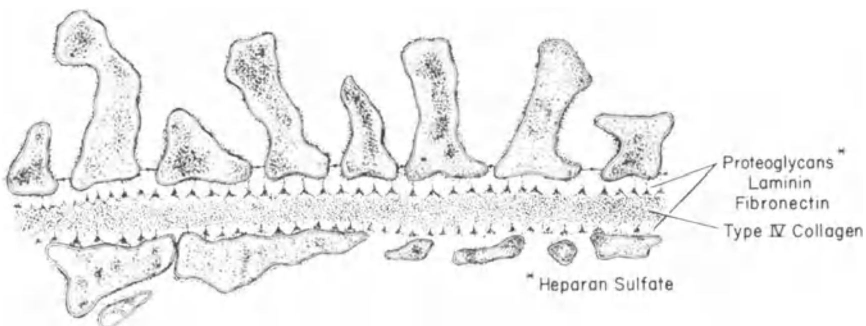


Figure 11-41. Diagram of GBM. (From Kanwar *et al.*, 1981.)

et al., 1980). For the most part these models have focused on the collagenous components, and they have considered the GBM as a uniform structure. They have *not* taken into account its multiple components and multilayered nature. Thus, the situation is undoubtedly infinitely more complex than indicated by any of the available models. Moreover, based on knowledge of the known interactions of the constituent molecules, e.g., fibronectin with collagen, actin, heparin, and cell surface receptors; proteoglycans with cell surface components, fibronectin, and collagens; laminin with cell surface constituents and heparan sulfate (see Chapters 4 and 10), it can be anticipated that multiple complex interactions occur between the glomerular matrix components and the cell surface and membrane constituents of the epithelial, endothelial, and mesangial cells.

To what extent can we relate the information available on the structure and function of the GBM to other basement membranes? As stated at the outset, basement membranes are the natural substrates on which all cells except connective tissue cells rest. Their basic layered organization appears to be similar from one location to another. The functions they perform include (1) to delimit connective tissue–nonconnective tissue boundaries, thereby maintaining the orderly organization of organs and tissues; (2) the attachment of cell layers to their associated connective tissue elements; (3) to provide a scaffolding for tissue regeneration and repair (Vracko, 1978); and (4) filtration of macromolecules, as stressed for the GBM. These can be considered general properties applicable to all basement membranes. For example, filtration of macromolecules must be a general function of all basement membranes regardless of their location. All epithelia and muscle cells are nourished by the interstitial fluid that percolates out from the capillaries. To reach these cells, circulating molecules must penetrate in sequence the endothelium, its basement membrane, the ground substance of the connective tissue, and the basement membrane of the cell layer in question. It follows that the ability of a circulating molecule to reach the cells depends on the permeability properties of all the layers between them and the circulation, which typically includes two basement membranes.

In some cases, the anatomical relationships of basement membranes are modified to facilitate a given function. A prime example is the glomerulus, where the filtration pathway has been dramatically shortened by fusion of the two basement membranes (endothelial and epithelial), thereby eliminating the interstitia. In other sites such as the skin, where the epithelial layers are subjected to considerable mechanical stress, an unusually tight epithelial–connective tissue attachment is required; additional elements known as anchoring filaments are often added to reinforce the construction along the dermal face of the basement membrane (see Farquhar, 1978).

Are basement membranes all alike? Morphologically, there is overall similarity among basement membranes, but this apparent organizational similarity at the electron microscopic level probably hides considerable chemical diversity, as manifest, for example, by the fact that there are qualitative and quantitative differences in the staining of basement membranes for different matrix components, e.g., for fibronectin (Scheinman *et al.*, 1980b; Courtoy *et al.*, 1981),

and by the fact that biochemical analyses have revealed differences in the relative amounts of collagenous and noncollagenous peptides in basement membranes from one location to another (Spiro, 1972; Kefalides, 1979). It can be anticipated that considerable molecular heterogeneity—perhaps to the point of ultimate specificity—may be detected in the future as cell biologists and biochemists probe more deeply into the molecular structure and chemical composition of basement membranes.

With the realization that proteoglycans are a general (probably universal) component of basement membranes, the definition of an important role for them in basement membrane biology and pathology can be predicted. Because of their many important properties, i.e., their ability to interact with all known components of basement membranes (collagens, fibronectin, laminin) as well as cell membrane components, their ability to influence the physical form of collagen assembly, their probable high rate of turnover in basement membranes in comparison to the collagenous proteins, and their established role in morphogenesis (Chapter 9), it is tempting to speculate that proteoglycans may play a key role in the molecular organization and specificities of basement membranes, as well as in their pathological derangements in disease.

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Chapter 12

Collagen and Embryonic Development

ELIZABETH D. HAY

1. Introduction

The possible importance of the extracellular matrix (ECM) to the development of the vertebrate embryo has been recognized since the turn of the century, but it was not until the fine structure of the molecule was fully appreciated that the first serious thought was given to a role of collagen per se in morphogenesis (see Gross, 1956). Collagen is initially detected in the vertebrate embryo at about the time of gastrulation (see Green *et al.*, 1968; Cohen and Hay, 1971; Manasek, 1975), that is, at the time the cleaving embryo begins to form the primary embryonic tissues (ectoderm, mesoderm, endoderm). In the avian embryo, basement (basal) laminae first appear under the ectoderm and endoderm when the primitive streak is forming, and the mesenchymal cells that migrate from the primitive streak between ectoderm and endoderm seem to move along these basement laminae (see Hay, 1968). As the primitive streak regresses, the notochord forms in front of it (Fig. 12-1). The first fibrillar collagen appears around the notochord and is produced by it (Carlson and Upson, 1974). Proteoglycans (PG) and other matrix molecules also begin to accumulate between the embryonic tissues at or somewhat before this time (Hay, 1973; Kosher and Searls, 1973; Wartiovaara *et al.*, 1980).

The notochord is a cylindrical, epithelial rod of mesodermal origin that prescribes the primary axis of the vertebrate embryo. It plays a role in induction of the neural tube and together with the neural tube induces the adjacent mesodermal mesenchyme to condense into somites, the paired epithelial balls that reflect the segmental plan of the embryonic axis (Fig. 12-1). The ECM is now rich in hyaluronate, fibrils, and interstitial bodies that contain fibronectin (see Chapters 9, 10), and it also contains type I collagen (von der Mark *et al.*, 1976). Further immunohistochemical studies are needed to trace the lineage of all the collagen types in the various vertebrate embryos, but the notochord sheath contains type II collagen in the youngest avian embryos that have been studied (von der Mark *et al.*, 1976) and it is likely that type III collagen appears early under the ectoderm. Basement laminae surround the notochord, neural

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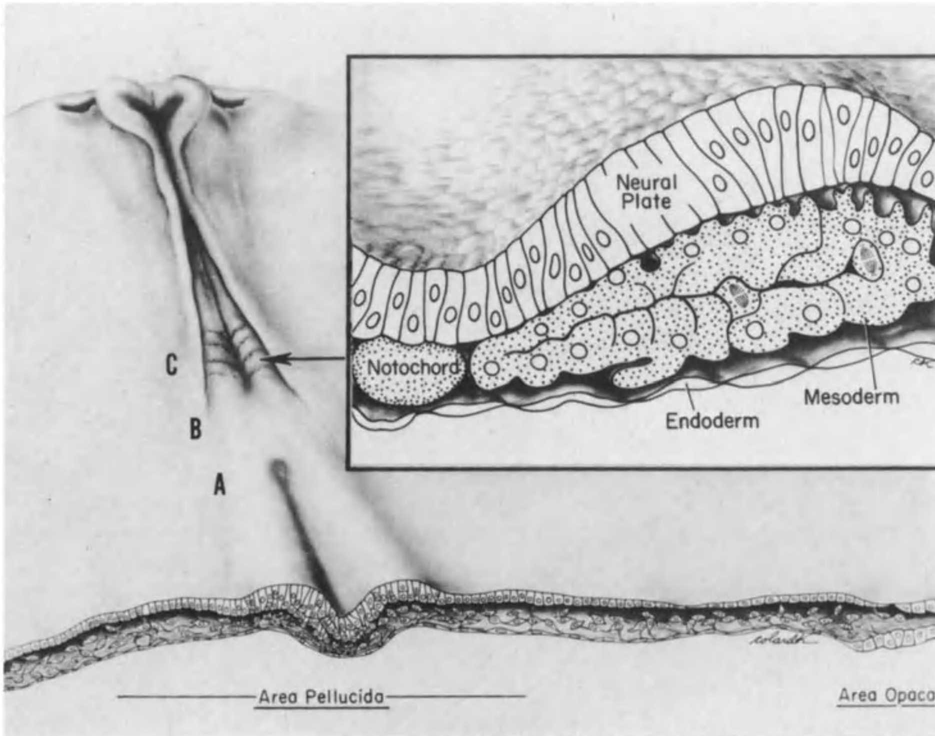


Figure 12-1. Diagram of a stage 8 (1 day old) chick embryo viewed from above and cut across the middle of the primitive streak. In the region of the streak, primary mesenchymal cells are detaching from the overlying ectoderm to migrate between ectoderm and endoderm. Some fuse with endoderm. In the area between A (Hensen's node) and B, the notochord has formed in the midline, and mesoderm is organized into somitomeres that will give rise to somites (Meier, 1979). In the region between B and C, the neural plate is starting to develop. At level C, three somites have formed, one of which is shown in cross section in the inset. Between levels B and C, the first definitive connective tissue fibrils are appearing, the basement laminae are thickening, and the matrix between neural plate and mesoderm is becoming rich in sulfated GAG. (From Hay, 1973.)

tube, and somites (Cohen and Hay, 1971) and are well developed under the ectoderm and endoderm; they contain fibronectin (Chapters 4, 10) and probably also type IV collagen and laminin in most embryos (Wartiovaara *et al.*, 1980). Subsequently, the ECM becomes more elaborate as the definitive organs form. As we shall see in this chapter, its collagenous components are implicated in the development of glands from ectoderm and endoderm, skeletal structures from mesoderm, and stromal structures from corneal ectoderm.

In this chapter, we will first review the evidence for a role of collagen in tissue interactions, or so-called embryonic inductions, such as have been alluded to above. This will lead us into a discussion of the relation of collagen to the cell's cytoskeleton and the possible role of cell shape and ECM in con-

trolling growth and synthetic activities of embryonic and regenerating tissues. We will also give some attention to the role of collagen in cell adhesion and migration, *in vivo* and *in vitro*. Finally, we will speculate on the function that collagen might play in binding molecules like morphogenetic factors that may promote or stabilize differentiation of embryonic tissues. Our discussion will be limited to vertebrates, but the principles should also apply to the invertebrates that contain collagen.

While the subject of this chapter is collagen, it will become clear that what has been said in the preceding chapters of this book about PG, glycoproteins, and other matrix molecules will have to be kept in mind, for none of these molecules is an isle unto itself; ultimately, we must understand the interaction of matrix molecules with each other as well as with the cell if we are to understand completely their role in embryonic development.

2. Tissue Interaction in the Developing Embryo

One of the first things that must be appreciated about the cell biology of matrix function in morphogenesis is the basic dichotomy of tissue organization in the early vertebrate embryo. The cells can be grouped into two classes: epithelia and mesenchyme (Fig. 12-2). An epithelium is composed of con-

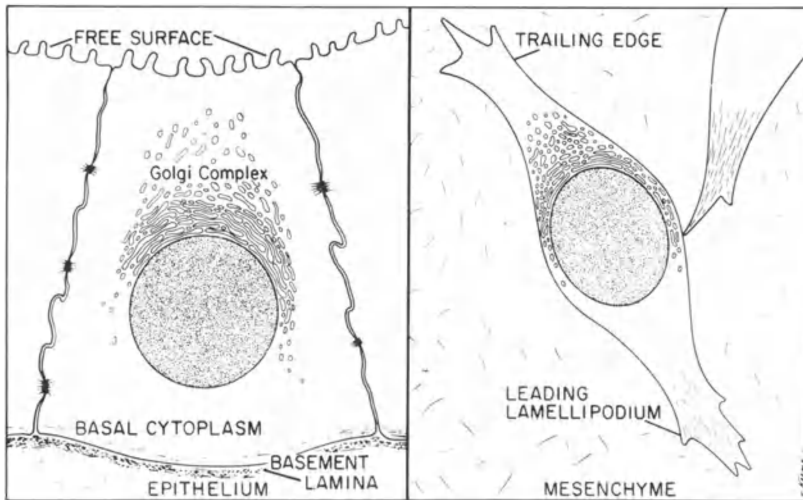


Figure 12-2. Diagram showing the major differences between an epithelial cell (left) and a mesenchymal cell (right). The epithelial cell sits on top of a basement lamina, under which connective tissue fibrils polymerize. The mesenchymal cell resides in the ECM and if it is migrating, it has a trailing edge containing the Golgi complex. In the epithelial cell, the Golgi complex is usually in the apical cytoplasm, the free surface is specialized for dealing with the outside world, and the basal surface is designed to connect to the underlying ECM; the lateral surfaces form numerous junctions with adjacent cells. Only occasional junctions are seen between mesenchymal cells.

tiguous cells, closely linked by cell junctions, that sit on top of ECM; the cells facing the outside (or an internal lumen) have a specialized free surface usually containing microvilli and a distinctive glycoprotein coat; the basal surface faces the basement lamina. Mesenchymal cells migrate into ECM and may be polarized in the sense that Golgi zones tend to occupy trailing ends (Trelstad, 1977). Epithelial cells do not invade ECM, even when isolated and recombined with matrix, but mesenchymal cells plated on ECM *in vitro* do invade the matrix (Overton, 1977). It is quite likely that the mesenchymal cell surface has a basic structure (organization of receptors, etc.) that differs from the epithelial cell surface. Moreover, the basal epithelial surface usually abuts a specialized basal cytoskeletal mat, and it is well known that the basolateral and apical epithelial membranes are quite different in many of their properties.

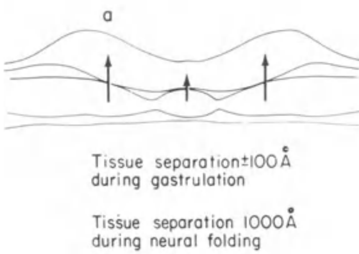
Keeping these points in mind, it is useful to classify embryonic tissue interactions as epithelial–epithelial, epithelial–mesenchymal, and mesenchymal–epithelial, indicating by the first member of the pair the tissue believed to be inducing the other (Fig. 12-3).

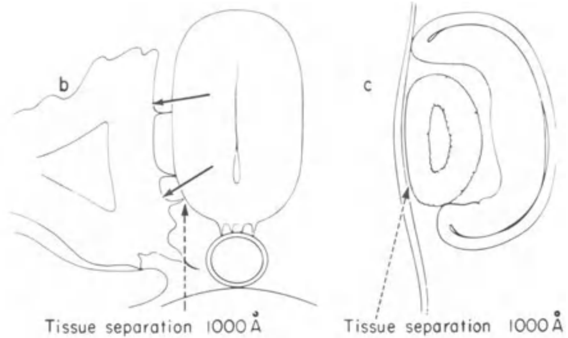
2.1. Mesenchymal–Epithelial Interactions

In 1955, Grobstein called attention to the possible role of ECM in embryonic tissue interactions as a result of experiments he had undertaken using trypsin to isolate the epithelium and mesenchyme of the developing mouse salivary gland. He classified the mesenchyme as the inductor in this interaction (class 3, Fig. 12-3), because the epithelium was unable to assume a glandular morphology when grown *in vitro* without the mesenchyme. Because the effect could be transmitted across a Millipore filter into which the inducing tissue secreted stainable ECM, Grobstein (1955), with remarkable intuition, predicted that matrix–cell interaction is active in the events he termed embryonic induction. He said (1955, p. 234), "I would like to define embryonic induction as developmentally significant interaction between closely associated but dissimilarly derived tissue masses. . . . Under this general definition, which deliberately avoids any reference to cellular determination, we leave our minds open as to mechanism." That the "mechanism" revealed by his tissue isolation approach involves ECM has been amply demonstrated since then; the interaction that Grobstein studied between mesenchyme and glandular epithelium, however, has been shown to be somewhat more complicated than he envisioned, as we shall now see.

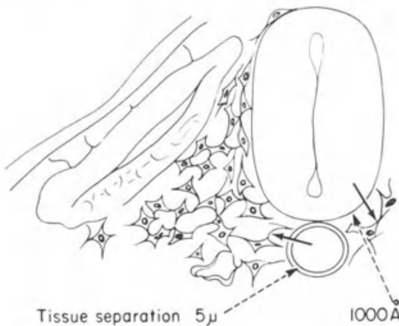
Bernfield and his collaborators have accumulated evidence (for review see Bernfield and Banerjee, 1978; Chapter 9) that mesenchyme promotes branching of an epithelial tube or acinus by removing glycosaminoglycan (GAG) from the basal surface of the epithelium at the tips of developing lobes. The GAG-poor areas are then able to grow out and new clefts develop. Clefts subsequently seem to be immobilized by collagen, which also stabilizes GAG (see David and Bernfield, 1979). The theory requires that considerable information be built into the epithelial cell surface, because new clefts would have to form on the

Class 1. Epithelial — Epithelial





Class 2. Epithelial — Mesenchymal



Class 3. Mesenchymal — Epithelial

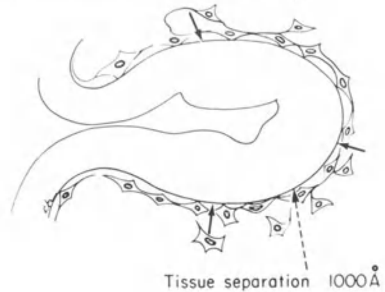


Figure 12-3. Tissue interactions in the embryo can be classified as epithelial–epithelial (class 1), epithelial–mesenchymal (class 2)., and mesenchymal–epithelial (class 3). The classical example of embryonic induction is the effect of notochord and mesoderm on the neural plate, causing it to fold (a). At this time the space between the interacting tissues is at least 100 nm, due to formation of ECM, probably contributed mainly by the inducing tissue (chordamesoderm). The neural tube influences somite development (b); again the tissues are separated by ECM. The lens induces corneal differentiation (c); here, there is evidence that ECM is involved (see text). The example of class 2 depicts neural tube and notochord, which induce the adjacent somite-derived mesenchyme (sclerotome) to become cartilage; both collagen and PG are involved in the interaction (see text). The example shown of class 3 is the induction by mesenchyme of gland morphogenesis (discussed further in Fig. 12-4). (From Hay, 1968.)

epithelial outgrowths even as they are being exposed to the putative hyaluronidase synthesized by the mesenchyme (Fig. 12-4). That collagen derived from the mesenchyme mainly accumulates in the forming clefts (see Wessells, 1977; Spooner and Faubion, 1980) also implies a localized control of its polymerization. If collagen, GAG, and enzyme can be transmitted across a Millipore filter from mesenchyme to epithelium, as required to explain Grobstein's results, then it must be the epithelium that knows what to do with the molecules.

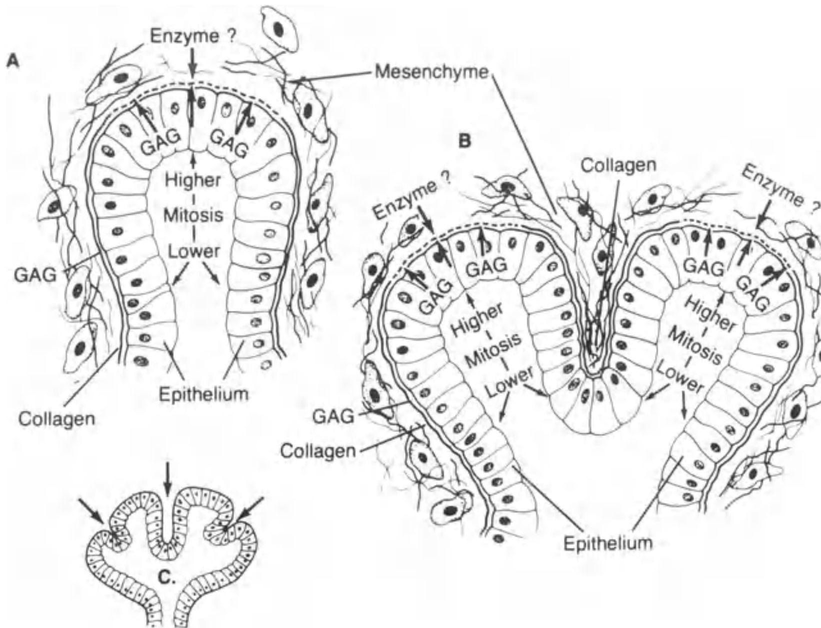


Figure 12-4. The effect of mesenchyme on branching of an epithelial gland is diagrammed here. (A) depicts a stage similar to that shown in class 3, Fig. 12-3. At this time, the mesenchyme seems to produce hyaluronidase, removing GAG; these areas of epithelium tend to grow out. In (B), a cleft has formed; in this region, GAG and collagen are not turned over and seem to stabilize the cleft. Mitosis is higher at the outgrowing tips. The gland shown in (B) then takes on the appearance shown in (C). Compare with Figs. 9-13, 9-14, and 9-15. (From Wessells, 1977.)

The state of "determination" of the reacting tissue thus does have to be taken into account. Grobstein (1955, and quote above) merely asked that it not be necessary to show that the responding tissue was "undifferentiated" in order to term an interaction between two developing tissues "embryonic induction." In fact, the responding tissue is almost invariably predifferentiated to some degree in tissue interactions. If we define "differentiation" as involving both changes in cell shape (A, Fig. 12-5) and enhancement of specific protein synthesis (B, Fig. 12-5), then salivary gland and pancreatic epithelia are predifferentiated in the sense that they are coded to exhibit particular branching patterns (Bernfield and Banerjee, 1978) and already contain small amounts of specific gland proteins (Rutter *et al.*, 1968). Because tissues are predifferentiated to some extent even at gastrulation, it is probably not useful to confine the use of the term "induction" to describe events that transform "undifferentiated" tissues to "differentiated" tissues. Rather, what we are trying to understand in embryonic development is the importance of tissue interactions (Fig. 12-3) in expression and stabilization of the genome. Cells which are predifferentiated would be expected to respond differently to the same "inducer" molecule, be that molecule ECM, growth factor, or other substance.

The question of requirement of a particular gland epithelium for a specific mesenchyme during embryonic induction has also attracted a great deal of study (see Ball, 1974; Cunha and Lung, 1979). It seems fair to conclude that in mesenchymal–epithelial interactions involving gland epithelia, there is not as restrictive a requirement for a specific mesenchyme as was once thought. It will be interesting to explore the question further in the future as we begin to learn more about the cell biology of mesenchymal cells. Not all fibroblasts are making the same ECM molecules (or enzymes, for that matter), and they may even modify the type of collagen they are producing in response to change in the environment (see Conrad et *al.*, 1980; Hay, 1980). Moreover, early mesenchymal cells derived from the primitive streak (primary mesenchyme) form little or no ECM; they differentiate into mesodermal epithelia (somites, nephrogenic and lateral mesoderm) that have the capacity to form definitive epithelia (e.g., kidney tubules) or secondary mesenchyme (as, for example, the sclerotome component of the somite). Secondary mesenchymal cells, which in the case of the salivary gland derive from ectoderm (neural crest), secrete ECM and give rise to fibroblasts, odontoblasts, chondrocytes, and osteocytes. They may also give rise to muscle (see Hay, 1968).

It is beyond the scope of this chapter to consider all the other examples of this class of tissue interaction that have been studied by developmental biologists (see Cunha and Lung, 1979; Dürnberger and Kratochwill, 1980). The interaction of mesenchyme with developing muscle cells is not strictly a mesenchymal–epithelial interaction, but it needs to be mentioned because it was the classic work of Konigsberg and Hauschka (1965) that first drew attention to the possibility that the effect of mesenchyme in certain tissue interactions could be mediated by collagen produced by fibroblasts. The effect of collagen is to promote attachment and fusion of myoblasts, which then develop

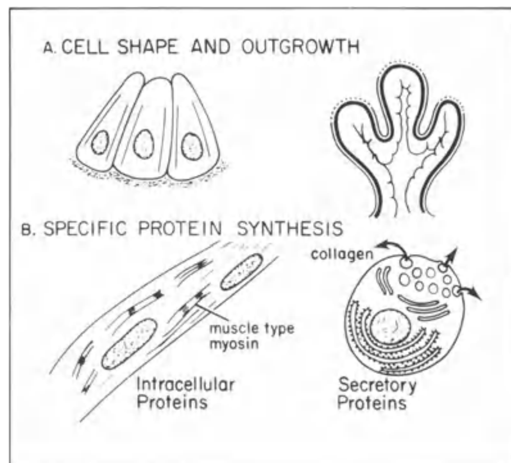


Figure 12-5. The effects of inducers can be divided into two major groups. On the one hand (A), inducers influence cell shape and outgrowth, sometimes referred to as "morphogenesis." On the other hand (B), inducers may enhance specific protein synthesis, sometimes referred to as "differentiation." The role, if any, of ECM in causing epithelial cell elongation (upper left) has not been demonstrated, but ECM is known to be involved in gland branching (upper right). ECM can enhance muscle differentiation (lower left) and cartilage differentiation (lower right).

striated myofibrils. A serum protein is required (Hauschka and White, 1972) that seems to be equivalent to fibronectin (Chicquet *et al.*, 1979); any type of collagen can mediate the effect (Ketley *et al.*, 1976), and while fibronectin is required for initial attachment of myoblasts to collagen, exogenous fibronectin delays fusion (see Kleinman *et al.*, 1981a,b; Chapter 10, this volume).

Finally, it has to be mentioned that treatment of cultured tissues with collagenase and inhibitors of collagen synthesis can interfere with tissue interactions; these studies are difficult to interpret because the effect may not be specifically on collagen, and, moreover, collagen synthesis may even be the end product of the differentiative process under study (see Kleinman *et al.*, 1981a,b; Wessells, 1977).

2.2. Epithelial–Mesenchymal Interactions

It is useful to consider epithelial–mesenchymal interaction in a separate category, because the structure of the tissues (Fig. 12-2) is distinctive. In mesenchymal–epithelial interaction, the base of the epithelial cell, which is normally covered by a basement lamina, is reacting to products of mesenchymal cells that may become part of the basement lamina and its underlying substratum or that may modify the basal lamina by removing some of its components (Fig. 12-4). In epithelial–mesenchymal interaction, the reacting mesenchymal cells are usually not polarized in this sense and their entire cell surface is more or less directly exposed to "inducing" molecules. The most extensively studied epithelial–mesenchymal interaction, that of the induction of vertebral cartilage by notochord and neural tube, happens also to provide one of the best cases for a direct role of ECM in promoting and stabilizing protein and polysaccharide synthesis during differentiation (Kosher *et al.*, 1973; Kosher and Church, 1975; Lash and Vasan, 1978).

During the morphogenesis of the somite, its ventromedial wall disperses into a mesenchyme, termed the sclerotome, which interacts with the notochord and neural tube (class 2, Fig. 12-3). Normally, this mesenchyme migrates around the notochord and neural tube and then differentiates into a definitive cartilage shaped like a vertebra. In culture, on agar (Fig. 12-6), or on Nucleopore filter, the mesenchymal cells of the sclerotome, but not the dermamyotome, eventually differentiate into cartilage (Gordon and Lash, 1974; Cheney and Lash, 1981). In this system *in vitro*, chondrogenesis is measured by the level of GAG synthesized (Fig. 12-7A), the size of PG produced (Fig. 12-7B), and/or the appearance of metachromatic nodules in the aggregated somite mass (Fig. 12-6). Notochord, neural tube, PG, and collagens speed up the appearance of cartilage and enhance the level of GAG synthesis (Fig. 12-7A); removal of ECM from notochord decreases its inductive ability (Kosher and Lash, 1975).

The effect of ECM on sclerotome differentiation can be said to be autocatalytic, in that the same collagen the presumptive chondrocytes eventually produce, type II collagen, is slightly more effective in elevating the level of GAG

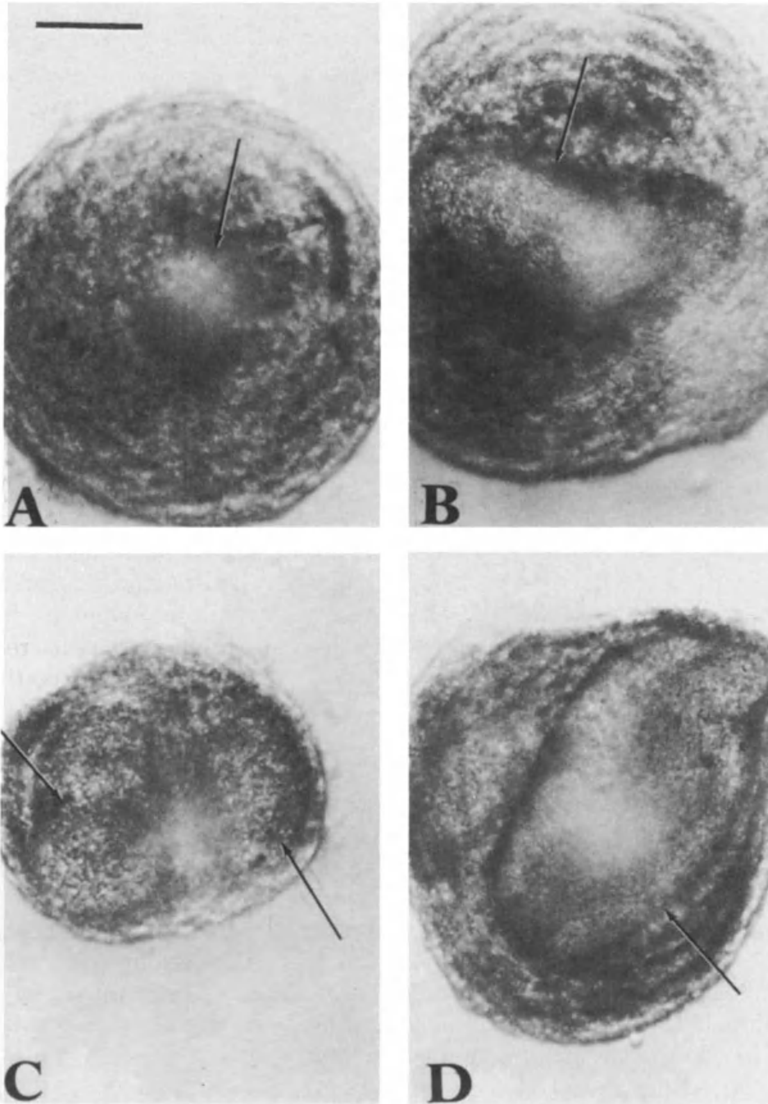


Figure 12-6. Photographs of living somite explants grown for 7 days on nutrient agar. Each clump is a cluster of 8–10 somites from stage 17 chick embryos. The anterior somites (A, B) derive from somites cephalad to the wing bud. The posterior somites (C, D) are the youngest. Typical refractile cartilage nodules appear in anterior somite plus notochord (B) on day 3, whereas they form by day 2 in vitro from posterior somites plus notochord (D). Anterior somites alone (A) rarely form cartilage, but posterior somites alone (C) usually start forming cartilage on day 4. Thus, the effect of notochord is to enhance an inherent ability of somites to produce cartilage. Arrows, cartilage nodules. Bar = 100 μm. (From Gordon and Lash, 1974.)

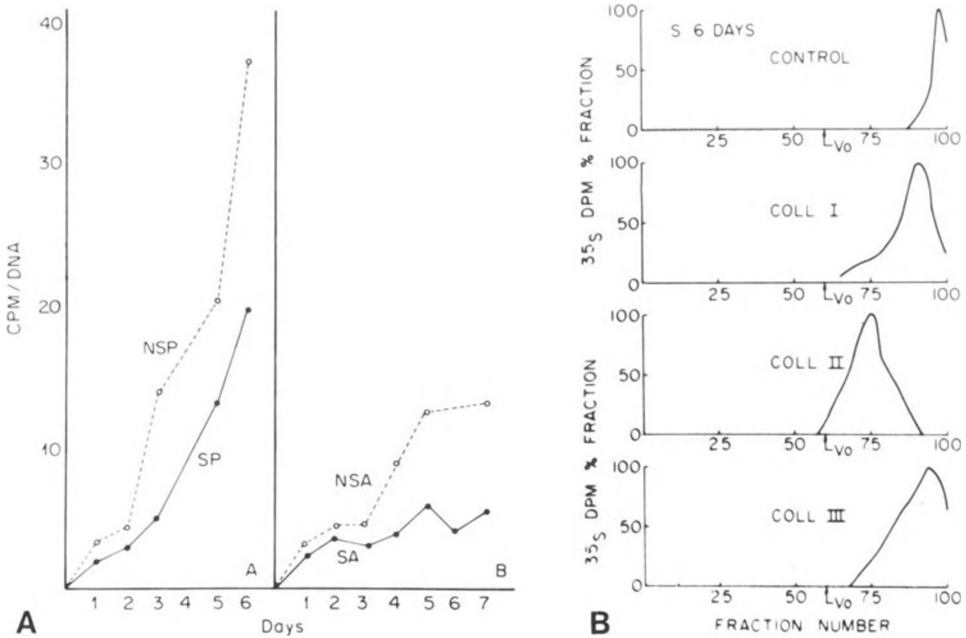


Figure 12-7. Biochemical parameters parallel morphological ones (Fig. 12-6) during the differentiation of anterior (SA) and posterior (SP) somites on agar (Fig. 12-7A). In the presence of notochord (NSA, NSP), sulfated GAG accumulation increases markedly on day 2 (NSP) or day 3 (NSA) *in vitro*. After day 4, posterior somites alone (SP) begin to increase GAG production, but anterior somites alone (SA) usually do not produce cartilage. Added to cultures of somites alone, collagen mimics the quantitative effect of adding notochord shown in Fig. 12-7A. Collagen also has a qualitative effect, as does notochord, because it induces larger PG to be produced by the somites as judged by molecular sieve chromatography (Fig. 12-7B). In both regards, collagen type II is slightly more effective than the other collagens. (A, from Gordon and Lash, 1974; B, from Lash and Vasan, 1978.)

(Kosher and Church, 1975) and in increasing the size of PG produced (Lash and Vasan, 1978). The effect of PG and GAG is similar to that reported in 1972 by Nevo and Dorfman (see Chapters 5, 9), who studied sternal chondrocytes: GAG production is accelerated. The presumptive chondrocytes are predifferentiated, as we already noted (Cheney and Lash, 1981), and it is tempting to think that ECM molecules, alone or in consortium, interact with preexisting receptors on the cell surface (Chapters 9, 10). Kosher (1977) postulated that collagen interacts with a receptor in the cell membrane that activates a second message, cyclic AMP. What he found was that high intracellular cyclic AMP levels inhibit collagen-induced somite chondrogenesis. On the other hand, cyclic AMP derivatives stimulate chondrogenesis of limb bud mesenchyme (Kosher *et al.*, 1979). The effect of collagen per se on limb chondrocytes is unknown. In another epithelial–mesenchymal interaction that has been studied, namely, induction by pigmented neural retina of neural crest chondrogenesis,

collagenous ECM has been reported to have a stimulatory effect (Newsome, 1972). The effect of purified collagen has not been investigated.

In the next section, we shall examine still another example of the autocatalytic effect of ECM on ECM synthesis in the very early embryo, but before turning to that, another subclass of epithelial–mesenchymal interaction needs to be mentioned and that involves the transformation of primary mesenchyme into epithelia. In one well-studied case, embryonic epithelium (e.g., ureteric bud, spinal cord) has been shown to cause the condensation of a primary mesenchyme (nephrogenic mesenchyme) into an epithelium (kidney tubules). Cultured transfilter to epithelial inductor, the formerly laminin-free mesenchyme acquires laminin in a spotty pattern as determined by immunofluorescence (Ekblom et *al.*, 1980; Ekblom, 1981). After aggregation, the developing kidney epithelial cells become polarized, in that they only exhibit laminin on their basal surfaces. We will return to discuss the role of ECM, including collagen, in establishing epithelial polarity in a later section devoted to the question of cell shape and cytoskeleton.

2.3. Epithelial–Epithelial Interactions

Epithelial–epithelial interactions are characterized by the fact that the basal surfaces of two epithelial tissues face each other during the interaction. Such interactions are typical of the early embryo (Fig. 12-3, class 1), but most epithelial tissues are separated from each other by secondary mesenchyme in older embryos. In one such early interaction, the induction by lens of corneal stroma production by corneal epithelium (upper right, Fig. 12-3), it has been postulated that the inducing epithelium (lens) begins to make ECM before the reacting tissue (corneal epithelium) and thus stabilizes the basal surface of the corneal epithelium by contributing collagen and PG to its basal lamina (see Hay, 1980). If the corneal epithelium is separated from the embryo by ethylenediaminetetraacetate or by trypsin–collagenese, its basal surface (but not its lateral or apical surfaces) blebs, attesting to the complete removal of ECM (Dodson and Hay, 1971, 1974). When such an epithelium is placed on frozen-killed lens, or other nonliving collagenous substrata (Fig. 12-8A), it produces a new corneal stroma (Fig. 12-8B) containing striated collagen fibrils (Dodson and Hay, 1971), both type I and type II collagens (Linsenmayer et *al.*, 1977), basal lamina (Hay and Dodson, 1973), and GAG (Meier and Hay, 1973). It does not produce a new stroma when grown on Millipore filter, but merely continues to bleb (Fig. 12-9B).

Meier and Hay (1974) compared the effect of a variety of noncollagenous substrata on avian corneal epithelial differentiation, as measured by production of polymerized corneal stroma (Fig. 12-8B) and isotope-labeled collagen (Fig. 12-8C) and GAG. When the latter data are quantitated, it can be seen that the epithelia produce a baseline level of ECM when grown on Millipore filter, glass, plastic, or albumin, and a stepped-up or "induced" level when grown on lens capsule, acellular corneal stroma, or purified chondrosarcoma collagen (Fig.

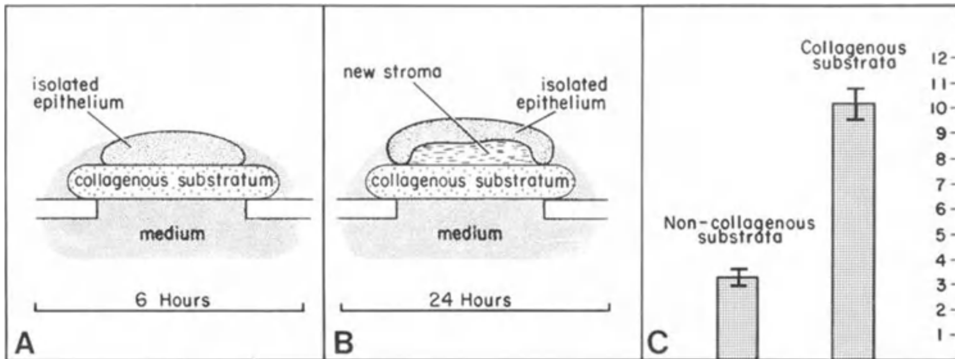


Figure 12-8. Diagrammatic summary of the experiments of Dodson and Hay (1971, 1974) and Meier and Hay (1974) on the production of corneal stroma by embryonic avian corneal epithelium. The corneal epithelium is isolated from the embryo using EDTA or enzymes and placed on a collagenous substratum (A). Within 6 hr, the basal cell surface is completely smooth, and by 24 hr, the epithelium has produced a new corneal stroma (B). The graph on the right expresses stromal synthesis as cpm ($\times 10^{-3}$) [^{3}H]proline in collagen produced by eight epithelia in 24 hr. The effects of lens capsule containing type IV collagen, rat tail tendon gels containing type I collagen, and purified chondrosarcoma type II collagen are the same (C). Epithelia grown on noncollagenous substrata (Millipore filter, plastic, glass, agar, albumin, or keratin) produced a small "baseline" amount of collagen. Morphologically detectable corneal stroma could not be detected in these latter cultures, and the basal surface of the epithelium continued to bleb. Compare Figs. 9-1 and 9.2.

12-8C). The stepped-up level is associated with extensive stromal polymerization, as viewed by electron microscopy (Fig. 12-8B). The effect of collagen can be obtained in serum-free medium and is not associated with significant ECM turnover (Meier and Hay, 1974). The effect of GAG is only on GAG synthesis and can be mediated by soluble GAG. "Soluble" collagen seemed not to stimulate collagen and GAG synthesis to the same degree as lens capsule and various collagen gels (Meier and Hay, 1974), but collagen is difficult to solubilize and so these results are under reinvestigation (Sugrue and Hay, 1981). The effect of the several collagen types is similar and is not lens specific; it is proportional to cell surface area in contact with collagen and does not seem to depend on ingestion of ECM by the epithelium (see Hay, 1977, for further review).

The influence of ECM on the basal epithelial cell surface and cytoskeleton is under current investigation in this system (Sugrue and Hay, 1981). As we noted above, the immediate effect of removal of underlying ECM from the corneal epithelium is extensive basal blebbing (Fig. 12-9B); the basal surface smooths out after several hours in contact with ECM and the basal cytoskeleton condenses again, taking on an appearance like that *in situ* (Fig. 12-9A). To test the hypothesis that collagen is interacting directly with the cell membrane, rather than exerting a "physical" effect, type I collagen was solubilized by heat denaturation and added to the medium under blebbing epithelia that were supported on Millipore filters. Within 2 hr, blebs began to withdraw and be-

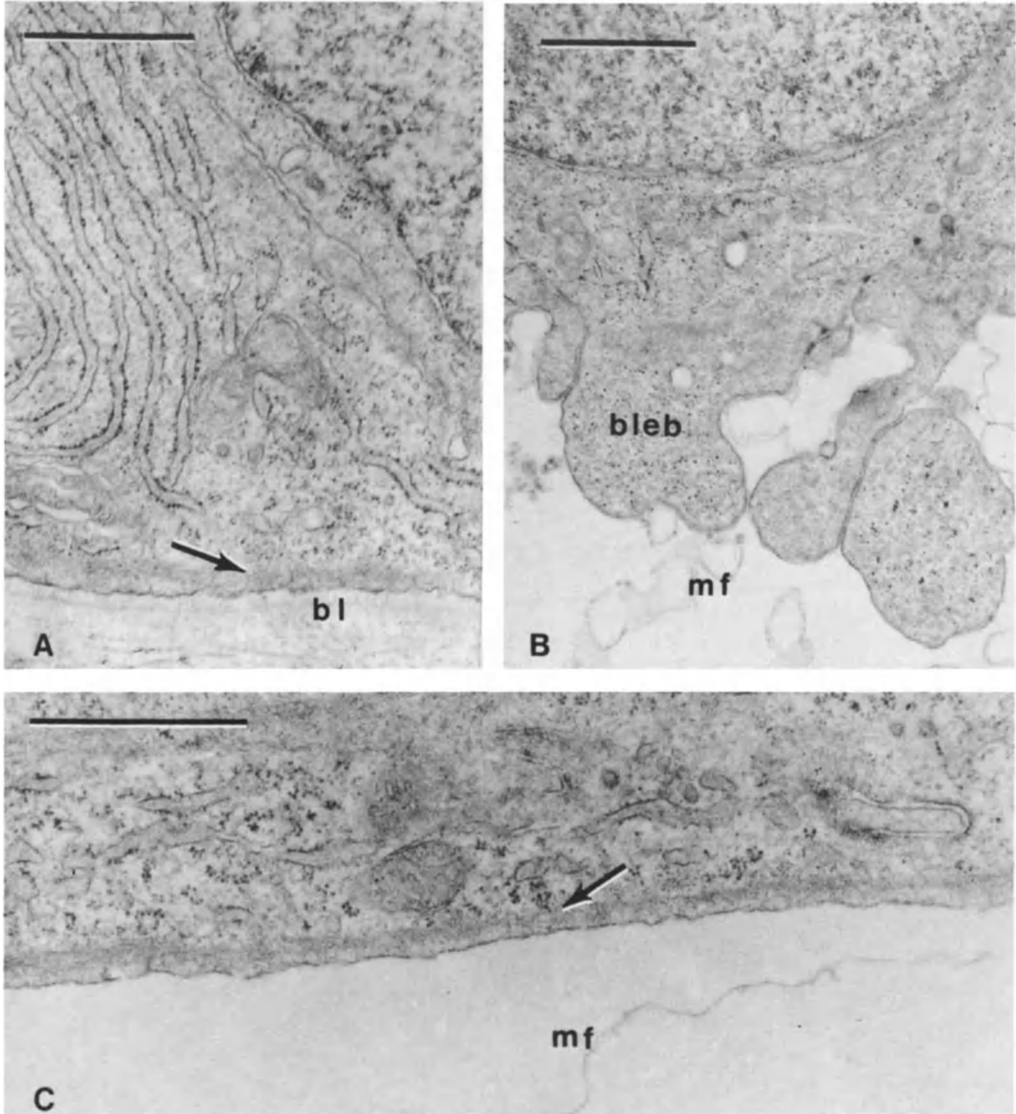


Figure 12-9. Electron micrographs showing the basal surface of the embryonic avian corneal epithelium *in situ* (Fig. 12-9A), *in vitro* on Millipore filter (Fig. 12-9B), and *in vitro* on Millipore filter 4 hr after adding solubilized $\alpha 1$(I) chains (100 μg/ml) to the medium (Fig. 12-9C). *In situ*, the epithelium sits on a basement lamina (b1) and exhibits a dense basal cortical cytoskeleton (arrow, Fig. 12-9A). The epithelium begins to bleb as soon as its basement lamina is removed. The blebs disrupt the basal cytoskeleton (Fig. 12-9B). Six hours after adding soluble collagen, the blebs have been withdrawn into the cell and the basal cell surface once more is flat, even though no polymerized matrix is visible; the basal cytoskeleton also has reorganized (arrow, Fig. 12-9C). mf, Millipore filter. Bars = 1 μm. (From Sugrue and Hay, 1981.)

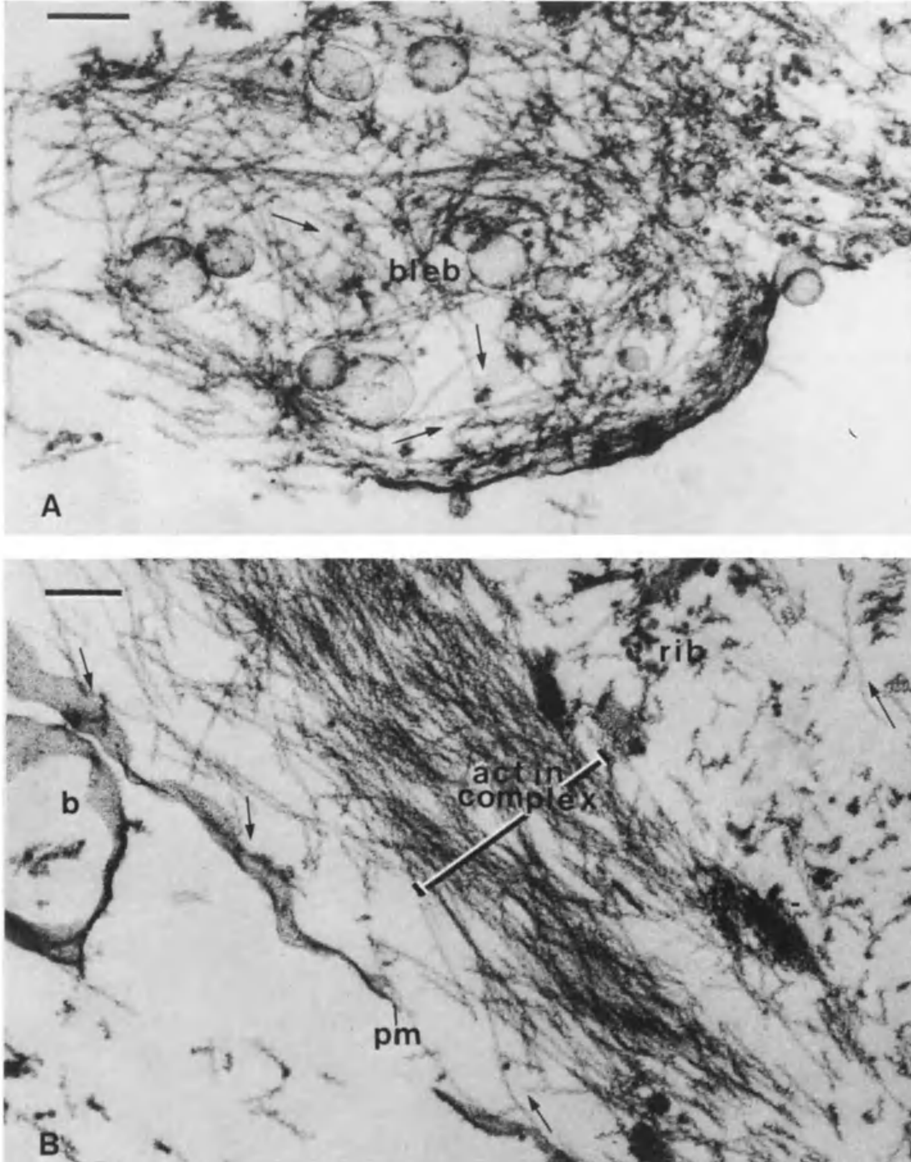


Figure 12-10. Electron micrographs of the basal cytoplasm of corneal epithelia treated as in Figs. 12-9B,C but labeled with S-1 fragments of heavy meromyosin to decorate actin filaments. The direction of the S-1 arrowheads is indicated for some of the filaments by small arrows. In a bleb (Fig. 12-10A), the actin filaments are disorganized. After addition of solubilized collagen, the basal surface smooths out and the actin complex that comprises the basal cytoskeleton re-forms (Fig. 12-10B). The preparations were treated with Triton X-100 so the basal plasmalemma (pm) is disrupted (permeabilized) by the treatment, making it difficult to evaluate its exact relation to the actin filaments. Some S-1 debris is present in the basal extracellular space (lower left, Fig. 12-10B); no visible ECM has polymerized in this time period (cf. Fig. 12-9C). b, blister (remnant) of Triton-solubilized membrane; rib, ribosomes associated with actin filaments in the basal cytoplasm. Bars = 200 nm. (From Sugrue and Hay, 1981.)

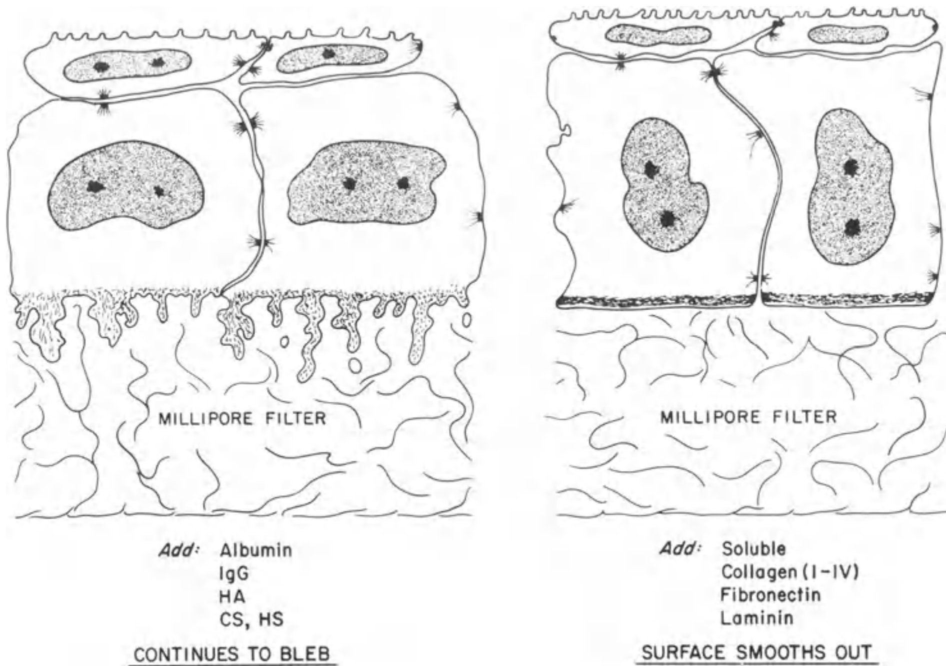


Figure 12-11. Diagrammatic summary of experiments testing the effect of a variety of molecules on the basal corneal epithelial cell surface. The isolated epithelium is placed on a Millipore filter (cf. Fig. 12-9B). The basal surface continues to bleb after addition of soluble albumin, IgG, HA, CS, and heparan sulfate (HS) in various concentrations. The basal surface smooths out and the basal cytoskeleton reorganizes in 2–6 hr after adding 10–100 μg/ml of solubilized collagens, fibronectin, or laminin. The ECM molecules are interacting with the plasmalemma, even though a visible subepithelial ECM has not yet re-formed. (From Sugrue and Hay, 1981.)

tween 4 and 6 hr disappeared completely (Fig. 12-9C). Purified type II and IV collagens had the same effect as type I collagen. The blebs contain actin (Fig. 12-10A), and as they withdraw, their microfilaments seem to merge with disrupted cytoskeletal components of the basal cytoplasm to recreate the cortical cytoskeleton (Figs. 12-9C, 12-10B). Soluble laminin and fibronectin have a similar effect on the basal surface, perhaps by stabilizing or capturing the collagen being secreted at low levels by the epithelium; cycloheximide in preliminary experiments inhibited the effect of fibronectin, but not of laminin (see Sugrue and Hay, 1981). Non-ECM proteins have no effect on the basal cell surface (Fig. 12-11).

What is emerging is the idea that the epithelial cell surface and cytoskeleton are stabilized by interaction with collagen (and other ECM molecules) via putative receptors in the cell membrane (see Fig. 12-16A). The idea is attractive because of evidence (to be reviewed in the next section) that cyto-

skeletal organization can affect cell metabolism. Thus, it is tempting to speculate that corneal stromal synthesis by epithelial cells is controlled in part by organization of the secretory machinery by cytoskeletal elements. In other types of epithelial–epithelial interaction, as for example in the case of neural tube folding induced by notochord and mesoderm (upper left, Figs. 12-3, 12-5), an effect on the neuroepithelial cytoskeleton (microtubule and microfilament organization) is the main result of the tissue interaction. It would be interesting to reinvestigate neural tube induction along these lines, because one of the events taking place at this time is the initial accumulation of ECM in the embryo, much of it directly under the neural plate.

3. Cell Shape, Growth, and Polarity *in Vitro*

The shape of an epithelial or mesenchymal cell can be modified by its external environment, and such modifications can be shown to affect cell metabolism *in vitro*. Anchorage-dependent fibroblasts removed from the substratum and cultured in suspension stop making mRNA and protein; when allowed to reattach and spread, the cells start making protein first and then mRNA (Benecke *et al.*, 1978). Penman and his colleagues have suggested that the cytoskeleton might affect protein synthesis by dictating the arrangement and organization of the polyribosomes that are associated with cytoplasmic microtrabeculae (Lenk *et al.*, 1977; Fulton *et al.*, 1980). Another example of the effect of culturing anchorage-dependent cells in suspension is the terminal differentiation of isolated basal epidermal cells in suspension culture; the suspended cell forms a cornified envelope and stops making DNA (Green, 1977).

Several cell lines, endothelial cells, and fibroblasts stop making DNA when the cells become round in shape in crowded cultures; transformed cells tend to escape such cell-shape controls on growth (Folkman and Tucker, 1980). Folkman and colleagues developed an interesting approach to show that the growth decrease is due to the round cell shape, not to cell density. They grew these untransformed cell types in sparse culture on poly(HEMA)-coated dishes, using different concentrations of poly(HEMA) to form films on the dishes. At high concentration (thick films), cells are very defective in attachment to the substratum, become round, and decrease growth. Flat, well-attached cells on dishes poor in poly(HEMA) have high levels of DNA synthesis, and there is a gradient of levels of DNA synthesis between the two extremes of cell shapes (Folkman and Moscona, 1978). Moreover, the taller the cells, the more serum (growth factor) is required to stimulate proliferation (Folkman and Tucker, 1980).

Studies of bovine adult corneal epithelial cells in culture also suggest that the effects of mitogenic agents (e.g., EGF, epidermal growth factor; FGF, fibroblast growth factor) can be related to cell shape, and moreover, they suggest that epithelial cell shape in turn might be regulated by ECM (see Gospodarowicz

et al., 1978). Dissociated basal corneal epithelial cells cultured on plastic form monolayers rich in microfilaments and respond to FGF by increased proliferation; EGF has no effect, even though EGF receptors are still present (Gospodarowicz *et al.*, 1978). As it was known that other epithelial cells maintained on collagen (Liu and Karasek, 1978) or feeder cells (Rheinwald and Green, 1977) respond to EGF, these investigators cultured bovine corneal epithelium on collagen gels. Now, the corneal epithelium grew more actively than on plastic and increased its proliferation in response to EGF, but not FGF (Gospodarowicz *et al.*, 1978). The basal cells became round instead of flat, and eventually the epithelium stratified. Gospodarowicz *et al.* (1978) speculate that a flattened morphology predicts sensitivity to FGF and a round or columnar shape, to EGF.

Vascular and corneal endothelia normally form monolayers *in vivo*. *In vitro*, they remember their original polarity and produce ECM, including collagen and fibronectin, on their basal surfaces, facing the plastic substratum (Birdwell *et al.*, 1978; Greenburg *et al.*, 1980). They require FGF for growth, but do not respond significantly to EGF. Therefore, these normally flattened cells are following the EGF–FGF rule mentioned above (Gospodarowicz *et al.*, 1978). Moreover, if vascular endothelium is cultured without FGF, the endothelial cells multilayer, surround themselves with ECM, and resemble mesenchymal cells (Greenburg *et al.*, 1980). Platelets, which previously did not bind to the apical surface, now attach to it. The effect can be reversed by conditioned medium from normally polarized endothelial cells, suggesting that "normal" cells produce a factor (of unknown nature) that helps them to maintain their polarity (Greenburg *et al.*, 1980).

Direct evidence for a role of collagen in establishing epithelial polarity comes from studies of cultured thyroid follicle epithelium. Mauchamp *et al.* (1979) report that when dissociated hog thyroid epithelial cells are plated on plastic, they grow off the surface of the dish as domes in which the cells present an apical surface toward the medium. If not allowed to attach, the cells form hollow spheres, and again their apical surfaces face the medium. This polarity is the opposite of what is observed in thyroid follicles *in vivo*. If, however, denatured collagen (gelatin) in solution is added to the medium, dissociated cells revert to *in vivo* morphology, forming follicles with all the apical surfaces facing the central cavity. Nitsch and Wollman (1980) report that in collagenase-isolated follicles, cells within the same follicle can reverse their polarity in response to serum, shifting their apical poles from the center to the outside of the follicle, which is now called a cyst because apical poles are on the outside instead of the inside.

Collagen substrata have been modified by Michalopoulos and Pitot (1975) to form floating gels that promote the epithelioid configuration of hepatocytes better than collagen-coated plates. On floating collagen gels, mammary epithelium assumes its *in vivo* polarity and characteristic biochemical responses to hormones (Emerman *et al.*, 1977). One of the advantages of the floating gel may be that it contracts to fit the contour of the cells (Sattler *et al.*, 1978), thus facilitating ECM regulation of cell shape.

4. Cell Adhesion and Migration

Since the initial observation of Ehrmann and Gey (1956) that collagen gels enhance the growth of many cell types *in vitro*, it has become commonplace to maintain cells *in vitro* on collagen or gelatin-coated substrata (for review see Kleinman et *al.*, 1981a,b). In most of these studies, it is difficult to separate the effect of collagen on growth from an effect of adhesion to the substratum. Moreover, the effect on adhesion could be direct or be mediated via collagen-bound factors such as fibronectin, laminin, or chondronectin (Chapters 4, 10). The experimental approach that is used to quantitate the degree of adhesion of cells to a substratum (Fig. 12-12) does not usually include correlated analysis of growth and cell shape. In the previous section, we speculated that increased adhesion causes cell spreading, which in turn causes increased cell growth (Folkman and Tucker, 1980); this is likely to be true, but adhesion per se was not quantitated in those studies. Considerable additional work is needed to tie together these various studies on the role of cell shape, ECM, and adhesion in controlling cell growth and differentiation.

The studies of adhesion of cells to collagenous substrata that will be briefly reviewed here have mostly employed the following experimental approach. Culture dishes coated with collagen are preincubated with medium containing the material to be tested (Fig. 12-12). Cells are added, the dish is incubated for a given period, and the unattached cells are washed off and discarded. Then, the attached cells are removed (usually by enzyme treatment) and counted (Fig. 12-12). Using this assay, investigators have accumulated data suggesting that fibronectin improves and may even be required for attachment to collagen of myoblasts, fibroblasts, and cells from a number of *in vitro* cell lines (see Chicquet et *al.*, 1979; Kleinman et *al.*, 1981a,b; Chapter 10). The receptor for fibronectin-mediated adhesion to collagen might be a ganglioside (Kleinman et *al.*, 1979). Linsenmayer et *al.* (1978) argued that a direct mechanism of attachment to collagen in addition to a serum-mediated effect might exist in the cell line they studied. To prove no fibronectin is needed for attachment, however, one must rule out an endogenous source of fibronectin, that is, one must treat the assayed cells with inhibitors of protein synthesis. Even this approach, which is coming into common use, is not foolproof. Because the assayed cells have usually been trypsinized, cycloheximide, the most commonly used inhibitor, might also interfere with regeneration of plasmalemma receptors for collagen. Indeed, Goldberg (1979) has published evidence that fibroblasts can possess surface receptors for collagen per se. It is perhaps not unreasonable to keep our minds open to the possibility of variation, even among fibroblasts, in the mechanism of cell–collagen adhesion.

Smooth muscle cells adhere to type V collagen in the presence of cycloheximide without fibronectin, but fibronectin enhances their adhesion to other types of collagen; the data indicate that smooth muscle cells bind to type V collagen via a membrane receptor that is trypsin insensitive and can be blocked by lectin (see Kleinman et *al.*, 1981a,b). The attachment assay (Fig. 12-12) has

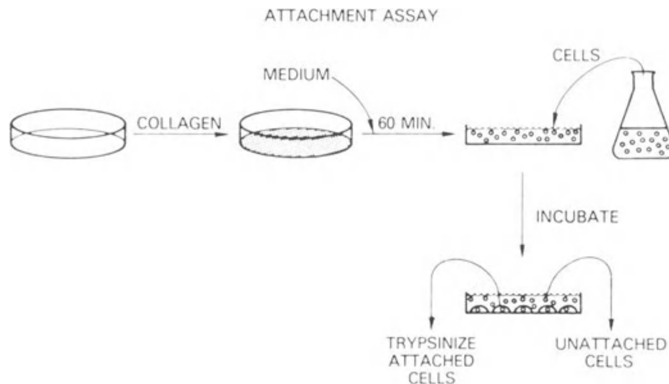


Figure 12-12. Diagram of the attachment assay used to evaluate adhesion of cells to collagen. The dish is coated with collagen, e.g., in the form of a neutralized gel. Freshly isolated cells are added in culture medium with or without serum or other factors. The dish is incubated at 37°C for a prescribed time, e.g., 2 hr. Then the unattached cells are decanted, the plate washed several times with saline, the attached cells removed with trypsin-EDTA, and counted. (From Kleinman et *al.*, 1981b.)

been used to study chondrocytes, and it has been reported that they adhere preferentially to type II collagen and use chondronectin instead of fibronectin to mediate the adhesion (see Kleinman et *al.*, 1981a,b; Chapter 4). Platelet and macrophage attachment to collagen seems to involve fibronectin (see Chapter 10).

For certain epithelial cell types (epidermis, mammary and yolk sac epithelia, EHS sarcoma cells, PAM 212 cells, and TERA cells) that have been studied *in vitro* by the attachment assay, a generalization is emerging: they do not synthesize fibronectin and they attach preferentially to type IV collagen using laminin (Wicha et *al.*, 1979; Kleinman et *al.*, 1981a,b; Terranova et *al.*, 1981). Antibodies against laminin can block attachment of such epithelial cells. Cycloheximide inhibits attachment of PAM 212 cells, which normally synthesize their own attachment factor (laminin) and require over 6 hr to reach maximal attachment (Terranova et *al.*, 1981).

Not all epithelial cell types, however, require laminin for attachment to collagen. Some hepatocytes can use fibronectin (Berman et *al.*, 1980). A liver-derived epithelial cell line synthesizes fibronectin (Foidart et *al.*, 1980), and fibronectin enhances collagen synthesis by this cell line (Foidart et *al.*, 1980). Rat hepatocytes need no helper proteins to attach to collagen and they adhere equally well to all collagen types that have been tested (Rubin et *al.*, 1981). They also adhere to collagen-like synthetic peptides such as $(\text{Gly-Pro-Pro})_n$ and $(\text{Gly-Pro-Hyp})_n$. Rubin et *al.* (1981) suggest that the cell-binding sites for collagen are mobile in the plane of the membrane and attach to multiple copies along the collagen molecule.

It is clear that in addition to the effects on cell shape, growth, and differentiation, adhesion to a substratum also affects cell migration *in vitro* and *in vivo*. On an artificial substratum with a gradient in adhesiveness, cells move in the direction of increasing adhesion until they become so firmly attached that movement is impossible (see Grinnell, 1978, for review). The idea of contact guidance by an adhesive substratum composed of natural ECM molecules *in vitro* has not been directly tested, but many studies call attention to seemingly adhesive contact of migrating cells with fibronectin (Chapter 10), GAG (Chapter 9), and collagen (see Bard and Hay, 1975; Bunge and Bunge, 1978; Löfberg *et al.*, 1980).

Collagen has been shown to affect the morphology of migrating fibroblasts rather dramatically. Corneal fibroblasts grown on plastic ruffle (Fig. 12-13B), as do other fibroblasts. In collagen gels (Elsdale and Bard, 1972; Bard and Hay, 1975), fibroblasts assume a bipolar shape (Fig. 12-13C), similar to that of corneal fibroblasts *in vivo* (Fig. 12-13A). Fibroblasts viewed in the intact cornea by Nomarski optics exhibit contact inhibition (Fig. 12-13A), and it has been postulated that they move into the cell-free center of the cornea because migration in the other direction is contact inhibited (Bard and Hay, 1975). They do use the orthogonal collagenous lattice created by the corneal epithelium as a substratum, for they distribute themselves along the plies (see Trelstad and Coulombre, 1971; Hay, 1980; Chapter 7). Interestingly, fibroblasts placed on top of trypsinized orthogonal collagenous lattices have also been observed to stop ruffling, and in this case they burrow into the matrix (Overton, 1977).

Fibronectin and hyaluronate (HA) increase in amount at the time of fibroblast invasion into the cornea (Figs. 9-2, 10-11) and may facilitate movement of cells along the collagen fibrils; the idea has not been tested *in vivo*, but is compatible with some *in vitro* studies (Chapters 9, 10). Fibronectin and HA, however, do not stop membrane ruffling of fibroblasts *in vitro*; the effect of ECM on fibroblast ruffling, blebbing, and adhesion is currently under more detailed investigation (see Tomasek and Hay, 1981).

Fibronectin and collagen have been shown to be chemotactic for cultured cells studied *in vitro* in a Boyden chamber (see Kleinman *et al.*, 1981a,b, for review). The Boyden chamber contains a filter with pores (usually 8 μm in diameter) large enough to allow cells to cross it. Cells are placed on top of the filter and the attractant under it; after a suitable period, cells that have migrated to the bottom of the filter are counted. The filters need to be coated with collagen in order that fibroblasts can adhere to them. Within the limits of the system, it can be said that collagen-derived peptides and type I, II, and III collagens (Postlethwaite *et al.*, 1978) and fibronectin (see Kleinman *et al.*, 1981a,b) placed in the lower well of a Boyden chamber are chemotactic for fibroblasts. Neural crest cells also find fibronectin chemotactic, and it has been proposed that fibronectin influences the movement of these cells on collagen fibrils *in vivo* (see Chapter 10). Chemotactic factors acting over even longer distances have been described in some migrating systems (see Wylie *et al.*, 1981, for review).

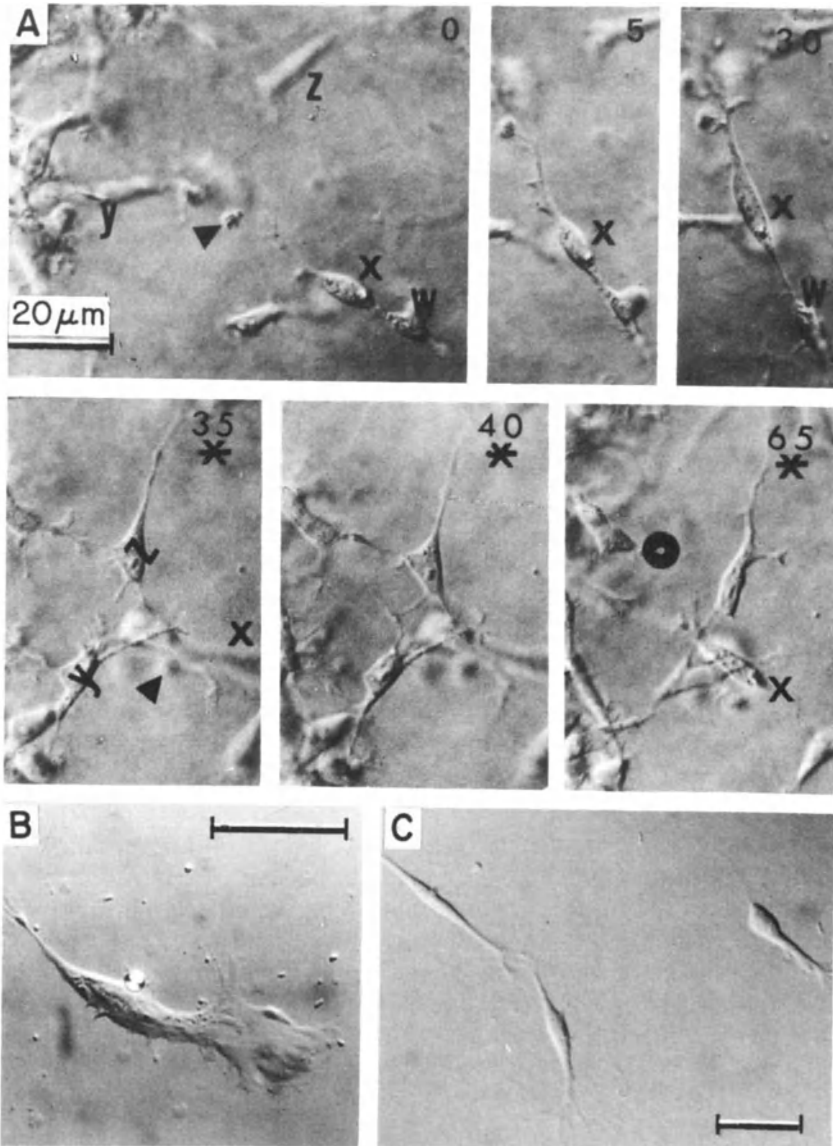


Figure 12-13. Light micrographs of living avian corneal fibroblasts photographed in situ as they move into the cornea at 7 days of development (Fig. 12-13A) or after being removed from the cornea and grown on glass (Fig. 12-13B) or in a collagen gel (Fig. 12-13C). The ruffled border characteristic of fibroblasts on glass or plastic disappears when they are coated with gelled collagen; now, the cells resemble their *in situ* counterparts. The *in situ* sequence shown here follows two recently divided cells, X and W, moving away from each other (5 min, 30 min), and two cells, Y and Z, that move toward each other (0 min), collide (35 min), and exhibit contact inhibition (40 min, 65 min). The arrowhead (0 min, 35 min) points to the same debris; the asterisk-labeled photos are focused on a slightly different plane than the first row of photographs and are mounted with a slightly different orientation. At 65 min, cell X moves past the debris labeled by the arrowhead in frame 0 and another cell (circle) moves away from cell Z. Bars = 20 μm. (From Bard and Hay, 1975.)

5. Dedifferentiation and Collagen Type Transitions

In the vertebrate embryo, the movements of mesenchymal cells come to a halt when these cells reach their destination (e.g., neural crest) or become crowded due to contact inhibition (e.g., cornea). Migration seems enhanced in the HA-rich embryonic matrices and inhibited by CS-rich matrices (Chapter 9); buildup of collagen seems to stabilize mesenchymal cell differentiation (e.g., sclerotome) and eventually to inhibit migration (Armstrong and Armstrong, 1980). It is of some interest, then, to find that in regenerating amphibian limbs, collagenase appears soon after amputation (Chapter 8). As the collagenous components of the matrix disappear, differentiated cells are released from preexisting cartilage, bone, and muscle; these cells acquire the characteristics of mesenchymal cells and proliferate to form a blastema that later redifferentiates to form a new limb (see Hay, 1974). During the proliferative period, HA is produced in the blastema, but prior to redifferentiation it is removed by hyaluronidase (Chapter 9).

The term "dedifferentiation" can be used to describe the transformation of muscle and skeletal cells to blastema cells during limb regeneration because the cells lose specific products that characterized their previous state of differentiation and take on a relatively undifferentiated appearance (Hay, 1974). Moreover, they may redifferentiate into a tissue other than the one of origin (Namenwirth, 1974; Wallace, 1981). Linsenmayer and Smith (1976) studied the collagen type transitions that occur during the regeneration of the larval amphibian limb. The amputated limb contains a cartilaginous skeleton rich in type II collagen. As the cells are released to enter the blastema, however, they switch their collagen type. The dedifferentiated mesenchymal cells of the growing blastema synthesize type I collagen; type II does not reappear until overt cartilage redifferentiation is observed. In this regard, the mesenchymal cells of the regeneration blastema resemble those of the original limb outgrowth, all of which at first synthesize type I collagen (see von der Mark, 1980, for review).

A similar collagen type transition can be observed in monolayer cultures of chondrocytes. At confluency, the formerly polygonal chondrocytes assume the shape of fibroblasts. The chondrocytes can be said to dedifferentiate, for they stop making type II collagen. They start making type I and III collagens and $\alpha 1(I)$ trimers (see Mayne *et al.*, 1976; Benya *et al.*, 1978; von der Mark, 1980). Another *in vitro* induced collagen type transition is the turning on of type III collagen synthesis by avian corneal fibroblasts, which normally produce type I collagen but not type III (Conrad *et al.*, 1980). In the latter case, the switch occurs as soon as the fibroblasts are isolated from the corneal epithelia and is associated with cessation of keratan sulfate synthesis by the cells. The end product in both cases is a "dedifferentiated" mesenchymal cell producing type I and III collagens. Whether or not "redifferentiation" can be induced in the *in vitro* system, or by returning the cells to an *in vivo* environment, is unknown.

A rather dramatic collagen type transition has been described in cultures of mouse tooth mesenchyme by Hata and Slavkin (1978). Tooth mesenchyme normally gives rise to odontoblasts that synthesize type I collagen but not type

II. When combined with avian limb bud ectoderm, mouse tooth mesenchyme becomes cartilage *in vitro*, synthesizing characteristic type II collagen (Hata and Slavkin, 1978). This is an aberrant kind of induction, because the apical limb ectoderm normally promotes outgrowth rather than differentiation of the avian limb mesenchyme (Ede *et al.*, 1977). Other aberrant effects of epithelia in inducing chondrogenesis have been reported (reviewed by von der Mark, 1980), and these are certainly worth reexamination.

The possibility that factors bound to the ECM might be active in inducing nonchondrogenic mesenchyme to become cartilage derives from studies of "bone morphogenetic protein" (see Urist, 1970). This putative inducer seems to be attached to the collagenous component of long bones. Bone matrix is prepared by lyophilizing demineralized rat tibias and extracting them in chloroform; the bones are bisected and placed in culture with minced embryonic muscle (Fig. 12-14) or inserted into the abdominal musculature. The end result is that in less than a week, fibroblasts (see Nogami and Urist, 1974; Reddi and Anderson, 1976) and myoblasts (Nathanson *et al.*, 1978) grown on bone matrix

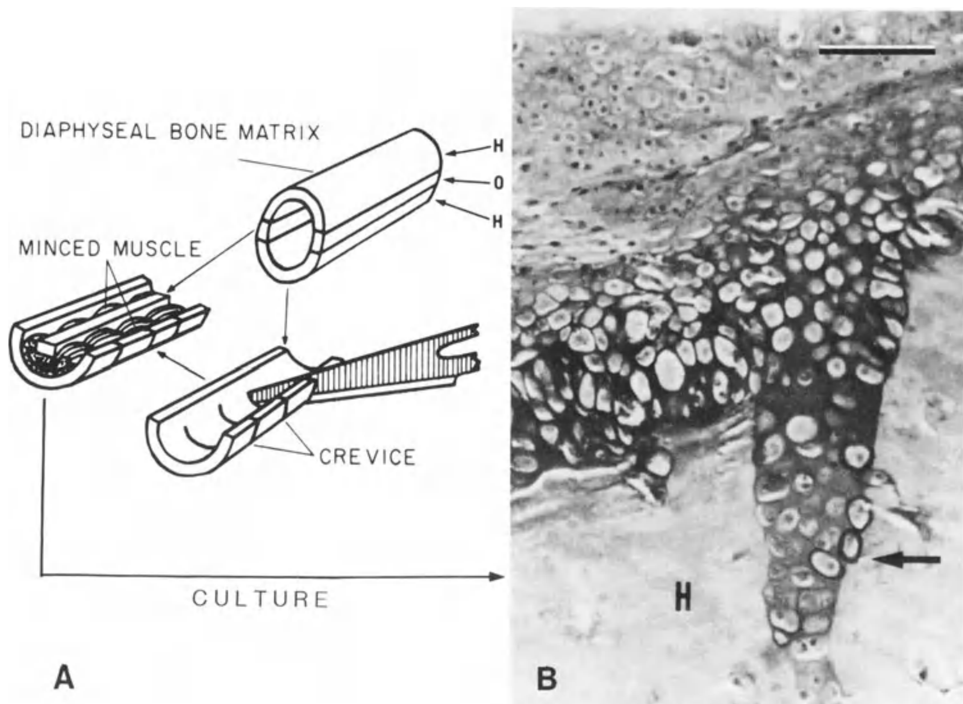


Figure 12-14. The bone matrix system for organ culture developed by Urist and others is illustrated here. (A) Long bone fragments are demineralized and chloroform-extracted, cut into hemicylinders (H) and overlays (O). Muscle is minced and placed in the hemicylinder as shown. Crevices cut into the bone provide increased surface area. (B) A typical result is shown in this section showing hyaline cartilage formed from minced embryonic rat skeletal muscle. H, hemicylinder bone matrix; arrow, newly formed cartilage in a crevice. Bar = 100 μm. (A, from Nogami and Urist, 1974; B, from Nathanson *et al.*, 1978.)

differentiate into cartilage and begin to synthesize type II collagen (Reddi et *al.*, 1977). In the case of myoblasts, they transform into fibroblastlike cells before differentiating as chondrocytes (Nathanson and Hay, 1980). Fibronectin is present throughout the transformation (Weiss and Reddi, 1981). The effect is not due to collagen, but is due to a factor unique to bone; control cultures of the same muscle treated in the same way but grown on collagen gels redifferentiate only into muscle (Nathanson and Hay, 1980). The factor may be a glycoprotein (Urist et *al.*, 1979).

The chondrogenic effect of bone matrix collagen, as we have just noted, seems to be due to an unknown factor that is bound to collagen. It is a particularly intriguing matrix effect, because the responding cells do something they would not normally have done. The induction seems to involve the kind of "mesenchyme-specific" factor that Grobstein (1967) postulated was needed to complement the less specific inductive effects of ECM molecules themselves. Examples of specificity of inducer and induction of transition in cell type during embryonic tissue interaction are not common (see Hay and Meier, 1978); the molecules that might be involved would have to pass through the matrix and might even be bound to collagen in some cases, as for example in neuromuscular synapse differentiation (Burden et *al.*, 1979).

6. Concluding Remarks

In this chapter, we have tried to give examples of all the roles that have been proposed for collagen in the vertebrate embryo, and some that have been demonstrated in cultured cells that seem relevant to morphogenesis. In closing, it seems worthwhile to attempt to integrate these thoughts about collagen with some of the ideas expressed in other chapters on the effects of ECM on cells. Figures 12-15 and 12-16 summarize the major thinking along these lines.

A recurrent theme is the possibility that collagen and some of the other molecules of the ECM affect the organization of the cytoskeleton (Fig. 12-15). Looking at embryogenesis chronologically, as we did at the beginning of the chapter, we see that one of the first events in the laying down of the primary tissues is the formation of basal laminae on which primitive streak mesenchymal cells seem to migrate. Data on cells *in vitro* that we discussed later on in the chapter make it seem reasonable to conclude that by adhering to ECM,

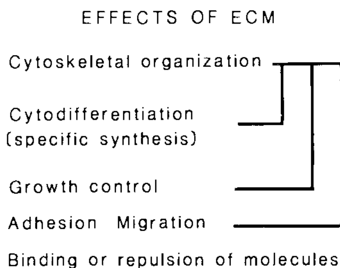


Figure 12-15. Some of the effects of ECM on cells that have been discussed in this book are listed here. Cytoskeletal organization may be involved in some of the effects of ECM on cytodifferentiation, growth, adhesion, and migration; hence, the connecting lines. By binding molecules like bone morphogenetic protein or repulsing others, ECM is in a position to mediate the cell microenvironment, in addition to the other more direct effects it can have on cell metabolism and cytoplasmic organization.

cells can organize actin and myosin components and extend their leading cell processes. The migration of these mesenchymal cells, and others later in development, is facilitated by HA (Chapter 9) and possibly by various structural glycoproteins (Chapter 10), through mechanisms that might also involve the cytoskeleton.

The primary mesenchymal cells give rise to mesodermal epithelia that interact with overlying ectoderm. The formation of the neural tube involves cytoskeletal rearrangements that could be influenced by attachment to the underlying ECM secreted by notochord and adjacent mesoderm. Control of cytoskeletal organization (Fig. 12-15) is also a major component of tissue interaction in the developing salivary gland, where cytoplasmic motility is required for branching and cytoskeletal rigidity for cleft formation. A balance between cell surface GAG and collagen removal and accumulation is critical (Chapter 9). The epithelial basal surface extends blebs or protrusions when the basement lamina is removed (Fig. 12-9). Removal of lamina may be an early step in carcinogenesis, following which the cells invade underlying cellular domains (Liotta *et al.*, 1979).

Cytoskeletal organization could also be involved in the effect of ECM on cytodifferentiation (specific synthesis, Fig. 12-15). We saw in this chapter how the corneal epithelium responds to collagen by organizing its basal surface and cytoskeleton, and then stepping up stromal synthesis; fibronectin and laminin play a role in the interaction (Fig. 12-16A). The corneal stroma is subsequently invaded by fibroblasts that begin to produce particular GAG, possibly in response to stromal ECM. Another example of cytodifferentiation we discussed in this chapter was the formation of cartilage from somites under the influence of neural tube and notochord ECM. Perhaps the effect is mediated via the cytoskeleton. That is not to say that other ECM feedback mechanisms might not exist, particularly in the case of inhibition of synthesis (see Chapter 6 and Wiestner *et al.*, 1979).

We saw in this chapter how cell shape can affect RNA, DNA, and protein synthesis *in vitro*. Cell shape and ECM are also involved in the response of cells to growth factors and hormones. Thus, the cytoskeleton may have a role in growth control (Fig. 12-15). EGF and FGF affect cells differently depending on their cytoskeletal organization. EGF and FGF could be factors involved in the effects of fibroblasts on epithelial differentiation (Hay, 1980). In pancreas development, a mesenchymal growth factor interacts with the epithelial cell surface to stimulate growth during the same period that other matrix factors are promoting epithelial branching into a gland (Levine *et al.*, 1973). We are only beginning to understand the interaction of the cell with other morphogenetic factors, such as the putative bone matrix glycoprotein that transforms muscle to cartilage. What emerges, however, is a concept of the cell surface as a complicated structure that not only possesses receptors for these various factors but that also controls, by the organization of its cytoplasmic cortex and surface ECM, the manner in which such factors act.

We have reached the point where we would very much like to be able to visualize the relation of matrix molecules to their putative receptors in the

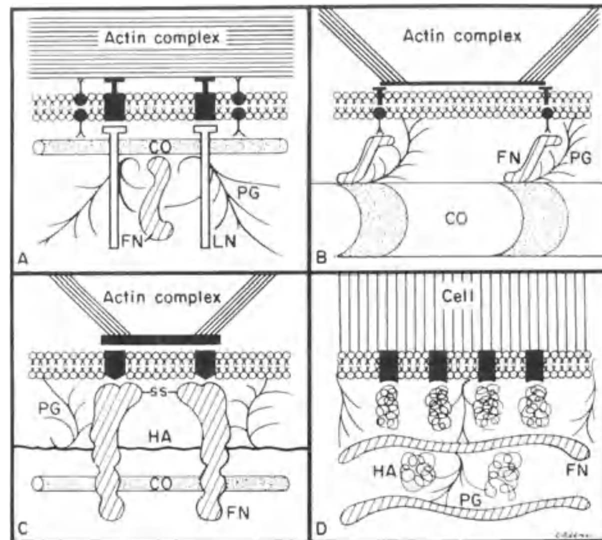


Figure 12-16. Diagrams of the possible relation of extracellular matrix to the cell surface. CO, collagen; FN, fibronectin; LN, laminin; PG, proteoglycan; HA, hyaluronate. All models envision receptors for one or more molecules in the plasmalemma, and A–C speculate on the relation of these receptors to actin in the cytoplasm. (A, unpublished drawing based on Sugrue and Hay, 1981; B, from Kleinman et *al.*, 1981a; C, after Hynes, this volume, Fig. 10-7; D, after Toole, this volume, Fig. 9-10.)

plasmalemma and to the cytoskeleton, and so we are beginning to construct models (Fig. 12-16). Figure 12-16A, based on Sugrue and Hay (1981), attempts to depict the surface of the corneal epithelium; 16B is similar to a drawing by Kleinman *et al.* (1981a) of cultured cells adhering to a substratum; 16C is from a drawing by Hynes in Chapter 10; and 16D is taken from a diagram by Toole in Chapter 9 of this book. The reader can see at once that the role of HA in the organization of the cell surface has received less attention from students of collagen and fibronectin than from students of GAG. It is not necessary, however, to postulate that all cell surfaces are the same. HA is not present in the primary avian cornea and would not be expected to relate to the cell surface there (Fig. 12-16A). HA might play a lesser role in substrate adhesion (Fig. 12-16C) than in aggregation (Fig. 12-16D) and so on. Moreover, epithelial cell surfaces may be quite different than mesenchymal surfaces; we are only beginning to understand the control of epithelial polarity and the factors that are involved in epithelial–mesenchymal transformation.

Obviously, time and more experiments will tell us whether or not any of these models of the relation of ECM to the cell (Fig. 12-16) bears some resemblance to the truth. Rapid advances can be expected in this area, however, as more cell biologists come to appreciate the importance of ECM and begin to contribute the additional experiments and interpretations that are needed to complete our understanding of cell–matrix interaction in the embryo, in the adult, and in transformed (Liotta *et al.*, 1978, 1979) cells.

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