HOMEOSTASIS IN EXTRACELLULAR TISSUES: INSIGHTS FROM STUDIES ON COLLAGEN

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INTRODUCTION

Homeostasis is manifest at many levels in biology – from intracellular processes to the interspecies relationships that underlie the present concern with ecology. Molecular biology has been centrally concerned with control mechanisms dependent upon reactions of macromolecules, and these studies have led to such concepts as: genetic transcription control through repressors;[1] inhibition of enzyme reactions through metabolite interactions;[2] and allostery.[3] The concept of matching surfaces – of molecular "fit" – is central to the mechanistic explanations of reactivity. The selective action of enzymes and the changes in activity of macromolecules brought about by conformation changes all relate to the critical shape and charge profile of surfaces.

The consequences of the appropriate fitting of two surfaces may be the lowering of energy barriers and consequent chemical reaction or the modification of the reactivity of one of the components of an interacting pair. Our knowledge is inadequate to describe the chemical balance of forces involved in such "fit" since such molecule to molecule interactions are compounded from the forces resulting from apposed, charged and uncharged groups in an aqueous system containing electrolytes; nevertheless, it has been possible to derive a qualitative understanding of interacting complementary surfaces through x-ray diffraction studies – for example, those of Wilkins and his co-workers on DNA, and of Johnson and Phillips on lysozyme.[4,5]

In this review attention will be directed to extracellular homeostasis, that between cells and the connective tissue that surrounds them. Such control mechanisms are of primary importance in the processes of differentiation in multicellular organisms, wound healing, tissue transplantation, and tumorigenesis, to name but a few. In the discussion it will be assumed that no new mechanisms of communication are involved and that in the extracellular tissues, as in the intracellular situation, interactions and reactivity involve the highly specific matching by "fit" of the surface of one molecule with that of another. However, several basic differences between intracellular and extracellular reactions may be expected. The complex sequence of enzyme-catalyzed, template-directed reactions involved in RNA or protein biosynthesis requires a series of

ternary collisions. These reactions can occur at a high rate only where the concentrations of reactants are high enough, but this requirement places no constraints on cell size. However, in uninucleate cells any nucleo-cytoplasmic reaction will be limited in rate by the rates of diffusion of the various reactants across the dimensions of the cell. Furthermore, the rate of uptake and discharge of metabolites depends critically on the surface to volume ratio of the cell. The high rates of hormone response or protein synthesis, for example, are related to cell size. Indeed, it seems plausible that the upper limit of cell size may be set when the rate of diffusion of inducers or co-repressors through the cell surface cannot initiate the nucleus-mediated response at a sufficient speed for homeostasis to be maintained. In contrast to the intracellular situation, homeostatic reactions between cells and connective tissue, and interactions between cells, take place across larger distances. Molecular messengers still appear to be the means by which one cell or tissue affects another (e.g., by hormone action), but all such effects that necessarily rely upon the diffusion of macromolecules or smaller specific molecules over dimensions larger than the intracellular dimensions must necessarily be slower than the reactions within the cell. Furthermore, since the molecular messengers rarely have direct access to the effector enzymes or the genome of the recipient cell, most such transcellular effects may be presumed to involve the action of a transduction mechanism at the cell surface.

Reactions directed from cell to connective tissue also differ in a special way from intracellular reactions: since reactions within the cell occur over a very limited domain, even a single effector molecule, by reason of the high collision rate, can bring about a change in a large number of substrate molecules. In cell-to-cell reaction, on the other hand, the action of a single effector molecule, at least in principle, can be amplified manyfold in the course of transduction across the membrane. In extracellular tissues, however, the rate of any reaction is limited by the distances across which diffusion processes must carry the effector molecule and also by the rate of successive reaction of the effector molecule – unless some mechanism exists for autocatalytic reactivity. In principle, it is therefore likely that any reactions in extracellular tissues are necessarily slow in comparison to those occurring between cells and within cells.

Since this article is a discussion and not a dissertation, we will not be concerned with semantics; for simplicity, we will exclude from consideration as extracellular tissues those superficial structures that are sited on the outer surface of a cell membrane but which appear to pertain directly to the cell viability, reactivity, and biosynthetic activity; for example, our interest is not in those immunogenic molecules on the surface of the cell whose populations change through the mitotic cycle. These structures pertain to the ongoing metabolism of the cell although they are located beyond the plasma membrane. Instead, our attention will be directed to those structures that are elaborated beyond the immediate domain of the cell and, in particular, those constituents of the intercellular matrix that are synthesized from the secretions of many cells and not simply of one.

In this article the structure and components of connective tissue will be described first; then the structure, biosynthesis, and reactivity of collagen, the best characterized macromolecule in connective tissue, will be discussed; and finally, how extracellular, presumably homeostatic, reactions change the collagen molecule and how these mechanisms probably reveal for us some of the basic processes of extracellular homeostasis will be discussed.

Homeostasis involves two processes, recognition and response. Although the macromolecules that build up the extracellular tissues are many and complex, it is clear that the permutations of their reactivities are more limited than those available within cells. We may presume, therefore, that the specific reactions that occur in connective tissues in response to changes of hormones, vitamin levels, tissue damage, or to developmental inducers and directives, are probably initiated by the cells of the connective tissue. Reactions in the extracellular milieu are then brought about by the secreted enzymes or effector molecules whose action occurs at a distance from the cell. To insure the specificity of such reactions and the appropriate response, the extracellular macromolecules must be structured appropriately during biosynthesis. Insofar as the same molecules may serve more than one purpose (e.g., in different tissues), it appears likely that the pluripotency of the structure of the component macromolecules is derived by modifying the reactivity of the macromolecule by

postsynthetic decoration, such as methylation, hydroxylation, or glycosylation.

From these preliminary considerations it seems profitable that we should look at the components of connective tissue from the point of view not only of "how do these components serve a particular purpose?" but also of "how do cells control the establishment, growth, reactivity, and catabolism of these components by action at a distance?" The structure of macromolecules in extracellular tissue must reflect both of these purposes.

If these deductions are valid, we may anticipate that diseases and defects in the connective tissue metabolism may reflect changes or mutations at one of three levels: at the primary sequence of the extracellular macromolecules; in the mechanisms that modify the protein molecule for its various roles in distinguishable tissues; or in the extracellular enzyme systems that modify and react with the molecules in the extracellular matrix. Examples of some diseases of connective tissue will be discussed later in this article.

CONNECTIVE TISSUES

In a broad sense, two classes of tissues may be distinguished in multicellular organisms: those formed of immediately juxtaposed cells, such as the brain, and those where the cells are in part or completely surrounded by extracellular tissue. In the extreme case of "connective tissues" such as cartilage or tendon, the cells may be very few in number, and the extracellular material may comprise more than 90% of the total tissue.

Cartilage and tendon are two examples of what is called dense connective tissue. Many such tissues, on microscopic examination, demonstrate a highly organized array of extracellular fibrous components. For example, tendons are built of bundles of long, parallel collagen fibers. Collagen fibers typically are cylinders or ribbons 1 to 12 μm wide, that appear granular in histologic section under the light microscope since they are built from 0.3 to 0.5 μm fibrils embedded in a continuous matrix. The material of the matrix is distinguishable from the collagen of the fibrils because it is digestible by trypsin. The cells in the tendon fibers appear to be arranged in columns one above the other in crevices between the fibers, while their processes spread in thin sheets between the fibers.

In contrast, in loose connective tissues, such as the fascia, serous membranes, and the subdermal tissues, large or small numbers of cells appear to be scattered among an array of fibrils (in which little order is perceptible) and surrounded everywhere by an all-pervading "ground substance."

Fibers

The fibrous components of connective tissue are the elastic, reticulin, and collagen fibers. The elastic fibers are built from protein, and they are remarkable for their resistance to chemical attack. As their name implies, these fibers endow the tissues with elasticity. They are generally thinner than collagen fibers and in bulk have a yellow tint. Unlike collagen, elastic fibers appear to be not granular but homogeneous in transverse histologic sections. Such tissues as blood vessel walls and the ligaments are rich in elastin. Elastic fibers are built of protein chains that are linked into a three-dimensional matrix by interchain cross-links. It is believed that the subunit protein is secreted from cells in the form of a tropo-elastin (also called a pro-elastin) molecule. The presumed precursor of elastin has been isolated by selective extraction from the aortas of copper-deficient swine.[6] The physical characteristics of tropo-elastin have not been adequately studied, but sequencing work has begun.[7] Cross-linking compounds capable of joining two, three, or four polypeptide chains are synthesized from lysine residues in these chains by the action of enzymes, and the high degree of cross-linking that results is responsible for the insolubility of this protein. Two of these cross-linking compounds, desmosine and isodesmosine, appear to be unique to elastin. Some of the chemistry of elastin has been reviewed recently by Franzblau and Lent, and Gallop et al.[8,9]

The reticulin fibers are thin filaments that may form an anastomosing network through many loose connective tissues, and they are also found in basement membranes. They are particularly prominent in embryonic tissues and in healing wounds, and they appear to precede the formation of collagen fibrils. They often appear in histologic sections to be continuous with collagen fibers, but they are distinguished from collagen by their failure to swell in acid and by their staining characteristics, particularly their argyrophilia. Stains indicate that these fibers have a high sugar content. Although these fibers do not show the characteristic collagen banding under the electron

microscope, they are resistant to trypsin but susceptible to collagenase attack, just like collagen. It is therefore generally concluded that the reticulin fibers are built from collagen molecules that contain a large number of bound carbohydrate residues.

The third of this category of fibrous structures, the collagen fibers, are straight or slowly weaving filaments, and under the microscope they are seen to have a characteristic banding pattern with a 67 nm period that permits their ready identification. The structure of the collagen fibrils and of the constituent macromolecules will be discussed at length in a later section.

Examples of the arrays of fibrils in connective tissue are shown in Figures 1 and 2.

Proteoglycans and Glycoproteins

The continuous matrix that interpenetrates the fibrous components and cells of connective tissue comprises a sol or gel of protein-polysaccharides in an aqueous phase that includes dissolved cell nutrients and catabolites, salts, vitamins, and some of the serum proteins. This hydrated gel matrix is the path between the blood vessels and the cells of the connective tissues through which all metabolites must pass.[9a] The serum proteins appear to be in dynamic equilibrium with the continuous body fluids and blood and are not particular to the connective tissues; the characteristic constituents of the ground substance of connective tissue are the proteoglycans (or glycosaminoglycans). Histologically the ground substance is characterized by a metachromatic staining with toluidine blue, acridine orange, or the pinacyanol dyes. This property reflects the π-π orbital overlap of the dye molecules that become bound in an ordered manner to the array of negative charges along the polysaccharide chains; these chains comprise a large fraction of the ground substance.

With the possible exception of hyaluronic acid, the polysaccharides of the ground substance are protein bound. Hascall and Sajdera distinguish two classes of protein-polysaccharides: the proteoglycans, that consist predominantly of polysaccharide chains bound to protein chains that may comprise only a few percent of the composite macromolecule; and glycoproteins, in which the major components are polypeptide chains to which are attached short lengths of polymerized carbohydrate residues in straight or branched arrays.[10]

The glycoproteins of connective tissue have been investigated only infrequently; they appear to aggregate and associate with the proteoglycans (perhaps through disulfide bonds and collagen).[10, 11]

In the proteoglycans the polysaccharide chains contain from 20 to 120 residues in most molecules, but in hyaluronic acid the polymer may comprise some thousands of sugar residues. In the proteoglycans several chains may be bound to one polypeptide, usually through O-serine linkages, and the polypeptide component of the proteoglycan macromolecule may be very small and its properties overshadowed by the carbohydrate moiety. The polysaccharide chains may be cleaved from the protein backbone by alkali or protease treatment, and such preparations, largely protein free, isolated from various tissue sources, have been fractionated to yield the commonly recognized polysaccharides of connective tissue, hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin. Hyaluronic acid contains the longest polysaccharide chains; this molecule probably imparts the high viscosity to synovial fluid.

These polysaccharide chains of the proteoglycans are usually unbranched, and they are, for the most part, built of repeating disaccharide units. The chains are strongly negatively charged since many of the alternating glycosidic units are hexuronic acids (glucuronic acid or iduronic acid). Hexosamines (glucosamine or galactosamine) are the alternating units of each polysaccharide, but the amino group is often acetylated or sulfated. This regular array of carboxylic or ester and amine sulfate residues, which in the tissues are always associated with their counter ion, sodium, gives a greater stiffness to these polysaccharide chains than that normally found in carbohydrates, and, as a result, these complex macromolecules impart the characteristic of a highly hydrated gel to the ground substance. Through this ground substance diffuse soluble proteins and cell metabolites.

The combination of this hydrated matrix and the fibrous components generates the rigidity and resilience demanded of the connective tissue. Presumably the varied organization of the fibrous components of the tissue and the various permutations of the proteoglycans impart the range of properties demanded of such specialized tissues as

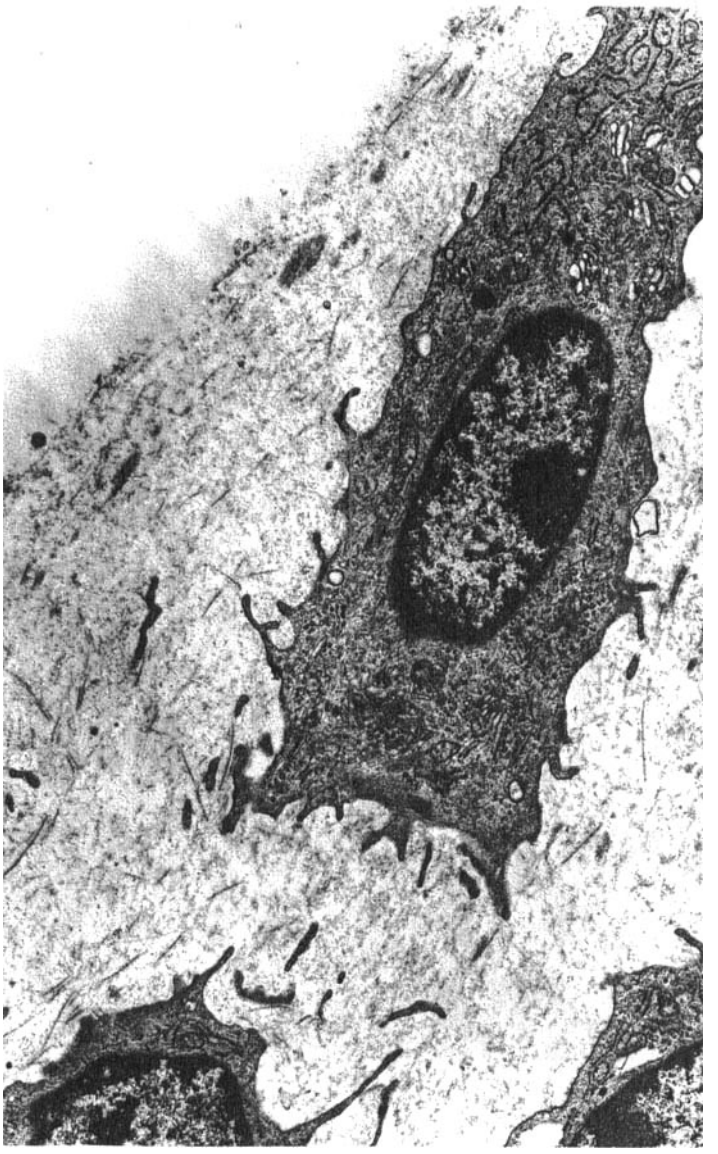

FIGURE 1. Section through the surface of bovine embryonic hyaline cartilage. Parts of three chondroblasts are seen with a matrix that includes collagen fibrils, singly and in small bundles. Magnification x 15,000. (Micrograph provided by Dr. E. A. Balazs.)

nasal cartilage, articular cartilage, bone, tendon, or dura mater, to name but a few.

For further discussion of the structure and synthesis of proteoglycans and glycoprotein, the reader is referred to Schubert and Hamerman, Spiro, Rodén, and Kobayashi.[12-15]

Basement Membranes

A very thin, diffuse layer that may be distinguished histologically beneath sheets of cells, particularly epithelial layers, is termed a basement membrane. Such lamellae underlie the cells of the capillary wall or the glomerulus of the kidney, for example. Under the electron microscope, these membranes appear to contain a felt-like mass of fine fibrils, approximately 4 nm in diameter, embedded in an amorphous matrix that stains somewhat denser than the surround. In the case of Descemet's membrane in the cornea and the anterior lens capsule, the arrangement of the fine

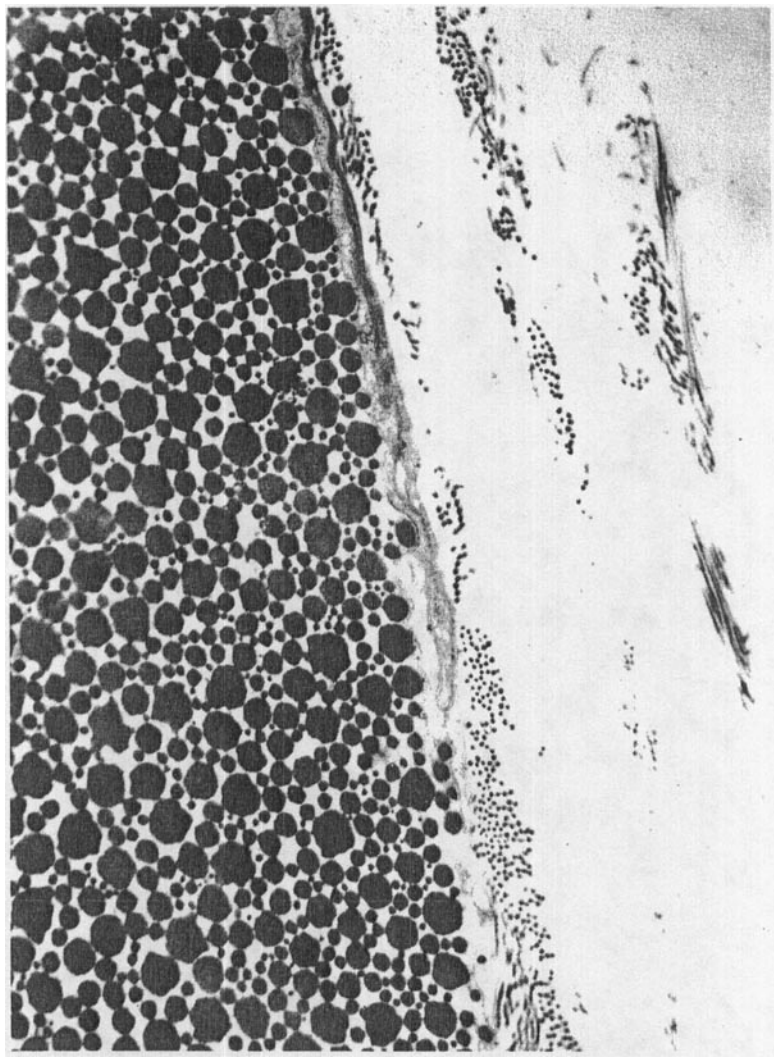

FIGURE 2. Cross section of the edge of a rat tail tendon fiber. Magnification x 25,000. (Micrograph provided by Dr. E. A. Balazs.)

fibrils may be highly ordered, but in these cases the use of the term "basement membrane" is controversial.

Basement membranes appear to be secreted by the cells they underlie but they may be separated by several hundred angstroms from the cell surfaces. These membranes are thought to have a supportive or a selective filtration function, and they are distinguished from other connective tissues not only in their organization and disposition but also in the nature of the collagen that is present. The collagen that can be extracted from basement membranes has a very high carbohydrate content, shows no regular banding under the electron microscope, and differs chemically from the collagen of many other connective tissues. The chemistry of basement membranes has recently been discussed by Kefalides.[16]

The Cells of Connective Tissues

In the embryo the earliest connective tissues are produced by mesodermal cells which secrete acidic polysaccharides into the intercellular spaces that are formed as the cells divide and move. The cells and their secretions comprise the mesenchymal tissues. In the developed organism the cells that inhabit the intercellular tissues are mostly of mesodermal origin, but not all of them are involved in biosynthesizing the extracellular constituents. In the case of basement membranes,

they are secreted by cells of the epithelium, and the earliest collagen laid down in the cornea is secreted also by the epithelial layer.

In the developed organism the connective tissue components appear to be synthesized by characteristic connective tissue cells, the fibroblasts, or their differentiated descendants, the osteoblasts and chondroblasts. Many other types of cells besides these are commonly found in the connective tissues; some of these cells, particularly the macrophages, probably serve to protect the tissues against infection or invasion and to scavenge dead or damaged cells and components. The presence of these cells is significant because the connective tissue is the site of the inflammatory response to infection or wounding. Fat cells also commonly occur selectively distributed through connective tissue.

In the discussion that follows we will be largely concerned with biosynthesis and homeostasis as they relate to the collagen molecule. None of the other major components of connective tissue has been as well characterized as collagen, and it is a major component of most connective tissues – with the major exception of blood and synovial fluid. The biosynthesis of many of the constituents of connective tissue has been studied, but their subsequent fate is more difficult to elucidate except in the case of collagen. All of the proteins and probably all of the polysaccharides of the connective tissue are synthesized intracellularly and subsequently excreted from the cell. In the proteoglycans both protein and polysaccharide are synthesized in parallel, and puromycin inhibits the production of both. Labeling experiments in tissue culture preparations have demonstrated that hyaluronic acid and collagen are biosynthesized by the same cell and that their synthesis can be separately controlled.[17] Thus it has been observed that mouse fibroblasts, for example, secrete more hyaluronic acid in the course of fast growth in tissue culture, whereas collagen preponderates in the stationary phase of growth.

Both the hexuronic acids and hexosamines can be tagged by supplying radioactive glucose to cells. Studies in vitro and in vivo have shown that much of the hexose of the proteoglycans is reutilized; relatively little of the degradation products of chondroitin sulfate, for example, is excreted. It has been determined that the average turnover rate of the proteoglycans is considerably faster than that of the fibrous components of connective tissue, but the discrimination of the turnover rates among the various categories of the proteoglycans has barely begun. The biosynthesis of chondroitin sulfate in the chondroblast and its appearance in the connective tissue have been studied by measuring the uptake and distribution of organic ^{35}S-sulfate. This label is taken up by the cells, becomes incorporated into chondroitin sulfate chains, and after a short residence time in the cell, is secreted. Such studies and others have been reviewed by Dorfman.[18] However, these investigations have mostly been concerned with turnover in the polysaccharide moieties of the proteoglycans: the polysaccharides in themselves display a forbidding complexity. Although discrete families of the polysaccharide moieties, such as dermatan sulfate and chondroitin sulfate, have been recognized after cleavage from the proteoglycans, preparations of the polysaccharides from different tissues differ in their sulfur content and in the presence of minor sugar residues so that Meyer has applied the term "microheterogeneity" to these populations.[19] How much greater, therefore, is the potential heterogeneity of the intact proteoglycans where members of such polysaccharides are bound to one or more protein chains? To what degree this diversity is significant in the biological sense and to what degree it reflects the vagaries of modifying enzymes within the cell, or subsequent enzymatic activity in the extracellular matrix, is not known. However, the complexity of the proteoglycan is so great that the discrimination of individual species of molecules has barely begun. Therefore, a description of the biosynthesis and turnover of any class of the proteoglycans – as opposed to their polysaccharide moieties – cannot yet be attempted.

In contrast to these components, collagen has been well characterized, and it has useful structural markers, hydroxyproline and hydroxylysine, by which it can be recognized in the course of biosynthesis and degradation.

Although it is known that most of the constituents of connective tissue are biosynthesized within the cells, the mechanisms of secretion of the various macromolecules, of the nucleation and accretion of macromolecules to fibrils in their growth, and the disposition of the proteoglycans around the fibrils are unknown. It seems likely that in many tissues the biosynthesis of the fibrils occurs close to the surface of the secreting cell and that the cells may thus decide the alignment of the

fibrils, but it also seems probable that much subsequent growth of fibrils occurs at considerable distances from the cell of origin. Certainly any secretion of collagen by epithelial cells must result in fibril formation beyond the basement membrane, which is several hundred Å from the cell surface. However, while it seems likely that the fibroblasts may move to designate the orientation of elements of the connective tissue (because fibroblasts are certainly motile cells in tissue culture), the mechanisms by which cells control the orientation and disposition of structures beyond their immediate domain are not known. This problem is certainly relevant to the continued synthesis of cartilage since most of the chondrocytes become enclosed within lacunae in the tissue and their interaction with the greater part of the matrix can only be from a distance.

In the discussion that follows, our attention will be directed toward mechanisms that may conceivably operate at a distance from the cell in which the macromolecules originate. Certainly, homeostatic mechanisms are continually at play since anabolism and catabolism are balanced to accord with the organism's needs, but our understanding of these homeostatic processes is limited. Thus the ratio of polysaccharide to protein and the hydration of the connective tissues varies through the life span of the organism; but to what degree this change reflects a change in the rate of catabolism or anabolism of either component and to what inducing signals the secreting cells are responding have yet to be discovered.

Many of the constituents of connective tissue have lifetimes significantly longer than the average; Mellman et al. recently showed that the average turnover time for protein in cultured human diploid cells is less than 30 hr;[20] yet, in rats, turnover times of collagen molecules of longer than a year have been measured. Nevertheless, at least some of the constituents of connective tissue are in a state of flux in all the tissues, as is shown by age-related changes in morphology and composition. Some tissues have considerable turnover rates, as is readily demonstrated by the profound changes that occur in them in response to a depletion of vitamin C in the diet with the onset of the symptoms of scurvy. All tissues retain the capacity for homeostatic response and for appropriate biosynthesis and remodeling, as is shown by the tissue reactions to wounding. We will attempt to examine the mechanisms of extracellular homeostasis using collagen, the best characterized component of connective tissue, as an example.

COLLAGEN

In the sections that follow, we will discuss the structural characteristics of collagen and their relationship to the secretion, fiber formation, and catabolism of fibrils in connective tissue.

The collagen molecule appears to be secreted primarily by fibroblasts or epithelial cells. Sequence studies have shown that the collagen molecules extracted from the major connective tissues (skin, bone, tendon – even dentine) of any particular species have the same primary polypeptide structure except for a tissue-specific pattern of hydroxylation or glycosylation. Recently, however, another form of collagen has been detected in skin, as a minor component, and yet other forms of collagen have been detected in fetal and very young animals and in cartilage. Collagen is the major protein of tendon and skin and, *in toto*, comprises perhaps 30% of the protein of a mammal.

It was demonstrated in the laboratories of Orekovitch and of Schmitt that highly asymmetric molecules could be extracted by acid or salt solutions from collagenous tissues such as tendon and skin, and Schmitt and his co-workers showed that from such aqueous solutions of soluble collagen the protein molecule could be precipitated in the fibrillar state, closely resembling that seen by electron microscopy in native fibers. In the 20 years since those discoveries, the molecule has been characterized both physically and chemically, and the culmination of that study has been the recent determination of almost the complete amino acid sequence of one of the polypeptide chains that comprise the molecule. Many aspects of the structure of the collagen molecule that will not be discussed here are described in the volumes by Ramachandran and Gould and in a recent review by Traub and Piez.[21-23]

Collagen fibrils examined in sectioned tissue or teased and displayed on an electron microscope grid usually show a marked regular banding. The same periodicity can be detected in x-ray diffraction photographs, and reliable measurements can be made without the intrusion of shrinkage or fixation uncertainties. In undried tissues the period is 67 nm; however, this distance is about one fourth the length of the soluble collagen

molecule. These observations were reconciled when Gross, Highberger, and Schmitt deduced that the fibrils of collagen in an animal tissue are formed by the accretion of molecules staggered at one quarter of the molecule length in a polarized, linear array.[24] Since the mode of precipitation is sensitively determined by the conditions of pH and salt concentrations in the solutions of the soluble collagen, it is probable that the mode of aggregation is precisely dictated by electrostatic interaction between the molecules. The name tropocollagen was given to the molecule that was postulated to be secreted by the cell and to aggregate in the native fibril. Since Gross and his colleagues were able to reconstitute fibers apparently native in appearance from the soluble form of collagen in extracts of tissues, they deduced that the soluble form was the precursor to the native fibril. It has more recently been recognized that a sequence of events follows the secretion by the cell of a molecule that is significantly larger than the final component of the fibril; therefore, the name tropocollagen is falling into disuse, and instead the word collagen is used to define the subunit protein that gives rise to the collagen fibril. In this text we will refer to the extracts from the tissues as soluble forms of collagen, using the word collagen to describe the protein rather than the tissue.

The quantity of collagen that can be extracted from a collagenous tissue by appropriate, nondenaturing solvents varies with the tissue and the age of the animal. It has commonly been noted that progressively smaller amounts of soluble collagen can be extracted from the tissues of an older animal, but experiments have shown that the fraction of readily extracted material may correlate more directly with the rate of growth of the tissues than with the age. Thus it would appear that collagen becomes progressively less soluble in a time-dependent reaction subsequent to biosynthesis. The mechanisms of these subsequent changes in the connective tissues will be discussed later.

It is now known that the length of the collagen molecule is more than four times the period of the native fibril in which it occurs and that therefore there is a space between the collagen molecule and its successor in line in the fibril. The fibers are not built by end-to-end linked monomers, but by a succession of partially overlapping chains. The relationship between the individual elongate macromolecule and the fibril that it forms in its organized, aggregated state is demonstrated in Figure 3.

When solutions of soluble collagen are heated or are treated with denaturants such as urea, thiocyanate, or guanidine hydrochloride, the viscosity of the solution falls dramatically, the optical rotation is changed, and the polypeptide chains that constitute the molecule can frequently be separated. The smallest polypeptide chain that is found normally in such a denatured solution is the α chain, the single chain that runs the length of the collagen molecule. In addition, there are normally present the so-called β components and γ components and higher aggregates that are respectively two, three, or more α chains linked covalently. From the x-ray crystallographic studies of Ramachandran and his colleagues and of Crick and Rich (see Ramachandran[21]), it was deduced that the native collagen molecule is built from three colinear α chains; each chain comprises a polyproline type II helix, and the three chains are wrapped together in a triple helix of opposite sense from the helix of the individual chains. The chains associate through hydrogen – and probably hydrophobic – bonds that can be broken by heating or by chemical denaturants. Upon removing the denaturant, the separated constituent chains may fold, as is demonstrated by a change in the optical rotation in the solution; the α chains may fold upon themselves in dilute solutions, or they may associate one with another to form aggregates. In solutions of high concentration, the aggregating chains may form a gel. Except in the cases where the three chains constituting the molecule are covalently linked (γ components) or under carefully controlled renaturing conditions, the triple helix structure of the native molecule is not regained.[25,26]

Piez et al. showed by chromatographic fractionation that there are two chemically distinct α chains present in mammalian skin collagen, and subsequent studies have extended this finding to the collagens from bone and tendon.[27] These chains, which have been termed $\alpha 1$ and $\alpha 2$ chains, are present in the ratio 2:1. It was widely assumed that this ratio implies that each molecule is built from two $\alpha 1$ and one $\alpha 2$ chains; this assumption has recently been validated.[28]

Piez and his colleagues[23] explored the scission of these α chains with cyanogen bromide, and the sequencing studies by these workers and by others

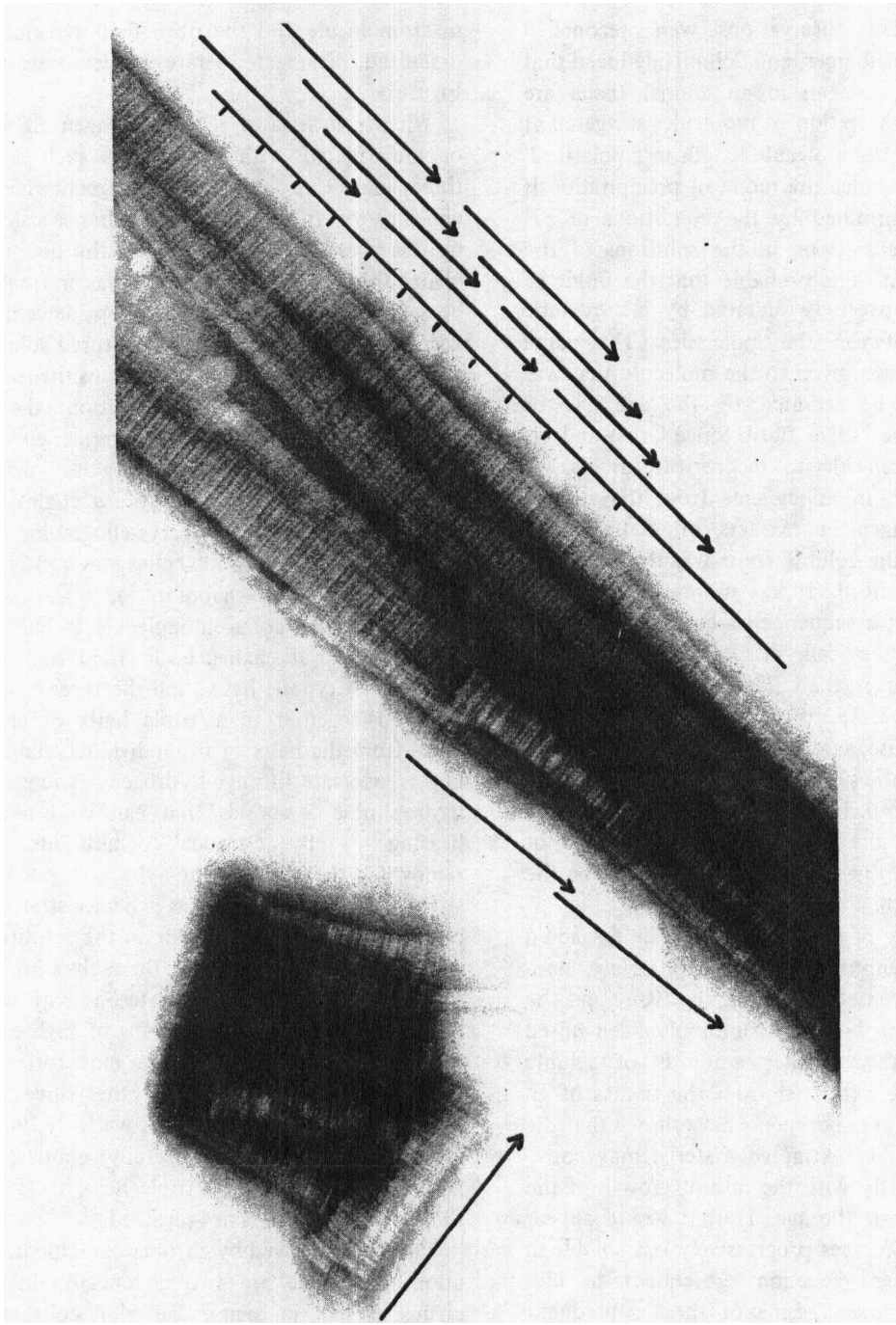

FIGURE 3. Reconstituted collagen fibrils with 67-nm periodicity together with some free SLS crystallites and some segments cohering to the surface of the fibrils and revealing the mode of packing. The staggering of the molecules in the fibril is shown diagrammatically. Magnification x 124,000.

have culminated in the determination of almost the complete sequence of the $\alpha 1$ chains for calf skin and chicken and rat skin and bone collagen. In view of the strong sequence homologies, a complete sequence for a composite $\alpha 1$ chain has been compiled (Gross, J., private communication, 1972).

Mention has been made of other chemically distinguishable collagens that have been identified in the mammal in addition to the major collagen that is characteristic of skin, tendon, dentine, and bone. These newly distinguished collagens have been identified in cartilage, fetal tissues, basement membranes, and as a minor component in skin.[29-32] All of these collagens are readily differentiated from the major collagens because they have only the single form of α chain that can be distinguished by electrophoresis or chromatography. For example, the α chain of cartilage collagen is eluted from a carboxymethylcellulose column in the position of an $\alpha 1$ chain of skin, but they differ in chemical constitution. The cartilage α chain was named $\alpha 1$ type II, and the collagen molecule can be represented as $[\alpha 1\ (II)]_3$. With the increasing number of different α chains now being reported, this terminology is becoming clumsy.

Most of these newly distinguished collagens have not yet been investigated adequately to be sure that each chain in the molecule is identical, but they do not contain the easily distinguished $\alpha 2$ chain. Some of these collagens have been discriminated from the major collagen by the population of peptides released from the chains by cyanogen bromide treatment. Both Miller and Kefalides have demonstrated that the cyanogen bromide peptides derived from cartilage or basement membrane collagens have a different chromatographic profile from that shown by the peptides isolated from the major collagens.[33-35] Furthermore, the analyses reported by Kefalides for basement membranes from the glomerulus, the anterior lens capsule, and Descemet's membrane suggest that each of these tissues has distinguishable collagen molecules.[31] We may conclude that the mammalian genome can code for minimally four and possibly more collagen α chains.

It already has been noted that there is considerable sequence homology between the $\alpha 1$ and the $\alpha 2$ chains of any particular mammalian collagen. Tkocz and Kühn showed the similar banding pattern of stained SLS segments formed from renatured $(\alpha 1)_3$ and $(\alpha 2)_3$ molecules: these observations imply a similar distribution of charged residues along the chain length.[36] Other evidence that the several collagens encoded in the genome are strikingly related and probably arise by genetic duplication is discussed in the section on phylogenetic stability. In view of the limited sequence studies and structural investigations that have been made thus far on the minor collagens, the subsequent discussions will relate mostly to the major collagens characteristic of skin, tendon, bone, and dentine.

Features of the Collagen Molecule

The collagen molecule is distinguished in several features of its chemistry and structure in addition to its unusual size and shape. A certain fraction of the proline and lysine residues in the isolated molecules is hydroxylated, but the extent of hydroxylation differs in various tissues. Galactose and glucosylgalactose are bound through an O-glysidic linkage to the hydroxy residues of hydroxylysine at certain points along the molecule, but again the pattern of the glycosylation varies with the tissue from which the protein was isolated. Cross-links may be detected between various pairs of the α chains of any one molecule; or two or more intramolecular cross-links may join all three chains. A large number of chemically distinguishable cross-linking structures have been detected in soluble as well as in insoluble collagen. Intermolecular cross-links are also formed in the tissues, and these account for the insolubility of the greater part of most collagen in such solvents as are used for the isolation of soluble collagen. Some intermolecular cross-links persist in the extracted, soluble collagen since physical evidence demonstrates that polymeric forms of the molecule are present in the solution.[37,38]

The greater part of the collagen molecule appears to be unusually resistant to proteolytic enzymes. Studies by Nishihara and Miyata and by Schmitt and his co-workers demonstrated that small regions of the collagen molecule are in fact accessible to common proteolytic enzymes and that the removal of these labile "telopeptides" changes the solubility and aggregation properties of the collagen molecules to a degree quite disproportionate to the small fraction (1 to 5%) of the molecule that is removed by the enzymes.[39-42] Most, if not all, of the inter- and intramolecular cross-links between the collagen

peptide chains are formed on the telopeptides, and hence many of the cross-linked chains are separated by the action of proteases.

The telopeptides appear to be the primary immunogenic sites on the collagen molecule. It was shown several years ago that the reactivity of collagen-induced antibodies (as measured by complement fixation) could be reduced or abolished if the homologous collagen were pretreated under native conditions with pepsin or other proteases. In view of the susceptibility of telopeptides to proteases, it was concluded that these were the targets for the antibodies.[43] The disposition of the telopeptides on the molecule was the subject of debate for several years, but it now is apparent that there are both amino- and carboxy-terminal, enzyme-labile, immunogenic peptides on both the $\alpha 1$ and $\alpha 2$ chains of the major (skin) collagen, and probably also in cartilage and other classes of α chains (e.g., see References 44-47). It has been found that antibodies raised in individual rabbits may differ markedly by reacting to different sites on the molecule, and these sites may change, even in one rabbit, in the course of the immunization procedure.[48]

Sequence studies have now fully confirmed the fact that both the amino- and carboxy-terminal sequences of the α chains contain lengths of amino acids whose sequence precludes any possibility of the formation of the normal collagen helix.[49] Other investigators have shown that species-specific as well as nonspecific antibodies can be raised in animals immunized with injected collagen; the antibodies that do not differentiate collagens from different species apparently are directed to regions of the triple helix, while the species-specific antibodies are directed toward the telopeptides. These observations demonstrate clearly that the telopeptide sequences on the collagen molecule are distinguishable in animals of different species.

In view of the enzyme susceptibility of the nonhelical chains, the definition of the length of the native collagen α chain has been a matter of some difficulty. Bornstein showed that the amino-terminal peptide on the $\alpha 1$ chain extracted from rat tail tendon is four amino acid residues longer than that extracted from the skin of the rat.[50] It was at that time uncertain whether this fact reflected a difference in the biosynthetic mechanisms or the inadvertent cleavage of the labile terminal peptide in the course of the isolation of the α chains. More recently, Becker et al. have shown that the C-terminal telopeptides in soluble calf skin collagen may be obtained in various lengths.[45] They demonstrated that one of two forms of the carboxy-terminal peptide of calf skin collagen ($\alpha 1$-CB6) was shorter by 18 amino acids than the other. Subsequent experiments showed that extraction of the collagen chains in 8 *M* urea rather than in acetic acid gave only the large form of the terminal peptide, and, moreover, the chain isolated by this procedure contained an additional dityrosine peptide beyond the previously characterized carboxy-terminal amino acid. These results indicate that two or more scissions may occur in the terminal regions of the chains in the course of collagen isolation; and there is no guarantee that the peptides isolated in the presence of urea are undamaged. In view of this uncertainty about the telopeptide lengths and in view of the reactivity of these chains and the influence they have on the associative properties of the collagen molecule, it would be premature at this time to conclude any listing of the properties and characteristics of the collagen telopeptides (e.g., see Reference 51). The amino-terminal telopeptides, at least, are the remnants of a longer chain that is cleaved subsequent to biosynthesis; this process of peptide cleavage and its significance and consequences will be discussed later. These findings confirm and amplify the original deductions by Hodge and Schmitt and Rubin et al., who noted that the physical and aggregative properties of collagen preparations made under various conditions differed and who then surmised that proteolysis might be occurring in the course of isolating the collagen from tissues.[40,52]

In the sections that follow, we will discuss collagen biosynthesis, the postsynthetic modifications to the collagen molecule that are made, the secretion of the molecule from the cell, fibrillogenesis in the connective tissues, and cross-linking and catabolism. In all of these sections the major theme will be the attempt to differentiate the intrinsic function of the molecule as a supportive element in connective tissue from the structural features of the molecule that we may deduce pertain to homeostatic control mechanisms.

Collagen Biosynthesis

Collagen molecules are synthesized within cells.

Although it is commonly assumed that the major cell type involved in collagen biosynthesis is the fibroblast, and certainly fibroblasts in tissue culture can produce significant quantities of extracellular collagen, several other types of cells are capable of synthesizing this macromolecule. We have already discussed the fact that epithelial layers can produce the collagen of the basement membrane, and recently Pfeiffer et al. (Pfeiffer, S., personal communication, 1972) showed that a glial cell in tissue culture can synthesize collagen.

There was speculation for some years that the collagen molecule might be built from a series of covalently linked subunits.[53-55] However, the sequence studies on the peptides cleaved by cyanogen bromide from the constituent α chains made it clear that there was no internal repetition in the molecule; and the precursor molecule, in fact, appears to be built from an unreiterated amino acid sequence of approximately 120,000 mol wt. It must be concluded that the alkali or hydroxylamine-labile bonds that had been studied by Blumenfeld et al. and others are not ester linkages, as was deduced earlier, but alkali-sensitive asparagine-glycine bonds.[55-57]

The conclusion that each α chain is synthesized as a continuous polypeptide was confirmed by the experiments of Vuust and Piez, who followed the biosynthesis of collagen in calvaria of young rats in pulse-labeling experiments modeled after those of Naughton and Dintzis.[58,59] These studies demonstrated a continuous gradient of radioactivity along the linearly ordered cyanogen bromide peptides in the $\alpha 1$ and $\alpha 2$ chains. These experiments also indicated that the $\alpha 1$ and $\alpha 2$ chains are synthesized simultaneously and not successively and are completed in 3 to 7 min.

Many studies have been made aimed at characterizing the polysomes engaged in collagen synthesis. Early experiments discussed by Gould indicated that collagen biosynthesis was associated with very large polysomal aggregates.[22] On the basis of these experiments, some authors speculated that the whole triple helical structure might be biosynthesized on the same very large polysome. Other work on cell homogenates from other tissues has shown that biosynthesis can be detected in polysomes of a more usual size (e.g., 200 to 300 Svedbergs).[60,61] These findings are compatible with the probability that each collagen chain is synthesized *in toto* in a normal manner with several ribosomes translating from a single mRNA molecule. The production of skin, tendon, or bone collagen with its demand for two $\alpha 1$ chains for every $\alpha 2$ chain might be expected to entail some rate controls at the transcriptional or translational level of biosynthesis; of the many in vitro systems in which the synthesis of collagen has been studied, only Layman et al. have reported an apparent disproportionation, with an excess of $\alpha 2$ chains detected outside the cells.[62]

Hydroxylation and Glycosylation

In the course of the synthesis of the polypeptide chains that constitute the collagen macromolecule, a certain fraction of the proline residues normally becomes hydroxylated in either the 3 or the 4 position in the pyrrolidone ring. This hydroxylation is accomplished by enzymes that have been termed proline hydroxylases. It is presumed from studies on the specificity of hydroxylation effected by the isolated proline hydroxylase that the enzyme giving rise to the 3-hydroxyproline is different from that producing the 4-hydroxy derivative. A selective hydroxylation process also takes place on certain of the lysyl residues in the molecule.[63] This reaction is catalyzed by a third enzyme, lysyl hydroxylase.[64] Each of these hydroxylation reactions requires the presence of the enzyme, ferrous ions, α-ketoglutarate, and atmospheric oxygen; ascorbic acid usually increases the rate of hydroxylation, although its role in the reaction is not yet determined. Removal of any of these cofactors results in a decrease in the rate of collagen biosynthesis and a partial decrease in the rate, or a complete block, of collagen secretion by the cell. These facts have been exploited by many workers to explore the mechanisms and the sequential stages in the biosynthesis, hydroxylation, and glycosylation of the collagen molecule prior to its excretion by the cell.

Not all of the proline or lysine residues in the α chains become hydroxylated. This fact reflects, in part, the specificity of the enzyme that permits it to hydroxylate proline residues only in certain locations. In general, the hydroxylation of proline in mammalian collagens is restricted to residue Y of the characteristic collagen tripeptide sequence -Gly-X-Y- and only in those triplets that precede glycine. The most recent studies on the mechanisms, kinetics, and restraints on the process of proline hydroxylation have been published by Kivirikko et al.[65]

Very soon after sequencing studies were begun on collagen peptides, it was noted that proline and lysine residues in certain locations in the α chains were incompletely hydroxylated; i.e., some of the molecules in the extract from the tissue contained residues that had escaped hydroxylation. Similarly it was noted that collagens in different tissues demonstrate characteristically different levels of hydroxylation. Thus, Bornstein demonstrated that in the 36-residue peptide α1-CB3 from the major rat collagen, seven of the proline residues are hydroxylated if the collagen is extracted from skin, but only three in tendon.[66] However, when these peptides were treated with proline hydroxylase, in vitro, the two peptides became hydroxylated to the same degree.[67] We must conclude that there is a tissue-specific difference in the process of hydroxylation that is not a reflection of the collagen primary structure or the enzyme specificity (at least as manifest in the enzyme tested in vitro – although tissue-specific hydroxylases have not been disproved).

The collagen molecules that are found in different tissues contain carbohydrate bound through an O-glycosidic linkage to hydroxylysine. Hydroxylation is therefore a necessary preliminary step to glycosylation. From enzymatic digests of mammalian collagens from different tissues, various levels and ratios of galactosylhydroxylysine and glucosylgalactosylhydroxylysine have been measured;[68] and, as in the case of proline hydroxylation, there have been detected, even in a single tissue, differences in glycosylation in different fractions of the collagen successively extracted by solvents.[69]

In lower organisms other sugars are found linked to collagen, and bonding occurs through other amino acids (e.g., serine, in earthworm cuticle collagen);[70,71] but galactose and glucosylgalactose are the only significant substituents on mammalian collagens. The level of glycosylation is very low in the collagen of mammalian skin and tendon – only a single substituent on the α1 chain of the rat – but it is high in the basement membrane collagens.[72] In some invertebrates the major collagens are heavily glycosylated.[73]

We can conclude from the evidence summarized above that the collagen molecules extracted from any one tissue display a microheterogeneity with respect to a pattern of substituents along the three polypeptide chains and that in any one animal there are consistent, tissue-specific differences in the levels of substituents on the chains.

Microheterogeneity is a familiar observation for workers in the field of glycoprotein structure. In these proteins, where glycosyl residues are sequentially attached through enzymic mechanisms to a polypeptide chain, significant heterogeneity can appear in a bulk population of molecules that is analyzed. It remains to be determined whether this heterogeneity in the case of glycoprotein reflects a lax control over the activity of the sequence of enzymes involved in the glycosylation, and in the case of collagen, lax control over the proline and lysine hydroxylases and the glycosyl transferases. We will return later in this article to a discussion of the possibility of a purposive discrimination of populations of collagen molecules by a selective pattern of substitution on amino acid residues.

Control of Collagen Exocytosis

Prockop and his collaborators noted that if the hydroxylation of collagen is inhibited in vitro by the chelation of ferrous ions (e.g., by adding to the medium the iron chelator, α,α'-dipyridyl) or by anoxia, then the secretion of collagen is inhibited. They also showed by autoradiography that under these conditions proline-rich polypeptides accumulate in the cells.[74-76] This underhydroxylated collagen precursor was named protocollagen.

Subsequent work has shown that this blocking of collagen secretion is not absolute. Margolis and Lukens demonstrated that in the presence of the chelator hydroxyproline-deficient chains were excreted, but only at a fraction of the rate of the chains in cells grown in media supplemented with ferrous ions.[77] Ramaley and Rosenbloom have shown that the excreted chains are smaller than α chains.[78] The relationship of the excreted chains to normal α chains therefore is uncertain. The protocollagen retained in the cells in α,α'-dipyridyl-containing media would appear to be complete α chains or longer, i.e., pro-α chains, as will be discussed below.[79,80] Much of the protocollagen appears to be normal collagen, apart from the lack of hydroxylation, because when ferrous ions are restored to the culture, these accumulated polypeptide chains are quickly released by the cell and normal collagen molecules are detectable in the culture medium.[81] Therefore, the polypeptide chains can be released by the ribosomes after their synthesis is complete irrespective of their level of

hydroxylation, but in the course of normal biosynthesis, however, hydroxylation is begun, at least, while the polypeptide chain is being synthesized and is attached to the ribosome.[60, 61, 81a]

These experiments signify that there is some mechanism to prevent the secretion by the cell of incomplete collagen molecules. Most experiments on these in vitro systems have been concerned with the hydroxylation mechanisms, but since glycosylation must necessarily be preceded by hydroxylation, which of these steps is the critical one to open the way for exocytosis is not clear.

It is appropriate to discuss here the question of glycosylation and protein secretion by cells. Eylar in 1966 noted the widespread occurrence of glycosylated proteins in extracellular tissues and suggested that the attachment of sugars to protein was a necessary preliminary and recognition signal to permit its export through the cell membrane.[82] This hypothesis has recently been discussed critically by Winterburn and Phelps.[83] These authors define the limit of intracellular, as against extracellular, domains stringently and conclude that the intracisternal medium in the endoplasmic reticulum is *extracellular* in view of its potential or intermittent communication with the medium outside of the cell. On the basis of this definition, these authors conclude that the question whether a protein is made for export or is made to be retained is decided by the cell from the moment the ribosome becomes attached with its messenger RNA to the endoplasmic reticulum surface to make a membrane-bound polysome, as opposed to those polysomes that remain freely dispersed in the cytoplasm.

Winterburn and Phelps discuss the evidence that indicates that glycosylation of the typical extracellular protein occurs either as the polypeptide chain passes through the endoplasmic reticular membrane or immediately afterwards. The polysaccharide chains may subsequently be lengthened by successive additions of sugar through enzyme-mediated glycosylations within the endoplasmic reticular cisternae or the Golgi vesicles. Thus glycosylation is not the factor that dictates whether the protein should be secreted by the cell since, if these conclusions are correct, that fate was decided before glycosylation commenced. It has been noted, moreover, that nonglycosylated proteins *are* secreted by cells and some glycosylated proteins remain intracellular. The familiar extracellular proteins that carry no carbohydrates include serum albumin, trypsinogen, chymotrypsinogen, β-lactoglobulin, and α-lactoglobulin. Since some of these proteins are zymogens, it seems unlikely that any carbohydrate was originally attached to the polypeptide chains and was then excised. In view of these criticisms, the basis for Eylar's hypothesis now appears very insubstantial.

With this discussion in mind, let us reexamine the biosynthesis and secretions of collagen. While the growing peptide chain is bound to the ribosome, the size of the polysome would indicate that the completed length of the chains has not begun to fold into a triple helix; indeed, the association of two polysomes (for the α1 and α2 chains) through folding peptide chains would become a topological nightmare if more than one of the several nascent chains on the polysome began to associate. Since Lazarides and Lukens showed that partially completed chains attached to ribosomes are usually heavily hydroxylated, we may conclude that the selective hydroxylation that will ultimately distinguish the tendon from the skin collagen, for example, must normally occur on the polypeptide chain as it is unfolded or only folded in the polyproline II helix.[61]

This conclusion is consistent with the experiments on the hydroxylation of synthetic substrates by Kikuchi et al., who concluded that triple helices are poor substrates compared to single chains for the hydroxylase enzymes.[84] However, Kivirikko and his co-workers compared a large variety of polymers of the structure $(X\text{-}Pro\text{-}Gly)_n$ as substrates for proline hydroxylase, and they concluded that hydroxylation can be effected on triple helical structures.[65] As the latter workers have discussed, it is difficult to draw conclusions from these conflicting studies on synthetic polymers. More relevant are the observations of Rhoads et al., who found that the α1-CB3 peptide from rat collagen could be hydroxylated in vitro most effectively in the unfolded state.[67]

There is no unequivocal evidence yet to demonstrate whether or not the α chains are biosynthesized on the endoplasmic reticulum. If we can accept the probability that synthesis occurs on membrane-bound ribosomes, then, if current theories are correct, the completed α chains must accumulate in the cisternae of the endoplasmic reticulum and pass to the Golgi apparatus before secretion. Trelstad has observed elongated vesicles

with possibly fibrous contents in collagen-secreting cells of the chick corneal epithelium.[85] These vesicles may be closed elements of the endoplasmic reticulum containing separated chains undergoing hydroxylation. Observations confirming vesicular storage have been reported by Coulombre and Coulombre.[86] It would follow that the proline hydroxylases must be membrane bound or located within the cisternae. It has been noted that in cell extracts the enzyme appears to be soluble and unbound.

At present it is uncertain what condition the α chains are in within the cell when glycosylation and lysyl hydroxylation (a prerequisite for glycosylation) take place. Certainly both lysine and proline hydroxylation are separate and apparently necessary steps prior to exocytosis. In place of α,α'-dipyridyl inhibition of hydroxylation, Dehm and Prockop and Harsch et al. invoked a more selective inhibition by adding *cis*-hydroxyproline and dehydroproline, respectively, to cell cultures.[87,88] Proline hydroxylation did not occur, and protocollagen accumulated in the cells. Similarly, Christner and Rosenbloom observed a block in collagen secretion in cells treated with dehydrolysine.[89]

Glycosylation of collagen also occurs prior to exocytosis. Experiments by Grant et al. on basement membrane collagen synthesis by chick lens cells showed that the molecules persist within the cell 30 min after full glycosylation has been effected:[90] the delay in exocytosis may relate to slow proline hydroxylation or to some other factor.

It is not certain whether glycosylation occurs on the collagen molecules before or after triple helix formation. Bosmann and Eylar have isolated both galactosyl and glucosyl transferase enzymes from a line of fibroblasts.[91,92] The enzyme will specifically attach galactose to hydroxylysine residues in a collagen chain and subsequently add glucose to the galactose. The artificial substrate used by these authors for the assay of this reaction was prepared from guinea pig collagen that had been denatured through removing the hexose residues by periodate and acid hydrolysis. Since the collagen chains will refold in a cooled solution, it is possible that there was some polyproline type II helix folding in the chains prepared in this way, but it is most unlikely that any triple helix reformed. Most probably the chain was largely denatured under the conditions and temperature of the enzyme assay. However, these observations do not preclude the activity of the enzyme on triple helical chains. These authors also reported that the glycosylating enzymes are in the plasma membrane of the cell and not in the membrane of the smooth endoplasmic reticulum.

In view of the very limited action of the glycosylating enzyme on the substrate, substituting only one disaccharide unit per skin collagen α chain in vivo, for example, we must conclude that the site of glycosylation on the molecule is partly dictated by the enzyme specificity that selects a particular sequence on the chain.[93] Although such tissue-specific glycosylation patterns may be dictated, for example, by allosteric control mechanisms, it remains possible that in tissues producing more heavily glycosylated collagen chains there are different glycosylating enzymes. On the other hand, the same enzyme might bring about different levels of glycosylation when acting on collagen chains with distinguishable primary sequence (e.g., skin, cartilage, or basement membrane collagen).

From this discussion we may conclude that proline and lysine hydroxylation (and possibly glycosylation also) must occur before the nascent, helical collagen molecules are allowed to leave the cell. It is uncertain at what step the triple helix forms. The fact that cells inhibited by iron chelation slowly secrete heterogeneous protocollagen chains shorter than α chains suggests that the chains are vulnerable to proteolytic degradation that is initiated either within the cell, or during the passage through the membrane, or in the extracellular medium. Extracellular degradation would be a natural consequence of the release of unfolded, separate α chains because these are vulnerable to proteases. Hence, we conclude that the triple helix formation occurs late, possibly just prior to secretion.

In summary, we may conclude tentatively that the decision for exporting collagen is not predicated upon the glycosylation of the chains, but is made at the time the mRNA-ribosome associates with the membrane. The completion of chain synthesis is independent of the hydroxylation and glycosylation. Since hydroxylation almost certainly occurs on an unfolded polypeptide chain (attached to the polysome), it is unlikely that there are steric constraints, and the pattern of substitution must be dictated by the enzymes involved. Hence, we must look for the tissue

specificity of hydroxylating and glycosylating residues in the controlled activity of the modifying enzymes.

The newly synthesized polypeptide chains are probably held in vesicles that then are transported to the cell surface by a mechanism involving microtubules. These organelles have been implicated in many circumstances with intracellular transport, and reports by Ehrlich and Bornstein and by Dehm and Prockop[94,95] show that exocytosis is delayed in cells treated with antagonists to microtubules, the drugs colchicine and vinblastine.

The step in collagen secretion that demands hydroxylation (and possibly glycosylation also) is not defined. It might be a mechanism coupling helix formation to the action of registration peptides (see below) and reaction at the plasma membrane (where the glycosylating enzymes are reported to be bound); certainly the lack of hydroxylation would not appear, in theory, to be an impediment to chain folding. The incompleteness of our knowledge of the mechanisms controlling the decoration and secretion of collagen by a cell is highlighted by recent studies on a human hereditary condition resembling the Ehlers-Danlos syndrome. In these patients the collagen molecules (in certain tissues only) have an abnormally low content of hydroxylysine, but the tissues are not deficient in collagen.[96] Hence, in this condition a low level of lysine hydroxylation – and possibly, glycosylation – does not prohibit collagen exocytosis. Moreover, even in normal animals we do not know at what step in collagen production ascorbic acid is required. Any deductions we draw from experimental findings at present must be tentative. Further studies on mutant organisms may give insights into collagen synthesis that are not available from experiments that employ chemical intervention.

Procollagen

The sequence of intracellular events described above appears designed to regulate the biosynthesis and secretion of a collagen molecule whose primary sequence is appropriate to the tissue (tendon, cartilage, or basement membrane), whose proline and lysine residues have been hydroxylated and glycosylated to a degree also appropriate to the tissue, and whose chains have been folded into a triple helix configuration. Studies on the biosynthesis of collagen in cell or organ cultures demonstrated that a fraction of the population of newly secreted molecules could be found that was longer by 10 to 20 nm than the molecule that is extracted from the native fibrils (Figure 4). The ratio of these larger chains to the normal size chains in the tissue culture fluids varies; the work of Layman et al. on fibroblasts, of Church, Pfeiffer, and Tanzer on 3T6 fibroblasts, of Bellamy and Bornstein, and subsequent work by Ehrlich and Bornstein and Bornstein et al. on rat and chick calvaria, all provided evidence for a novel form of the nascent molecule with all three α chains elongated, but estimates of the size of the collagen precursor varied.[62,97-100] Subsequent studies by Prockop and his collaborators on biosynthesis by chondrocytes in culture seem to have provided a cleaner system for investigation, in the sense that most molecules appear in the elongated form that is now widely regarded as a precursor of the molecule that will make the collagen fibrils.[101,102] This precursor has been called procollagen.

In the flurry of activity subsequent to these discoveries, many tissues culture systems have been investigated, and an enzyme, procollagen peptidase, that can cleave the large amino-terminal peptides that exist on all three α chains has been discovered in the extracellular fluid of cultured calvaria and other tissues.[103,104] A very similar cleavage can be effected by treatment of the procollagen molecules at acid pH with pepsin. The susceptibility of these amino-terminal peptides to

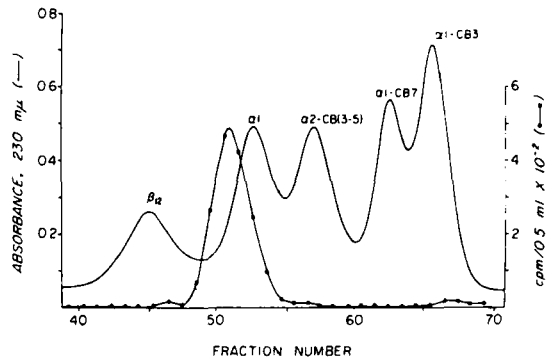

FIGURE 4. Elution diagram of collagen chains and collagen peptides together with nascent, pro-α1 chains from chick calvaria labeled biosynthetically with ^{35}S-cysteine. The order of elution from the molecular sieve column (4% agarose beads) is indicative of chain length: The pro-α1 chains are longer than α1 chains and contain cysteine. (Reproduced from Bornstein, P., von der Mark, K., Wyke, A. W., Ehrlich, H. P., and Monson, J. M., *J. Biol. Chem.*, 247, 2808, 1972. With permission.)

protease suggests that they do not have a triple helix structure, and analyses have confirmed this point; these peptides have a lower glycine and proline content than the main lengths of the α chain. The peptides do not appear to be compactly folded in a globular form since they can become aligned, giving an appearance of a linear extension of the chains when SLS crystallites of the molecule are formed. These crystallites are longer by 13 nm than the crystallites of soluble collagen, but the length of these appendages cannot be directly correlated with their weight without knowledge of the folding and structure within this terminal region.[105]

It has been speculated that these procollagen peptides serve to modify the solubility characteristics of the collagen molecule to permit its transport from the cell to the site of fiber growth, but clearly these peptides can also be involved in homeostatic control of fiber formation, and they might also have a role in initiating helix formation that, as we have discussed, might be related also to glycosylation and extrusion from the cell.

At the time of the first discovery of procollagen, Speakman was speculating that there might be terminal peptides on the collagen molecule that served to register or initiate the folding of the three chains into the triple helix.[106] He based his postulate on the apparent need for a mechanism to promote the rate of helix formation after biosynthesis. Annealing denatured α chains to restore a triple helix configuration is a slow process in vitro, but the rate-limiting step is probably the ordering of the three α chains appropriately for helix formation. Speakman suggested that the associative properties of the "registration peptides" might initiate chain folding. The necessary subsequent removal of these peptides by a proteolytic step is analogous to the conversion of zymogens to give active enzymes; it is becoming apparent that such proteolytic activation of proteins is a widely used metabolic mechanism (e.g., fibrinogen to monomer fibrin;[107] proinsulin to insulin;[108] immunoglobulin to light chains[109]).

Recent work on several biosynthesizing systems has shown that the amino-terminal peptides that can be cleaved from each procollagen α chain have a molecular weight of 20,000 to 35,000.[99,105] In several systems the three chains of procollagen have been shown to cohere despite denaturation, and Burgeson et al. have related this property to disulfide bonds formed through cysteine residues in the registration peptides.[110] Cysteine does not occur in the helical lengths of the α chains, a fact that emphasizes the several striking differences in composition between the registration peptide and the helical length of the chain. Analyses by Bornstein and his co-workers of procollagen from chick calvaria showed that the registration peptide is low in glycine but rich in serine; they also found no cysteine in the pro-α2 chain.[99] In this instance, therefore, the triple helix nucleation is not solely based on disulfide bridges, and a better understanding of the process must await fuller analyses. It is also to be expected that some differences will be reported by the several groups working in this area who are studying cells of different species and tissues.

The cleavage of the registration peptides probably occurs extracellularly. The experiments by Grant et al. on chick basement membrane collagen suggest that procollagen molecules may persist 1 hr after exocytosis before scission of the peptides takes place.[111] Similarly, the observations by Ehrlich and Bornstein showed an enhancement of nascent procollagen to nascent collagen chains under conditions expected to block transport of vesicles of procollagen to the cell surface, and these results are most simply interpreted by assuming that the cleavage of the peptides is normally extracellular.[112] Indeed, it appears likely that cleavage may be one step in the metabolic control over the site and time of the deposition of the collagen into a fibril. It is possible that selective proteolysis might remove the peptide in more than one step with appropriate changes of reactivity at each stage.[113,114] Since newly synthesized collagen molecules in extracellular tissue do not contain cross-links or aldehyde derivatives, it is also feasible that these peptides serve to inhibit the activity of any lysyl oxidase that has access to the molecules. It also is possible that these appendages serve to direct or modify appropriate interactions with other connective tissue constituents so that the deposition of the collagen molecules may correspond to the requirements of the tissue, e.g., to be deposited in appropriate locations near a wound or in regions of growth.

Although it may appear at first sight implausible, there can be no doubt that the terminal peptides on the collagen molecule drastically affect the solubility and interactive capacity of the molecules. Schmitt, Hodge, and co-workers noted

the change in the solubility character of collagen after the protease reaction on the native molecule.[41,115] Their work over several years established the existence of the telopeptides; and those at the amino-terminus, at least, are the remnant of the registration peptides on the $\alpha 1$ and $\alpha 2$ chains. For example, collagen treated with trypsin precipitates in an SLS crystallite that is symmetrical rather than polarized.[41] This observation implies that the telopeptides dictate the mode of lateral association of the molecules when they crystallize. These workers also observed that pronase-treated collagen is soluble over a far wider range of pH and ionic strength than the native molecules. Furthermore, although the fibrils containing a 670 Å spacing could be precipitated from pronase collagen under special conditions, the tendency of the molecules to precipitate in this manner under physiologic conditions no longer occurred. In the same vein, Leibovich and Weiss and Steven et al. have discussed changes in the mode of precipitation of collagen after it has been treated with various enzymes.[116,117] We may conclude that although the charge profile of the molecule is probably adequate to order the molecules in the quarter-staggered array of the native fibril, the tendency of the molecules to interact, and their associative properties, are significantly determined by these terminal peptides.

In view of its size, it is highly probable that the registration peptide does more than initiate helix formation and maintain the nascent molecule in a soluble state prior to its accretion to an extracellular collagen fiber and, as mentioned above, this amino-terminal appendage may be removed in steps that relate to stages of activation. It also is possible that the cleaved peptides, like the fibrinopeptides, have a metabolic function perhaps related to extracellular homeostasis.

Extracellular Fiber Formation

The collagen molecules that are secreted by the connective tissue cells either initiate the synthesis of new fibrils or add to the surface of existing fibrils to enlarge them. The manner of this accretion and the homeostatic mechanisms that govern the degree of accretion and the initiation of new filaments is unknown, but we will discuss possibilities that have been considered.

It has been noted that the planar array of quarter-staggered molecules that was first illustrated by Hodge and Petruska (Figure 3) cannot be developed into a space-filling, three-dimensional structure.[118] Smith has argued that the most plausible manner in which a close packing of collagen molecules into a fibril can be achieved is through the formation of a pencil of staggered molecules in a helical assemblage of five molecules.[119] He therefore pictures a collagen fibril as an aggregate of the protofilaments formed by the polymerization of this pencil. Veis and his colleagues have studied the aggregates of α chains that can be dispersed from insoluble collagen by heat or denaturant treatment, and they have evidence for a particular aggregate that is considerably larger than the three chains of one molecule and which they find compatible with the cross-linked chains that might arise from a four-unit packing assemblage of chains.[120] Recently Miller and Wray deduced from x-ray crystallographic studies that a protofilament exists in teased tail tendons of rats and that the dimensions of the protofilament are compatible with a five- (or possibly four-) membered protofilament subunit of structure.[121]

Confirmation of a protofilament substructure of collagen fibrils by electron microscopy is equivocal. In my own experience, long filaments of about 3 nm in diameter can be observed in reconstituted fibrils but not in teased and macerated dispersions of native tendon. On the other hand, Bouteille and Pease detected fibrillar organization wider than one molecule within fibrils of tendons.[122]

Some observations are not easily reconciled with a protofibrillar substructure in the fibrils. The organizational element described by Miller and Wray from their equatorial x-ray reflections measured about 3.8 nm in diameter.[121] The diameters of fibrils in a young tendon range down to 20 nm. If a 4-nm protofibril were added to a 20-nm diameter filament, the addition should result in a resolvable bump on the surface of the cylinder of the tendon. In contrast, most electron micrographs of tendons in cross section demonstrate a smooth circular profile with no evidence of an irregular surface. It is, moreover, not clear what weight should be given to the argument that the molecules are arranged to maximize the space-filling capacity of the molecules in their staggered array. The density measurements on collagen fibrils are not yet accurate enough to rule out structures that may be assembled with less than optimal packing.

This extended discussion is justified because it

is not yet clear whether individual molecules are added successively to build up the diameter of a fibril or whether nascent molecules first order themselves into a protofibril that in its turn binds to the fibril in appropriate register with the protofibrils assembled before it. The successive addition of separate molecules to an existing fibril would appear to be the simplest physical process. If this process is the normal one, it still remains unclear whether successive molecules add in any special sequence or randomly to those arrayed in the fibril. If the latter is the case, because the molecules are staggered, we may anticipate voids along the axis of the fibril that cannot accommodate intact molecules and are approximately one-, two-, or three quarters the length of the molecule. So long as adequate lateral cross-links are added to maintain the longitudinal stability of this association, it is plausible that such an association could serve the biological function of a tensile stress-bearing structure, and it is not necessary to assume that the lateral surfaces of the molecule juxtaposed to other molecules must be maximized. Indeed, such a structure with long voids would have a tensile strength per unit mass greater than the structure assembled from Veis' or Smith's protofibrillar elements if the strength of collagen fibrils is imparted more by covalent intermolecular cross-links than by the forces directing the lateral association of the molecules. The weakness of lathyritic fibrils (see below) would support this supposition.

If, on the other hand, successively added molecules are sequentially ordered to pack as densely as their dimensional constraints will allow, then we must search for the interacting mechanism. Speakman suggested that the registration peptide might fill the gap to the next molecule, providing an obvious means of successive addition.[106] At present, it seems doubtful that the peptides are long enough to bridge the gap; moreover, this process would require that peptide cleavage occur after assembly into the fibril, but molecules sheared of the registration peptide are found in the supernatant fluid – at least in the unnatural circumstances of organ or cell culture.

Whatever the mechanism of fibril growth, whether by individual molecules unit by unit or by the addition of aggregates of molecules, the primary topic of concern in this review is the mechanism by which the body controls the site and the mode of deposition of collagen in the extracellular tissues. It appears at present that the molecules that constitute the fibers in subdermal tissues or in tendon, to take two examples, are identical in primary sequence and yet that the fibrils they form are disposed in very different ways. In and beneath mammalian skin collagen, fibrils are disposed in a loose and poorly ordered manner, while in the tendon the fibrils are arranged in a massive colinear array. Beneath amphibian skin, there are orthogonal lamellae of parallel fibers. The mechanisms that direct the ordering of the fibrils in these various tissues are not known, but possible mechanisms will be discussed in the section on homeostasis.

Cross-linking

After the collagen molecule has become aligned in the fibril (and possibly also before), it is subjected to further enzymic modification. Selected lysine and hydroxylysine residues concentrated in the amino- and carboxy-terminal telopeptides are oxidized by an amine oxidase (that has been called "lysyl oxidase") to produce aldehyde groups on the δ-carbon of the lysyl residue (i.e., derivatives of δ-adipic semialdehyde).[123,124] These aldehydes, by condensation with other aldehydes or with the ε-amino groups of hydroxylysine or lysine on other α chains, can form cross-linking aldol or Schiff base (aldimine) adducts. Another type of cross-link is formed by the Michael addition of an imidazole nitrogen in histidine to an aldol[125] or to merodesmosine or hydroxymerodesmosine: these products are capable of joining three or four peptide chains. Finally, the products of the condensation of a hexose aldehyde with the ε-amino group of lysine and hydroxylysine have been detected.[126,127] The hexose-containing compounds might link collagen chains to proteoglycans or glycoproteins, while the aldol, aldimine, and other more complex structures might cross-link two or more α chains within one molecule, or between molecules. Several investigators noted, before the structure of the more complex adducts was known, that an intramolecular aldol condensate might be a precursor of an intermolecular cross-link.[128,129]

As a result of the cross-linking processes, the newly deposited collagen molecules that were soluble in salt solutions of moderate ionic strength become insoluble except to reagents that can break the rather weak bonds that are formed by the aldehyde adducts. As a result of further

processes whose precise chemistry is yet unknown, the cross-links become stronger or are modified in character (e.g., by reduction) so that they no longer can be cleaved by mild reagents, and the result is a matrix of "insoluble" collagen molecules in which most of the constituent α chains are interlinked.

The stabilization of these aldehyde adducts can be effected by reduction, and if the reduction is carried out with ^{3}H-sodium borohydride, the bonds are not only stabilized but also labeled.[130, 131] The problems of isolating the labeled, reduced cross-links from 6 *N* or 3 *N* hydrochloric acid or 2 *N* sodium hydroxide hydrolysates and identifying them are described in recent reviews by Piez and Gallop et al.[9,131a] A number of laboratories have been engaged in characterizing the population of cross-links in native collagen, and the catalogue is large but still incomplete.

With the hydrolysis of some of the weak (unreduced) cross-links in strong salt solutions and in dilute organic acids, a fraction of the collagen molecules becomes soluble – the familiar "salt-soluble" and "acid-soluble" fractions. In the native state, however, it appears that all the molecules except a very small number of nascent molecules are cross-linked; their dissolution depends upon the cleavage of intermolecular bonds. The effects of solvents on the array of reducible bonds in collagen fibrils have been demonstrated by several groups of investigators. The solvent-cleaved intermolecular bonds can be at least partially reformed by incubating the collagen molecules after they have been reprecipitated in a quarter-staggered fibrillar array similar to that of the native fibers.[129,132] This interaction and intermolecular linking can be blocked by the reaction of thiosemicarbazides with the intramolecular cross-links.[133–135] The reaction with this semicarbazide probably involves the aldol cross-link. Therefore, these observations probably indicate that the condensation of an intramolecular aldol condensate with histidine and possibly lysine or hydroxylysine on another molecule (to give aldol-histidine, histidyl merodesmosine, or histidyl hydroxymerodesmosine) is the mode of formation of many collagen intermolecular cross-links. Other studies have suggested that Schiff bases, particularly those involving hydroxylysine, are significant intermolecular bonds, particularly in hard tissues such as bone.[136]

Tanzer showed that the re-formation of cross-links did not occur in molecules aggregating in a more random fashion (instead of the quarter-staggered array), a fact that demonstrates the high specificity of the interaction between adjacent molecules and the purposive distribution of the cross-linking residues.[137] This specificity is strikingly illustrated by the formation of a cross-link involving histidine. In a preliminary compilation by several authors (Gross, J., personal communication, 1972) of the complete amino acid sequence of skin α1 chains derived from data on chick, rat, and calf, histidine was found only in residues 89, 929, and 1034 (see Bruns and Gross for the numbering convention[138]). Clearly, any intermolecular bond formation to histidine is contingent upon precise lateral alignment of the molecules.

The process that produces these cross-linking allysine and hydroxylysine residues is the highly specific activity of the enzyme lysyl oxidase.[124, 139] This enzyme has been characterized and its activity studied. It is irreversibly inhibited by β-aminopropionitrile, an observation that explains the lack of cross-linking in collagen fibrils in animals fed this lathyrogen. The enzyme contains copper: animals fed on a copper-deficient diet show symptoms similar to those of lathyritic animals.

It is not known when the lysyl oxidase acts on the collagen molecule, but the enzyme is found mostly in the extracellular medium, and the following recent experiments suggest that aldehyde formation occurs only after the molecule is incorporated into the native fibril.[140] Only 0.1 to 0.25% of the collagen in the tail tendon of an adult rat can be extracted with neutral saline or acidic solutions if the tendons are first reduced with sodium borohydride.[141] Therefore, we may conclude that all but this small fraction of the collagen is associated in the fibril through cross-links that have been stabilized by reduction. On the other hand, if a native tendon is extracted with salt solutions, between 1 and 2% of the collagen can typically be dissolved. If this salt-soluble collagen (the newly synthesized molecules) is reduced with sodium borohydride, the level of radioactivity that can be detected in the material after hydrolysis and chromatography is low, only 20% or less of the activity in reduced native or acid-soluble collagen.[128,141] Therefore, this salt-soluble collagen has suffered very little attack at this stage by lysyl oxidase, and this conclusion is

confirmed by the fact that it consists almost entirely of α chains with very few β chains, showing that little intramolecular cross-linking has been effected. Nevertheless, the insolubility of this fraction in the reduced, native tendon shows that this small fraction of the collagen is bound, presumably then as the recipient of aldehyde cross-links.

We may conclude that, for the most part, each salt-soluble collagen molecule becomes associated in the appropriate orientation in the fibril (or protofibril) and bound by cross-links before the lysyl oxidase has produced any significant number of aldehydes on the molecule itself. It is plausible that the registration peptides preclude the action of the lysyl oxidase on the procollagen molecule; however, the intervals between peptide cleavage, the incorporation of the molecule into the fibril, and the appearance of allysine and covalent cross-links are not known. A large fraction of nascent molecules are cross-linked within 24 hr in vitro.

The observations of Lukens on $\alpha 1$ and $\alpha 2$ chain heterogeneity in the population of chains retained in a cell under α,α'-dipyridyl inhibition might be interpreted to show that lysyl oxidase acted on the chains intracellularly.[81a] In view of the arguments above, this conclusion appears unlikely; the heterogeneity more likely relates to the presence of collagen and procollagen chains.

It is possible that the concentration of lysyl oxidase is the factor that limits the rate of cross-linking. Pulse-labeling experiments have shown that the salt-soluble collagen comprises nascent molecules and its concentration is normally high in young animals. Detailed study has shown, however, that the level of salt-soluble collagen in a tissue reflects not age, but the rate of collagen synthesis; thus, the level is low in a starved young animal but high in a starved animal restored to a full diet.

After the rapid establishment of inter- and intramolecular cross-links soon after fiber formation, other changes in the number and character of the cross-links occur in a tissue in the course of months or years. These changes increase the number of cross-linked chains in tendons (e.g., Cannon and Davison[141]) and decrease the proportion of salt- and acid-soluble collagen in most tissues.[142-145] Some of these solubility changes indicate the stabilization of some previously acid-labile cross-links; the detection of reduced dihydroxylysinonorleucine in collagen suggests that one method of stabilization that occurs involves reduction in vivo.[146] It is questionable whether the slow changes in the cross-links indicate further enzyme activity because it is not clear what access an enzyme can have to molecules packed in close array in the center of a fibril. Some of the age-related changes probably relate to the progressive dehydration of connective tissue and an improved crystallinity in the packing of the collagen.

Mention has been made of the specific siting of the allysine and hydroxyallysine residues. Most of the aldehydes are located on the telopeptides. This fact is demonstrated by the removal of intramolecular cross-links from native molecules by pepsin or other enzymes that can attack only the telopeptides,[40,41] by the elimination of the aldehyde residues themselves from α chains by pepsin treatment of acid-soluble collagen (Davison, unpublished), and by the identification of the aldehydes on the amino-terminal peptide split by cyanogen bromide from $\alpha 1$ and $\alpha 2$ chains of acid-soluble collagen (Reference 147 and Patel, A. and Davison, P. F., in preparation). Few of these aldehyde residues are detectable in the native fibrils, but they are exposed when dilute acid, in dissolving part of the collagen fibril, hydrolyzes several complex intermolecular cross-links and also, possibly, interchain aldimines.

The terminal location of the cross-linking sites is shown also by direct analysis of the cyanogen bromide peptides of reduced, tritiated, acid-soluble collagen[147,148] or guanidine hydrochloride-denatured extracts (Davison, P. F., unpublished data, 1972). Kang found ^{3}H-hydroxynorleucine only in the amino-terminal peptides of the $\alpha 1$ and $\alpha 2$ chains from rat tail tendon;[147] Eyre and Glimcher confirmed that the amino-terminal site predominates.[148] In our laboratory we found, in addition, that 20% of the label is in the carboxy-terminal peptide. Stark et al. have also detected allysine residues in the carboxy-terminal peptide, and in this case they have defined the location to within the telopeptide region of the $\alpha 1$ chain.[149]

In contrast to these findings, Deshmukh and Nimni reported a significant population of aldehydes in cyanogen bromide peptides from regions far from the terminal segments of the $\alpha 1$ chain in a special fraction of collagen, the cysteamine-soluble, from rat tissues.[150,151] These aldehydes

were not detected in Kang's or our own experiments on rat acid-soluble collagen; either the cysteamine-soluble fraction is especially heavily modified by the action of lysyl oxidase, or these aldehydes are artifacts of the isolation procedure.[147] Shuttleworth and Glimcher reported cross-linking sites on rat skin collagen that were invulnerable to chymotrypsin, and, therefore, they concluded, not on the amino-terminal telopeptides; but they did not define the location further.[152]

Other evidence (Davison, P. F. and Patel, A., unpublished data, 1972) indicates that the *inter*-molecular cross-links, at least, are built entirely by the aldehyde residues located on the telopeptides. As mentioned above, when collagen is reduced in the native fibril, virtually none of the collagen molecules can be extracted by acid or salt solutions. On the other hand, digestion of such reduced fibrils with pepsin at 20°C results in the liberation of better than 90% of the collagen in a soluble form. This solubilization is the result of the cleavage of the amino- or carboxy-terminal telopeptides that are the only parts of the molecule vulnerable to pepsin under the conditions of the experiment. In the polarized, quarter-staggered array of a native fibril, the limited reach of the telopeptides precludes their forming intermolecular cross-links (except in a special case) to other telopeptides, and these cross-links are made to the triple helix body of an adjacent molecule. The scission of the aldehyde-containing peptide chains at any site short of the aldol adduct results in the separation and solubilization of the molecules previously bound by such cross-links, and the reduced aldehyde adduct is then found attached to the region of the recipient molecule to which the aldehyde reacted (Figure 5). Cyanogen bromide cleavage of α1 chains from such pepsin-solubilized, reduced native fibers indicates that adducts form to several positions along the helix with some preference for the carboxy-terminal peptide α1-CB6.

The exceptional intermolecular cross-link mentioned above would join amino-terminal to amino-terminal chains (or carboxy-terminal to carboxy-terminal) in the case of molecules juxtaposed in register. Evidence for such cross-linking has been reported by Zimmerman et al.[153] Such cross-links, as well as aldehydes not yet involved in adducts, could be excised completely by pepsin. On the other hand, intermolecular

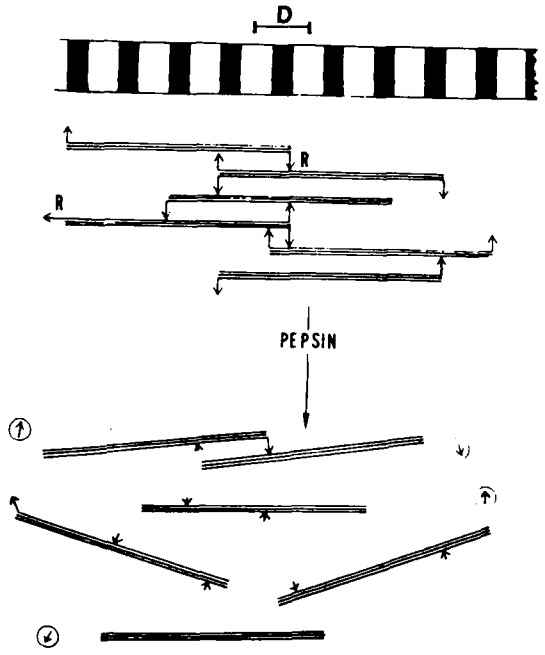

FIGURE 5. An illustration of the possible disposition of intermolecular cross-links formed through aldehyde residues on telopeptides is shown beneath a diagrammatic representation of a native collagen fibril with banding of 67-nm periodicity. Intramolecular cross-links are not shown.

Dilute acid treatment of the native fibril would hydrolyze some of the cross-linking adducts, and the solubilized molecules would then carry N- or C-terminal allysine or hydroxyallysine residues.

Borohydride reduction renders the aldehyde adducts invulnerable to dilute acid hydrolysis, but the cross-linked molecules can be solubilized by pepsin, which cleaves peptide bonds in the telopeptides. In the pepsin-solubilized preparations, some pepsin-resistant bonds (R) give rise to some polymeric forms that appear in solution. Some of the solubilized molecules now carry the reduced aldehyde adducts on the site to which the aldehyde had reacted. Uncoupled aldehydes ③ are excised (if the telopeptides are pepsin-labile) and appear as dialyzable aldehyde derivatives in short peptides.

The band periodicity in the native fiber is frequently assigned the length D; the collagen molecule has a length 4.4 D.[118]

cross-links formed from aldehydes situated in the triple helix length of the collagen molecule, as Deshmukh and Nimni described, could not be cleaved by the enzyme since neither a reduced Schiff base or aldol compound, nor the peptide chain in which they are situated, is vulnerable to pepsin.[151] The fact that reduced native tendon is so well solubilized by pepsin implies that such cross-links must be very few or absent from tail

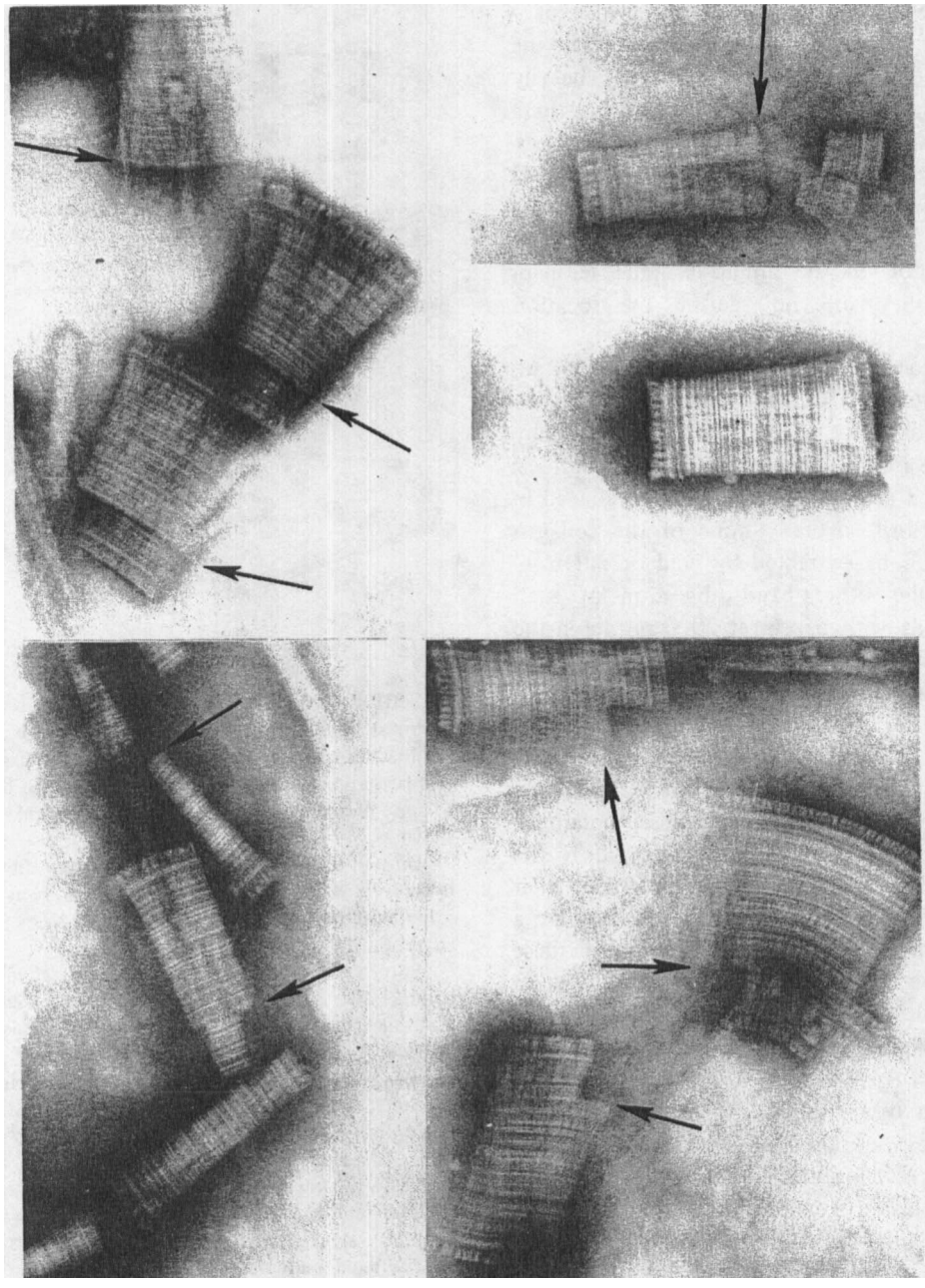

FIGURE 6. Segment-long-spacing crystallites of collagen digested by rabbit corneal collagenase are shown after negative staining: top left, collagen from human umbilical cord; top right, digested (upper) and control (lower) collagen from lamprey skin; bottom left, rat skin collagen; bottom right, calf articular cartilage collagen. The point of enzyme cleavage ↗ appears to be identical on all the collagen molecules. The two samples on the left are $(\alpha 1)_2 \alpha 2$ type, the others $(\alpha 1)_3$ collagens.

Under these staining conditions (phosphotungstic acid, pH 4.2), the banding patterns are similar, but all except the human and rat segments are distinguishable.

similar to that found in the involuting rat uterus.

The activity of the two rat collagenases exemplifies the differences in activity that have been observed in collagenases over several species including amphibia, but the significance of this difference is uncertain. On the one hand, the collagenase may produce a single cleavage at a point 75% of the molecule from the amino-terminus; on the other hand, other enzymes appear capable of producing other cleavages, particularly at points 62 and 67% of the length of the molecule from the amino-terminus. It may be noted, however, that some investigators have had to go to extravagant lengths to eliminate contaminating protease activity from tissue collagenases. At present the possibility cannot be dismissed that the several sites of attack on the molecule indicate the action of a cathepsin nibbling its way along the triple helix from the initial point of cleavage. The fragments complementary to the 62 and 67% cleavage points have not been found, only the 25% piece that is the complement of the 75% fragment.

Tokoro et al. reported that if the activity of tissue collagenase on mammalian collagens is studied at 25°C, the action of the enzyme produces a pronounced lowering viscosity but a minimal change in the optical rotation of the collagen – observations that imply that the length of the molecule is shortened but that the triple helix is not unwound.[181] At this stage electrophoretic analysis and electron microscopy indicate that the 75% and 25% pieces predominate. At higher temperatures and after longer periods, the 62% and 67% fragments become detectable; dialyzable peptides appear; and there is a progressive loss of optical activity. This further activity of the enzyme may indicate attack by the collagenase, or by contaminating proteases that are unable to attack the collagen molecule prior to cleavage or at temperatures where the helix is more stable.

Distinctions between collagenases by their mode of attack, as we have seen, must be interpreted cautiously until well-purified enzymes have been studied or until adequate control experiments have been made to exclude other proteolytic activities. A similar criticism must apply to discrimination based on kinetics, inhibitors, or molecular weight;[182] until the enzymes have been shown to be free of adsorbed tissue constituents (serum protein, for example), such discrimination must be tentative. Thus, Eisen et al. have noted that human granulocyte and human skin collagenase differ in their susceptibility to serum inhibition, yet possess identical major antigenic determinants;[183a] this latter criterion is probably the more crucial indicator of identity.

Early experiments by Gross and his collaborators that led to the detection of the first collagenase from tadpole tissue showed that the enzyme was not detected in extracts of the cells but was found in the fluid above a cell culture.[171] These experiments were first interpreted to indicate that the collagenase was synthesized and promptly excreted and that no level of enzyme was built up intracellularly. Subsequent studies on several tissues have shown that failure to detect enzymic activity may arise from two causes: on the one hand, the enzyme may be secreted as a zymogen for subsequent activation;[184-186] on the other hand, the enzyme may be inactivated by tissue or serum proteins that include α1-antitrypsin and α_2-macroglobulin.[179,187-189] These findings show that under appropriate circumstances the activity of the enzyme may be controlled by a variety of processes from the time of synthesis, through activation, to inactivation. The regulation of collagenase activity has been discussed recently by Bauer et al. and Eisen et al.[183,190]

The range of activity of tissue collagenase is controversial. Robertson and Miller reported that rabbit polymorphonuclear leukocyte collagenase and human gingival collagenase can digest skin collagen but not cartilage collagen.[191] Both collagens were in solution, and their activity was assayed by viscometry. Unpublished studies in our laboratories with rabbit corneal collagenase showed that solubilized bovine articular collagen and calf or rat skin or tendon collagen were cleaved by this enzyme;[192,193] in addition, the enzyme cleaved lamprey skin collagen (at an apparently identical position in the molecule, judging from the stained bands) (Figure 6). Lamprey collagen, like cartilage, contains only α1 chains (Reference 194 and Cannon, D., unpublished data, 1972). These investigations compare two collagenases from rabbit tissues (leukocyte and cornea). If the differences can be confirmed, this evidence would tentatively distinguish two collagenases from different tissues of the same animal. The human synovial enzyme

studied by Harris et al. is capable of digesting both tendon and cartilage;[177] these preparations contain two physically distinguishable enzymes, and the range of activity of each must still be clarified. The physical differences may relate to different states of aggregation of the enzyme with itself or with other tissue proteins so the number of different enzymes remains uncertain.

With the elucidation of the primary sequence of an $\alpha 1$ chain, and with the correlation of this sequence to the stained profile of the SLS crystallite (e.g., Von der Mark et al.[195]), it has become possible to locate the point of attack of the enzyme accurately. Studies in our laboratory pinpointed the site of collagenase scission by the enzyme from the cultured human cornea to between residues 219 and 225 of the α1-CB7 peptide. The only bond in that region that could release glycine and isoleucine as the terminal residues is the bond adjacent to residues 222 (Fietzek, P.P., Rexrodt, F., Hopper, K., and Kühn, K., personal communication, 1972) and it therefore is likely that this is the vulnerable bond.[196] In the $\alpha 1$ chains the peptide sequence in this region is consistently -(Gly-Pro-X)- so there is probably no interruption in the triple helix; however, until the appropriate sequence in the $\alpha 2$ chain has been determined, it will not be possible to reaffirm that the triple helix is complete in this area. As we have mentioned, the same point of cleavage is shown on cartilage and on lamprey skin collagen, and both of these collagens contain only $\alpha 1$ chains and no $\alpha 2$ chains. Unfortunately, the sequences of the appropriate peptides from these collagens have not yet been determined so that once again we cannot be assured that the triple helix is necessarily intact at this point, but it seems probable that the point of cleavage is delimited by the specificity of the enzyme rather than the deficiencies of the structure of the collagen.

In many instances the cells that produce the collagenase in connective tissues have not been identified. Studies by Eisen and Gross showed that in the resorbing tadpole tail the cells of the epidermis are responsible for collagenase production, while the hyaluronidase activity that is also necessary for the remodeling of the connective tissue is secreted by cells of the dermis.[197] In human skin the enzyme is secreted by cells of the dermis.[198]

It may be noted that the point of cleavage of the collagen molecule by collagenase is juxtaposed to the space between two successive collagen molecules in the staggered array in the native fibril. Whether this fact is significant with reference to the process of degradation is a matter of speculation, but certainly it would be expected that the ability of the enzyme to gain access to the point of cleavage of each collagen molecule is critical. In a tissue so close-packed as a collagen fibril, where adjacent molecules may be literally touching, the attack by an enzyme might well be limited by the accessibility of the site of cleavage.

The properties of human and animal tissue collagenases have been reviewed by Eisen et al. and by Seifter and Harper.[183,199]

HOMEOSTASIS AND THE COLLAGEN MOLECULE

In this survey of the properties of collagen, more attention has been paid to indications of specificity and reactivity than to physical chemistry because the aim of this review is to determine mechanisms of homeostasis that act upon the collagen molecule. It is clear that many of the reactions that collagen undergoes (e.g., cleavage of the registration peptides, fibrogenesis, the building of cross-links, and collagenase scission) are dictated by the precise primary sequence. On the other hand, the same primary sequence molecule builds connective tissue scaffolding with different structural organizations (e.g., tendon and dermis). How is such organization determined in the tissue?

All the biosynthetic processes that lead to the exocytosis of a native, decorated collagen molecule and the collagenase zymogen occur intracellularly. What directs the synthesis of the α chain or chains appropriate to the tissue from the several sequences encoded in the genome? And what constrains the activities of the hydroxylating and glycosylating enzymes? It is probable that the cellular environment, i.e., the surround of cells, proteoglycans, and other connective tissue constituents, directs the selection of the appropriate mRNA through controlling the level of metabolites, and possibly of specific messenger molecules that reach the cell. Thus, Layman et al. have reported that chondrocytes in organ culture produce cartilage $(\alpha 1)_3$ collagen, whereas in cell culture the product is $(\alpha 1)_2\alpha 2$ collagen.[200] Moreover, it is a common observation in tissue cultures that the preservation of particular cell clones, or

the differentiated character of individual cell types, depends upon the conditions of culture and the constituents of the culture medium (e.g., Holtzer et al.[201]).

It is also likely that the pattern of hydroxylation and glycosylation on the collagen molecules is directed by cellular metabolite levels – either by their invoking the biosynthesis of tissue-specific hydroxylating or glycosylating enzymes, or by their controlling (by allosteric mechanisms) the activity of enzymes that are common to all collagen-producing cells.

It is probable that collagen and collagenase synthesis are brought about by hormones or other chemical stimuli released in the course of growth induction or in response to wounding, for example. Many examples of direct hormonal influence on the connective tissues are known: the activation of the cells in cartilage in response to growth hormone; the uterus to estrogens; the cockscomb and the sexual skin of monkeys to androgens. Recently an effect of prostaglandins E_1 and $E_{1\alpha}$ on collagen synthesis was reported.[202]

The control of fiber formation or degradation subsequent to the secretion of collagen or collagenase involves the operation of homeostatic mechanisms about which we know little, but which merit discussion and speculation. Clearly, in seeking an explanation for tissue morphology through interactions between cells and extracellular constituents, we are following the observations, hypotheses, and experiments of generations of developmental biologists, and it is unlikely that any models will be truly novel. For broader discussions and wider references, the reader is referred to articles by Weiss, Hay, and Revel, and Fitton-Jackson.[203-205] However, the task of understanding cellular control over extracellular structures might be somewhat simpler than understanding cell-to-cell interaction. Moreover, our expanding understanding of molecular biology makes it a challenge to speculate on mechanisms that might apply to the special array of macromolecules in connective tissue.

To take some specific examples, bone growth at the epiphysis demands cartilage collagen resorption and bone deposition; and these processes involve the activities of chondrocytes, osteoclasts, and osteoblasts. Degradation of collagen in the resorbing tadpole tail calls for collagenase from the epithelium and hyaluronidase from the mesenchyme. In each case the cells secrete proteins – and probably other products – into the connective tissue, the matrix of which was synthesized by these or other cells. Subsequent events depend upon diffusion and the built-in reactivities of the proteins as they can be expressed in the presence of the proteoglycans and other matrix constituents.

What steps and control mechanisms appear necessary to direct the synthesis and degradation of an extracellular collagen fibril? To take the last topic first, it seems that appropriate cells respond to chemical stimuli to secrete a collagenase zymogen into the connective tissue. Examples of hormonal control include the resorption of the tadpole tail following thyroxine injection and the block of rat post partum uterine resorption following progesterone administration.[206] The collagenase zymogen is probably synthesized as required and not stored in the cells, because its appearance is blocked by puromycin.

The distribution of the secreted zymogen is apparently effected by diffusion, but its proteolytic activation is probably controlled locally, possibly by a site-specific tissue interaction that may involve a chain of processes akin to fibrinogen activation. Activation may invoke a specific interaction with a particular class of fibrils to bring about their breakdown selectively. Such a mechanism would appear to be necessary in view of the broad activity of some collagenases. It might have been anticipated that the remodeling that accompanies the conversion of cartilage to bone at the epiphysis, for example, would be facilitated by an enzyme that discriminated between bone and cartilage collagen. The demonstrated pluripotency of some enzymes (Figure 6) appears to demand either a selective and local activation, as discussed above, or else a highly constrained activity. Possibly the tissue digestion occurring in the course of replacing cartilage by bone is brought about only in the immediate proximity of the osteoclast, and the diffusion of the enzyme activity is contained by the inhibitory action of α1-antitrypsin or α2-macroglobulins among the serum proteins in the tissues. Fiber degradation probably does not require the digestion of the constituent macromolecules to small peptides since macrophages are capable of engulfing large chain fragments and presumably aid catabolism in many circumstances.

The feedback mechanisms that maintain collagenase synthesis until the appropriate substrate is destroyed are unknown, but it is feasible that the degraded chain fragments act as inducers to the secreting cells. These speculations on collagen catabolism are no more than extrapolations of well-studied enzymic mechanisms to the extracellular situation, although there is no explanation, at present, for the strikingly limited attack on the collagen molecule by collagenase.

In speculating on the mechanisms of fibrillogenesis, we must account for the fact that dispositions, density, and diameters of collagen fibrils are different in various tissues. How can the fibril morphology and organization be directed at a distance from the cell? The mechanisms that come immediately to mind involve either a specific collagen-to-collagen or collagen-to-ground substance interaction. In this context it seems probable that the reactivity of the collagen molecules dispersed in the arrays of proteoglycans and other constituents in the extracellular matrix will vary if the levels of hydroxylation and glycosylation are changed. It seems plausible, therefore, that the tissue specificity of collagen hydroxylation and glycosylation discussed earlier is purposive and not accidental.

The possibility that the pattern of substitution on the collagen molecule has high significance raises the question of the control of collagen exocytosis, which clearly is tied to hydroxylation and possibly glycosylation. The earlier discussion could not resolve which process, proline and lysine modification or chain folding, is the final key to open the collagen-filled vesicle to the extracellular medium. It does not appear likely that the cell has a screening mechanism (like that effecting DNA repair) to destroy inappropriately modified chains; protocollagen is retained, not catabolized. It seems more likely that one final step, possibly helix formation, is coupled to the completion of the modification program, and normal exocytosis follows only after the final step. Such a mechanism – based, for example, on the activity of lysyl hydroxylase, the proline hydroxylases or the glycosyl transferases – might involve the physical movement of the enzyme along the chains, the enzyme's presence prohibiting folding until its action is complete.

From this viewpoint we may ask how specific and significant is the pattern of modifying substituents along the chain? How much of the microheterogeneity is random, or what error is tolerable? Unless the battery of enzymes involved is cell- or tissue-specific (like the methylating enzymes that modify bacterial DNA), the different levels of substitution imposed by cells in different tissues presumably reflect allosteric interactions of metabolites with the enzymes or the collagen chains. How stringent is this control? The error rates in DNA replication and protein synthesis appear to be about 10^{-6} but these are template-directed processes. We may expect in the level of hydroxylation of any one proline or lysine residue variations significantly greater than 10^{-6} to result solely from imperfections in the hydroxylating system: nevertheless, the analytical data from any one tissue collagen show a high consistency, while the same analyses (summarized below) clearly differentiate the hydroxylation level in collagens from different tissues. That such differences exist between tissues shows that hydroxylation is not random; but such differences do not demonstrate that the decoration is purposive. We must examine the molecules in greater detail.

Gribble et al. showed that in rapidly proliferating fibroblasts in vitro, the collagen that is made first is hydroxylated to a low extent.[207] A trivial explanation in this situation where biosynthesis is proceeding very rapidly would be that there simply is not time for the newly synthesized molecule to become fully hydroxylated before it is excreted, or perhaps that there is an inadequately stocked commissary to meet the demands of the cell. Similar investigations by Bates et al. compared the hydroxylation of proline in collagen synthesized in vitro by 3T6 fibroblasts in logarithmic and stationary culture with and without ascorbate.[208] The level of hydroxylation varied with growth and ascorbate concentrations, leading to the conclusion that the hydroxylation process is responsive to various extracellular influences. Furthermore, while the hydroxylation level of proline may vary considerably before a block on secretion is invoked, the procollagen molecules differentiated by various extents of hydroxylation are subjected to cleavage at different rates by procollagen peptidase.[209] Similar studies on the effects of growth rate and ascorbate on hydroxylation have been reported by Peterkofsky.[210] These experiments clearly do not rule out the likelihood that the hydroxylation pattern is a significant determinant of the extracellular career of the collagen molecule, but they raise questions about

the mechanisms that dictate the pattern and control exocytosis.

Evidence for the precise allocation of hydroxyl groups comes from many studies. The work of Bornstein, quoted earlier, demonstrated consistent differences between rat skin and tendon in a region of the α1 chain (α1-CB2),[50] yet the amino acid sequence of these peptides from the two tissues appears to be the same. Butler has commented on specific differences between the levels of hydroxylysine in specific peptides from rat skin and dentine collagen.[211] Similarly, Miller et al.[212] have shown that the lysyl residue in chick bone α1-CB1 peptide is 50% hydroxylated, whereas Kang et al.[213] found no hydroxylysine in the same peptide in chick skin. The obvious example of a precisely limited hydroxylation step is the action of the enzyme that hydroxylates the single 3-hydroxyproline residue detected in calf skin and other α1 chains (e.g., Rauterberg and Kühn[214]). In contrast, basement membrane collagens contain far more 3-hydroxyproline – but the location of these residues is not known.[90] Barnes and his co-workers pointed out specific complements of hydroxylysine in N-terminal telopeptides from various chick and rat tissues;[215,216] they also observed that the levels of hydroxylation differed between very young and maturing skin.

The levels of hydroxylysine in the telopeptides have obvious functional significance. Several workers have noted that the hard tissues, bone and dentine, are rich in the cross-link dehydrodihydroxylysinonorleucine. Levene et al. related the increased solubility of collagen synthesized in vitro under ascorbate insufficiency to the lowered content of this cross-link and an increase in dehydro-lysinonorleucine (presumably a weaker bond).[217a]

The stronger bond requires the hydroxylation of telopeptide as well as appropriately sited main chain lysines, so we are led to conclude that the level of hydroxylation of an excreted molecule is a major factor in determining its cross-linking characteristics. Clearly, also, glycosylation of an otherwise available hydroxylysine residue will interfere with cross-linking. We also may deduce that certain of the properties of the hard tissues, for example, are determined by the pattern of cross-linking. Thus Katz and Li have reported a pronounced difference between the lateral packing of soft compared to hard tissue fibrils;[218] the close packing of the molecules in the soft tissue fibrils precludes the penetration of phosphate ions. While these conclusions are controversial, the argument illustrates the point that cross-linking, and possibly glycosylation, may determine or limit fibrillar function or interaction with other tissue constituents.

It may be noted parenthetically that while it is suggested than any particular cross-link is reported to be more stable than other Schiff bases, this stability is measured with respect to dilute acid hydrolysis, which is not a measure of bond strength in vivo. Indeed, we have no way of determining the bond strength or efficacy of any cross-links in vivo, whether these bonds are simple or complex aldehyde adducts, native or after in vivo reduction. Most assessments and age comparisons of bond character have measured solubility, a reflection only of bond hydrolysis under nonphysiologic conditions of pH and ionic strength. Direct measurements of tensile strength on tissues such as tendons in the native or heat shrunk state reflect not only changes in the cross-link population, but also changes in hydration, crystallinity, and in the ground substance.

To return to the main argument, we may conclude from the consistency of the hydroxylation pattern in different tissues that hydroxylation is a significant and controlled process. The same conclusion pertains to glycosylation. Spiro, Schofield, et al., Pinnell et al., and Morgan et al. have shown that the levels of glycosylation in collagens from various tissues or from fractions from one tissue may be readily discriminated.[68,69,218a,219] Again, the consistency of these differences that permits their analysis is evidence that these modifications are not haphazard.

The heterogeneity of modification demonstrable in collagen extracted from a single tissue (shown directly by Spiro[69] on successive soluble fractions, or implied by finding less than 100% hydroxylation on certain proline residues in the sequence studies) probably is the explanation for the chromatographic heterogeneity detected in soluble collagen by Kawasaki and Bernardi and Trelstad et al.[220,221] Undoubtedly, tissues such as the dermis contain populations of fibrils that are morphologically as distinguishable as populations in different tissues. Possibly different populations of fibers contain molecules with different levels of hydroxylation and glycosylation. On the other hand, these different molecules might come from

single fibrils, and the different pattern of substitution might reflect a meaningful change in the reactivity of the molecules along or through the fibril.

In summary, this discussion has provided sufficient evidence that the post-synthetic changes on the collagen molecule are controlled and determined that we may look for a purposive explanation of their presence. We already have discussed how decoration of the molecule can change its packing and cross-linking character; it also is possible that differences in the surface could lower the tendency of a collagen molecule to aggregate laterally and instead promote head-to-tail overlapping polymerization. In addition, it is probable that changes in the distribution of hydroxyl groups along the surface affect the balance of the collagen-to-collagen and collagen-to-matrix interactions. As a result, we may anticipate that, in an appropriate matrix, changes in collagen decoration might be the basis for the discrimination of those molecules destined to accrete to an existing fibril and those nucleating new fibrils in the course of fibrillogenesis.

A particularly challenging problem to consider is the establishment of the chick primary corneal stroma by the cells of the corneal epithelium. In the embryonic chick eye, the epithelial layer secretes successive layers of parallel fibers of uniform diameter;[204] each layer is orthogonally arranged;[222] and the relative orientations of the successive layers are defined with such precision that it is possible to detect a superimposed rotation upon the orthogonal arrangement.[223] The transparency of the cornea depends upon the fiber size and uniformity.[224] In many tissues it is not known how far from the secreting cell fibrillogenesis occurs, but in this instance the organization of the fibrils takes place beyond the basement membrane, at least 20 to 30 nm from the epithelium. How is order imposed on the assembly of macromolecules diffusing across this distance? The problem may be separated into control of fibril size and control of orientation.

Presumably the building of successive orthogonal lamellae demands some interruption in the process of collagen synthesis, and a plausible working hypothesis would be that the punctuation is effected by a change in the level of hydroxylation and glycosylation, and possibly a brief cessation in biosynthesis. Such a change in the surfaces of the molecules might stop them from accreting to the fibrils in the nearest layer and instead initiate the synthesis of a new layer. If, through this period, the secretion of proteoglycans displaced the completed fibril layer further away from the cell surface, then a later shift in the secretion product back to the conventional collagen molecules would promote accretion and allow the new fibrils to be built up, while the greater diffusion distance would limit further growth of the fibrils in the preceding layer.

While largely speculative, this idea is suggested by the following observations: a) reticulin fibers, which appear to be built from heavily glycosylated collagen molecules, are thought to be precursors of fibrils; b) electron microscopy has shown that collagen fibrils have a narrow core that stains with ruthenium red,[225] a reagent selective for glycoproteins.[226] This observation may indicate that heavily glycosylated collagen, or collagen and an associated glycoprotein component, initiate fibril formation; and, c) the fibrils of basement membrane collagen that is heavily glycosylated remain very thin and never grow to demonstrate the normal collagen banding pattern.[248]

Some relationship of glycosylation to the packing, and possibly to fibrogenesis, of the collagen molecules may also be inferred from the fact that the sole carbohydrate constituent in rat skin collagen $\alpha 1$ chains (in cyanogen bromide peptide $\alpha 1$-CB4) is positioned precisely at the terminus of an adjacent molecule in the quarter-staggered array and in the gap between that molecule and the succeeding one.[226a] In view of the bulk of the carbohydrate residues, it is likely that a more extensive glycosylation would limit the lateral association of the molecules.

Other explanations should be considered to explain the control of fibril dimension. For example, the sclera, the tissue adjacent to the cornea, in marked contrast to the cornea itself, contains fibers of widely varying diameter. The mechanism of the control of growth of structures poses a problem in many fields: in the assembly of a bacteriophage tail from protein subunits, what limits its length if there is no internal template? One might discuss sensitive parameters reflecting solvation and surface to volume ratio, but at this stage our ignorance is so great that the discussion would probably be unrewarding.

The second part of the problem of the organization of the corneal stroma is even more challenging. What determines the course of a fibril? In

less ordered connective tissues, it is possible that a fiber is delineated by the migration of the secreting fibroblast, but this process cannot occur in the primary stroma of the cornea. The relative rotation of successive lamellae could imply some spatially directed interaction with the preceding lamella. This influence would presumably be relayed through the ground substance since the collagen fibrils are not apposed. A directive role in morphogenesis for the proteoglycans has been discussed.[227] There is also evidence for collagen interaction with glycoproteins and phosphoproteins.[11,228] Such interactions might involve ionic (e.g., Langham et al.[228a]) or covalent bonds; but the explanation of if and how an orienting field could be established in the ground substance through such associations must await further investigation. Some directive influence of ground substance on fibril morphology may be inferred from the fact that in certain disease states affecting the polysaccharides and lipids, there are also changes in the coarseness of collagen fibrils. Weiss has discussed other mechanisms for the establishment of orthogonal collagen lamellae.[203]

It must also be considered that the cell may directly influence fibril orientation. Trelstad has pointed out that elongated vesicles (which are postulated to carry the collagen to the cell surface) are oriented in parallel within the epithelial cell.[85] It would require some further mechanism to maintain this orientation beyond the basement membrane.

In summary, if the foregoing deductions are relevant and justified, we may conclude that the heterogeneity of the collagen molecules extracted from any one tissue does not reflect the vagaries of a hit-or-miss hydroxylation system that employs several enzymes, some of which may not have time to reach the molecule; instead, the heterogeneity might reflect homeostatic control over collagen reactivity to bring about the building of fibrils with a required size and reactivity.

These speculations suggest that the population of distinguishable molecules in a tissue should vary with different phases of growth and organization. Toole et al. recently reported experiments characterizing collagen changes in endochondral bone formation: they noted a change in hydroxylysine content and discussed possible functions relating to control of calcification.[229]

Our discussion has centered upon outgoing instructions from the cell embodied in the exported molecule. Homeostasis demands a feedback response from the connective tissue to the cells. The molecules that are involved in this reaction are not known, but a possible candidate is part of the excised registration peptide.

It is likely that some of the changes occurring in connective tissue are not cell directed. The process of calcification of the hard tissues is probably an automatic response of chemically specialized fibrils to a critically balanced ion level in the fluids. The tendency of many tissues to calcify with age may reflect a change in ion level, fibril characteristics, or the composition of the ground substance. The slow changes in cross-linking that occur over months (in contrast to the initial cross-links that are apparent within hours of collagen synthesis) probably reflect more a change in bond character and solvent lability than an increase in number. These changes probably follow from spontaneous physical (e.g., dehydration, crystallization) and chemical reactions (some possibly radiation induced) and may not be enzyme mediated or cell directed.[141]

Turnover

Homeostasis in the prokaryote appears to depend upon a process of continuous protein anabolism and catabolism, and the proteins that replace those that are catabolized are induced (or not repressed) to refit the cell for the structural and enzymic activities that are necessitated by the environment. In the differentiated organism, relatively few of the complement of cells must respond directly to the requirements of the environment, but many cellular populations will respond to endogenous requirements through hormonal or other intercellular messages. In contrast to the prokaryote, the eukaryotic cell and the extracellular compartments contain proteins with lifetimes ranging from minutes to months. A question particularly pertinent to our interest is What is the purpose and significance of turnover among extracellular proteins when remodeling does not appear to take place? The very long survival time of collagen highlights the question whether or not the degradation of proteins is a selective one to eliminate "old" proteins, and if it is, then by what criteria the old proteins are recognized by the degradative machinery. Kohn has discussed the point that proteins may become denatured with time and therefore are likely substrates for the catheptic activity that pre-

sumably is designed to destroy ineffective proteins.[230] However, the known long lifetimes of many proteins in vivo suggest that the instability of the proteins in solution may be an artifact of the in vitro conditions; and, indeed, the only likely cause for a time-dependent decrement in enzyme activity, or in other reactivity, might be expected to result from the slow, spontaneous elimination of amide groups from asparagine and glutamine. However, in experiments measuring turnover in collagen, it frequently is found that there is greater catabolism of more recently synthesized collagen than of old. It has been suggested that older fibrils might be more resistant to collagenase because they are more efficiently cross-linked; but in such tissues is turnover really concerned with the disposal of outworn molecules? It seems more likely that turnover in most connective tissues (excluding the situations of wounds or remodeling) reflects reorganization and consolidation among the categories of fibrils, changes that are reflected in the age-related coarsening of collagen fibrils. Fitton-Jackson has illustrated growth of tendon fibrils and a concomitant change in ratio between interstitial and fibril components.[205] It may be concluded that the individual collagen molecule once incorporated into a fibril is never discarded, and it will be destroyed only if the fiber itself becomes redundant. The lifetimes of the polysaccharide moieties of the proteoglycans (and therefore, presumably, the whole proteoglycan molecule) are shorter than that of collagen.

The Phylogenetic Stability of Collagen

In the discussion of the process of fibrillogenesis, it was proposed that the precise pattern of decoration of proline and lysine residues in the collagen molecule is highly significant. It appears likely that the demand for a stringent control on the action of intra- and extracellular modifying enzymes on collagen will preclude any deviation in primary sequence of the collagen chain, at least over operationally important regions. From this viewpoint the remarkable phylogenetic stability of collagen over the greater part of its length may imply that the molecule serves other critical functions of which we are not presently aware.

Several groups have remarked that phosphotungstic acid and uranyl-stained SLS segments of collagens from animals of many phyla are almost indistinguishable.[43,231,232] These observations have covered a range from sea anemones through birds and fish to man. Chemical studies suggested that the positive staining (i.e., the stain retained by the specimen after washing with water) indicates the locus of lysine and arginine residues that bind the phosphotungstate stain, or of acidic residues that bind the uranyl ion. Therefore, the consistency of the staining diagram through these phyla indicates the preservation of the distribution of the charged residues along the collagen molecules.

The recent sequencing studies on the collagen $\alpha 1$ chain permit a comparison of the distribution of the charged residues along the chain with the PTA staining pattern. In an unpublished study we have mapped colinearly the distribution of residues with the reference band pattern published by Bruns and Gross.[138] A remarkably good correlation is observed. The $\alpha 2$ chain has not yet been sequenced, but since triple helices built from three $\alpha 2$ chains or three $\alpha 1$ chains annealed together give SLS segments that stain almost identically, it is probable that the charged residues occupy the same relative positions in both chains.[36] On this assumption it appears that certain bands reveal the position of only one or two close charged residues per α chain, i.e., 3 to 6 residues in register across the triple helix of each molecule. Therefore, the near identity of an enormous range of collagen molecules – invertebrates to man – implies a high resistance to mutational drift.

More detailed sequence work on peptides from $\alpha 1$ and $\alpha 2$ chains has shown that very few amino acid substitutions have occurred even among the nonpolar residues between chick, rat, calf, and human collagen (see References 9 and 23). Clark and Bornstein have noted a homology between chick and guinea pig skin collagen sequences, although the methionine groups in the latter molecule are not in homologous positions and the cyanogen bromide peptides, therefore, are not directly comparable.[233] Piez et al. studied the internal similarity between $\alpha 1$ and $\alpha 2$ chains of rat skin collagen by a computer search program.[234] Striking evidence of a homology in position and sequence was found. This finding, together with the similarity in SLS staining, suggests strongly that the two chains arose by gene duplication and have diverged only slightly in composition since that time.

Several laboratories have noted that lamprey skin collagen comprises $(\alpha 1)_3$ type (e.g., Bailey[194]

and Cannon, D., unpublished data, 1972), whereas the shark has $((\alpha 1)_2 \alpha 2)$ structure.[235] This observation suggests that gene duplication arose during approximately the evolutionary period of the elasmobranch fish.

The $\alpha 1$(II) chain that comprises chick cartilage collagen yields a pattern of cyanogen bromide peptides different from the $\alpha 1$(I) chain;[29,236] however, the work quoted above by Clark and Bornstein has shown that a novel distribution of methionine residues in a chain may conceal a significant homology, and Miller et al. have shown some consistent methionine locations.[157,233] The resemblance between cartilage and skin collagens is also shown by the similar (but distinguishable) SLS staining pattern.[236,237]

This similarity between skin and cartilage collagen suggests that here, too, gene duplication and limited divergence have produced a similar but distinguishable collagen molecule. It is not known how many representations of the collagen α chains are present in the mammalian genome, but as we have discussed, there are at least four.

Further evidence for the limited divergence in the composition of collagen chains in any one species and between species is provided by studies on collagenase susceptibility. The precise structure that enables the enzyme to cleave the molecule is not yet recognized, but it appears to be well preserved. We have observed that the point of cleavage effected by corneal collagenase is identical in lamprey skin collagen, human tissues, rat and calf skin and tendon collagen, and the calf articular cartilage collagen. Therefore, the composition of the corresponding regions of the main chains has not changed enough to eliminate the definition of this cleavage point.

Immunologic studies on the tissue collagenases by Bauer et al. have shown that baboon collagenase shows considerable cross-reaction with antiserum to human enzyme but that other mammalian species cross-react far less.[180] The factors resisting genetic drift in the collagenase molecule are clearly less effective than those that preserve the structure of collagen. The only sites of species specificity detected immunologically on the collagen molecule are the telopeptides.

DISEASES AND DISORDERS OF CONNECTIVE TISSUE

This discussion of homeostasis in connective tissue has been focused on collagen because our knowledge of the structure and turnover of the various proteoglycans and other components is too meager to justify much speculation. If we can define, however, even in a broad manner, the way that homeostatic processes build distinguishable connective tissues from a limited number of components subjected to extracellular enzyme modification and organization, then we may expect to achieve an improved understanding of some of the many toxic, disease, and heredity conditions that are manifest in connective tissue defects. The findings discussed in this article have shown that in a wide variety of connective tissues, in essentially every organ of the body, the same components are ordered in locally specific organizations to serve local tissue requirements. It is to be expected, therefore, that any disorder of the connective tissues will be a widely manifest disorder and that it may have its origin at any one of several levels. For example, a genetic disturbance to collagen function could reflect: a) a mutation in the primary sequence of one of the collagen chains; b) a mutation in one of the modifying enzymes or a change in the cellular concentration of one of the enzyme cofactors; or c) a change in the enzymes or extracellular tissue components that may respond to the special structures of the collagen molecule. Obviously, therefore, the identification of a particular lesion may require careful examination of the connective tissue components.

A genetic lesion in factors controlling synthesis or reactivity of ground substance components might show as widespread morphological and functional effects as factors affecting collagen. A study of such disorders would aid in understanding the inductive interaction between the fibrillar constituents and ground substance of connective tissues. Matalon et al. have reported recently that the basic defect in Hurler's syndrome is a deficiency in iduronidase.[238] Such a defect would satisfactorily explain the etiology of several of the diseases of connective tissues. Investigations of such conditions hold great promise for an understanding of normal connective tissue function. This approach is discussed by Waldenstrom and others.[239]

A simple explanation has been arrived at for the condition known as lathyrism. Classically, lathyrism developed in an animal fed or treated with β-aminopropionitrile or its derivatives. In this

condition the animals showed distorted bones, and weakened skin and blood vessels. A similar syndrome is brought about by injections with penicillamine or by maintaining the animals on a copper-deficient diet. All of these observations, it now appears, can be explained by the fact that the enzyme lysyl oxidase is a copper-containing enzyme that is irreversibly inhibited by β-aminopropionitrile.[139,240] The weakness of the collagen-containing tissues in such treated animals is good evidence that the strength of a collagen fibril depends far more upon intermolecular cross-links than upon the electrostatic and solvation forces that drive the molecule into the quarter-staggered array in the fibril.

Puleo and Sobel observed fluorescent changes in vivo in the skin of mice in hyperbaric oxygen, and they correlated these changes with cross-linking changes in collagen held under high oxygen pressures in vitro.[169] They speculate that aseptic neurosis on bone observed among diving personnel might relate to tissue responses brought about by oxidative changes in connective tissue, possibly involving cross-linking.

Great interest was aroused very recently by the discovery and description of a disease clinically similar to the Ehlers-Danlos syndrome, as was mentioned previously. In this condition, which has been referred to as a hydroxylysine-deficient disease, hyperextensible skin and scoliosis were seen.[241] In this case reduction of the collagenous tissues with borohydride and analysis for cross-linking compounds demonstrated an excess of allysine adducts and a large deficiency of hydroxyallysine adducts in skin and tendon, but a far smaller deficit in bone and cartilage.[241a] Further examination showed that skin was low in lysine hydroxylase activity.[96] In this instance it is not so easy to pinpoint the primary defect. Clearly, the enzyme lysyl hydroxylase is present in the tissues, but in skin it is acitve at only 10 to 20% of the normal level. Its diminished activity reflects one of the following: a breakdown in the homeostatic controls that call forth the biosynthesis of the enzyme; a mutation in the enzyme so that it hydroxylates some collagens but not others; or an unusually rapid rate of catabolism of the enzyme itself in the defective tissues, in what could be a manifestation of another aberrant control mechanism.

Mechanic recently used the approach outlined in the study of the "hydroxylysine-deficient disease" to screen a number of other Ehlers-Danlos syndrome patients.[242] One of these showed a deficiency in hydroxylysine. Studies such as these may enable the primary or secondary defects leading to disease to be more rapidly pinpointed and the diseases to be more meaningfully categorized.

Turning again to the report that the primary defect in Hurler's syndrome is the lack of iduronidase activity, we are left with an unanswered question: whether the enzyme is present but inactive (or of low activity), or whether it is not synthesized by the cell either because there is a defect in the gene coding for the enzyme, or because the controlling mechanism calling forth its synthesis is inoperative.

Eisen has reported an unusually high level of collagenase in skin of individuals suffering from dystrophic epidermolysis bullosa.[243] The basic genetic defect – whether lack or inactivity of inhibitors; abnormal enzyme; or ineffective repressor, for example – that causes the elevated enzyme levels has not been identified. Enhanced collagenase levels in several tissues suffering degenerative changes are known: middle ear,[244] ulcerating cornea,[176,192] and rheumatic joints.[177] Although the pathological processes correspond well in most cases to the collagenase level, the cause for the elevated collagenolytic activity has not yet been determined.

One feature shared by a variety of "collagen diseases" is an increase in salt-soluble collagen in the tissues.[245] This observation presumably indicates either a general interference in the process of cross-linking or an increased rate of biosynthesis. An increased biosynthetic rate would also entail more rapid catabolism. If there is a pattern of increased turnover in pathological tissues, we may conclude that homeostasis is disturbed in addition to whatever prime defect is present.[245a]

The explanation for pathological states involving abnormal calcification may not yet be within the reach of our understanding. From the manner in which calcium can be deposited and dissolved from bones under hormonal influences, it is apparent that the equilibrium governing the deposition of calcium apatite from soluble calcium salts in the tissue fluids is a complex one. It appears that in general the calcifying tissues such as bone and dentine are marked out for that function from the time of synthesis, but some

other tissues can and do normally become calcified with time; such a tissue is the cartilage of the aorta. What changes are responsible for the further appearance of calcium in disseminated tissues in a variety of pathological conditions is not known.

Two veterinary diseases have been described recently that appear to correspond to interruptions in the process of cleaving the registration peptide. The first example of this situation was noted among Belgian cattle, and the condition was termed dermatosparaxis.[246] The collagen fibrils in the tissues of the affected animal were thin and wavy although normally banded. The skin was weak. The abnormality relates to the extra amino-terminal appendage that can be seen in the molecule in SLS crystallites.[49] Whether this situation corresponds to the loss of the pro-collagen peptidase that should have cleaved these peptides from the molecule, or whether this is evidence for mutation within the peptide that diminishes susceptibility to the protease, is not clear. The fibrils themselves apparently are weakened in the dermatosparaxic animals, and this fact is possible evidence for the inhibition of lysyl oxidase activity by these peptides. Very recently a similar condition was reported among sheep in Norway.

The views and postulates expressed in this article are of value only insofar as they generate new ideas or experiments to test them. Thus, the speculation relating control of fibrillogenesis to hydroxylation and glycosylation suggests that the defects in scurvy relate not only to the slowing of collagen excretion (because hydroxylation is retarded), but also to abnormal extracellular consequences for the secreted molecules. The tissues affected by scurvy are presumably those active in collagen anabolism, particularly in wounds. Most tissues in which the fibrils are long-lived appear little changed by ascorbate depletion. On the other hand, collagen secretion is also slowed in starvation, but without the attendant disorders of connective tissue. The recognition of the implications of extracellular homeostasis means that the origins of genetic defects must be considered carefully and fully. The "hydroxylysine-deficient collagen disease" is in fact a disease of local hydroxylysine deficiency. The enzyme appears to be appropriately effective in cartilage so the inherent error is not as simple as a missing enzyme.

The diversity of the diseases of connective tissue, and the shading of their symptoms from one condition to another, reflect the fact that in a homeostatic system of high complexity a breakdown in one component can cause changes in many; in an elucidation of the cause and character of the disease, the fact that the connective tissues are normally in homeostasis (albeit showing a drift with aging) is a factor that cannot be neglected. An understanding of the homeostatic mechanisms might come through the use of selective chemical agents to interfere with biosynthesis (e.g., Coulombre and Coulombre[86]), but these investigations might be importantly supplemented by the study of mutant organisms. Such studies on clinical hereditary diseases or veterinary diseases might permit the discrimination of steps in extracellular homeostasis with a precision that chemical interference cannot offer.[247]

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REFERENCES

1. **Jacob, F. and Monod, J.,** Genetic regulatory mechanisms in the synthesis of proteins, *J. Mol. Biol.*, 3, 318, 1961.
2. **Umbarger, H. E.,** Feedback control by endproduct inhibition, *Cold Spring Harbor Symp. Quant. Biol.*, 26, 301, 1961.
3. **Monod, J., Changeux, J. P., and Jacob, F.,** Allosteric proteins and cellular control systems, *J. Mol. Biol.*, 6, 306, 1963.
4. **Wilkins, M. H. F.,** X-ray diffraction studies of the molecular configuration of nucleic acids, in *Aspects of Protein Structure*, Ramachandran, G. N., Ed., Academic Press, London, 1963, 23.
5. **Johnson, L. N. and Phillips, D. C.,** Structure of some crystalline lysozome-inhibitor complexes determined by x-ray analysis at 6A resolution, *Nature (Lond.)*, 206, 761, 1965.
6. **Smith, D. W., Weissman, N., and Carnes, W. H.,** Cardiovascular studies on copper-deficient swine. XII. Partial purification of a soluble protein resembling elastin, *Biochem. Biophys. Res. Commun.*, 31, 309, 1968.
7. **Sandberg, L. B., Weissman, N., and Gray, W. R.,** Structural features of tropoelastin related to the sites of cross-links in aortic elastin, *Biochemistry*, 10, 52, 1971.
8. **Franzblau, C. and Lent, R. W.,** Studies on the chemistry of elastin, *Brookhaven Symp. Biol.*, 11, 358, 1968.
9. **Gallop, P. M., Blumenfeld, O. O., and Seifter, S.,** Structure and metabolism of connective tissue proteins, *Annu. Rev. Biochem.*, 41, 617, 1972.

9a. **Gersh, I. and Catchpole, H. R.,** The nature of ground substance of connective tissue, *Perspect. Biol. Med.*, 3, 282, 1960.

10. **Hascall, V. C. and Sajdera, S. W.,** Proteinpolysaccharide complex from bovine nasal cartilage: The function of glycoprotein in the formation of aggregates, *J. Biol. Chem.*, 244, 2384, 1969.
11. **Anderson, J. C. and Jackson, D. S.,** The glycoproteins of tendon and their interaction with collagen, *Biochem. J.*, 128, 122P, 1972.
12. **Schubert, M. and Hamerman, D.,** *A Primer on Connective Tissue Biochemistry*, Lea & Febiger, Philadelphia, 1968, 53.
13. **Spiro, R. G.,** Glycoproteins: Their biochemistry, biology and role in human disease, *N. Engl. J. Med.*, 281, 991 and 1043, 1969.
14. **Rodén, L.,** Biosynthesis of acidic glycosaminoglycans, in *Metabolic Conjugation and Metabolic Hydrolysis*, Vol. 2, Fishman, W. H., Ed., Academic Press, New York, 1970.
15. **Kobayashi, S.,** Acid mucopolysaccharides in calcified tissues, *Int. Rev. Cytol.*, 30, 257, 1971.
16. **Kefalides, N. A.,** Chemical properties of basement membranes, *Int. Rev. Exp. Pathol.*, 10, 1, 1970.
17. **Bhatnagar, R. S. and Prockop, D. J.,** Dissociation of the synthesis of sulfated micropolysaccharides and the synthesis of collagen in embryonic cartilage, *Biochim. Biophys. Acta*, 130, 383, 1966.
18. **Dorfman, A.,** Differential function of connective tissue cells, in *Chemistry and Molecular Biology of the Intercellular Matrix*, Vol. 3, Balazs, E. A., Ed., Academic Press, New York, 1970, 1421.
19. **Meyer, K.,** Introduction: Mucopolysaccharides, *Fed. Proc.*, 25, 1032, 1966.
20. **Mellman, W. J., Schimke, R. T., and Hayflick, A. L.,** Catalase turnover in human diploid cell cultures, *Exp. Cell. Res.*, 73, 399, 1972.
21. **Ramachandran, G. N.,** Structure of collagen at the molecular level, *Treatise on Collagen*, Vol. 1, Ramachandran, G. N., Ed., Academic Press, London and New York, 1967, 103.
22. **Gould, B. S.,** Collagen biosynthesis, in *Treatise on Collagen*, Vol. 2, Gould, B. S., Ed., Academic Press, London and New York, 1968, 139.
23. **Traub, W. and Piez, K. A.,** The chemistry and structure of collagen, in *Advances in Protein Chemistry*, Vol. 25, Anfinsen, C. B., Jr., Edsall, J. T., and Richards, F. M., Eds., Academic Press, New York, 1971, 243.
24. **Gross, J., Highberger, J. H., and Schmitt, F. O.,** Collagen structures considered as states of aggregation of a kinetic unit. The tropocollagen particle, *Proc. Natl. Acad. Sci. USA*, 40, 679, 1954.
25. **Kühn, K., Engel, J., Zimmermann, B., and Grassmann, W.,** Renaturation of soluble collagen. III. Reorganization of native collagen molecules from completely separated units, *Arch. Biochem. Biophys.*, 105, 387, 1965.
26. **Beier, G. and Engel, J.,** The renaturation of soluble collagen. Products formed at different temperatures, *Biochemistry*, 5, 2744, 1966.
27. **Piez, K. A., Lewis, M. S., Martin, G. R., and Gross, J.,** Subunits of the collagen molecule, *Biochim. Biophys. Acta*, 53, 596, 1961.
28. **Nold, J. G., Kand, A. H., and Gross, J.,** Collagen molecules: Distribution of alpha chains, *Science*, 170, 1096, 1970.
29. **Miller, E. J. and Matukas, V. J.,** Chick cartilage collagen: A new type of α1 chain not present in bone or skin of the species, *Proc. Natl. Acad. Sci. USA*, 64, 1264, 1969.
30. **Miller, E. J.,** Isolation and characterization of a collagen from chick cartilage containing three identical α-chains, *Biochemistry*, 10, 1652, 1971.
31. **Kefalides, N. A.,** Isolation of a collagen from basement membranes containing three identical α-chains, *Biochem. Biophys. Res. Commun.*, 45, 226, 1971.
32. **Miller, E. J., Epstein, E. H., Jr., and Piez, K. A.,** Identification of three genetically distinct collagens by cyanogen bromide cleavage of insoluble human skin and cartilage collagen, *Biochem. Biophys. Res. Commun.*, 42, 1024, 1971.

33. **Miller, E. J.,** Isolation and characterization of the cyanogen bromide peptides from the α1(II) chain of chick cartilage collagen, *Biochemistry*, 10, 3030, 1971.
34. **Miller, E. J.,** Structural studies on cartilage collagen employing limited cleavage and solubilization with pepsin, *Biochemistry*, 11, 4903, 1972.
35. **Kefalides, N. A.,** Isolation and characterization of cyanogen bromide peptides from basement membrane collagen, *Biochem. Biophys. Res. Commun.*, 47, 1151, 1972.
36. **Tkocz, C. and Kuhn, K.,** The formation of triple-helical collagen molecules from α1 or α2 polypeptide chains, *Eur. J. Biochem.*, 7, 454, 1969.
37. **Davison, P. F. and Drake, M. P.,** The physical characterization of monomeric tropocollagen, *Biochemistry*, 5, 313, 1966.
38. **Ananthanarayanan, S. and Veis, A.,** The molecular parameters of monomeric and acid-soluble collagens. Low shear gradient viscosity and electric birefringence, *Biopolymers*, 11, 1365, 1972.
39. **Nishihara, T. and Miyata, T.,** Transactions of the 3rd Collagen Symposium of the Collagen Research Society, Japan, 1962, 84.
40. **Rubin, A. L., Drake, M. P., Davison, P. F., Pfahl, D., Speakman, P. T., and Schmitt, F. O.,** Effects of pepsin treatment on the interaction properties of tropocollagen macromolecules, *Biochemistry*, 4, 181, 1965.
41. **Drake, M. P., Davison, P. F., Bump, S., and Schmitt, F. O.,** Action of proteolytic enzymes on tropocollagen and insoluble collagen, *Biochemistry*, 5, 301, 1966.
42. **Schmitt, F. O.,** Biochemical dynamics of tropocollagen, *Fed. Proc.*, 23, 618, 1964.
43. **Davison, P. F., Levine, L., Drake, M. P., Rubin, A., and Bump, S.,** The serologic specificity of tropocollagen telopeptides, *J. Exp. Med.*, 126, 331, 1967.
44. **Timpl, R., Fietzek, P. P., Furthmayr, H., Meigel, W., and Kuhn, K.,** Evidence for two antigenic determinants in the C-terminal region of rat skin collagen, *FEBS Lett.*, 9, 11, 1970.
45. **Becker, U., Timpl, R., and Kuhn, K.,** Carboxyterminal antigenic determinants of collagen from calf skin, *Eur. J. Biochem.*, 28, 221, 1972.
46. **Furthmayr, H. and Timpl, R.,** Structural requirements of antigenic determinants in the aminoterminal region of the rat collagen α2 chain, *Biochem. Biophys. Res. Commun.*, 47, 944, 1972.
47. **Rauterberg, J., Timpl, R., and Furthmayr, H.,** Structural characterization of N-terminal antigenic determinants of calf and human collagen, *Eur. J. Biochem.*, 27, 231, 1972.
48. **Timpl, R., Furthmayr, H., and Beil, W.,** Maturation of the immune response to soluble rat collagen: Late appearance of antibodies directed to N-terminal sites of the α2-chain, *J. Immunol.*, 108, 119, 1972.
49. **Stark, M., Lenaers, A., Lapière, C., and Kuhn, K.,** Electronoptical studies of procollagen from the skin of dermatosparaxic calves, *FEBS Lett.*, 18, 225, 1971.
50. **Bornstein, P.,** Comparative sequence studies of rat skin and tendon collagen. II. The absence of a short sequence at the amino terminus of the skin α1 chain, *Biochemistry*, 8, 63, 1969.
51. **Eyre, D. R. and Glimcher, M. J.,** Evidence for a previously undetected sequence at the carboxyterminus of the α1 chain of chicken bone collagen, *Biochem. Biophys. Res. Commun.*, 48, 720, 1972.
52. **Hodge, A. J. and Schmitt, F. O.,** The carge profile of the tropocollagen macromolecule and the packing arrangement in native-type collagen fibrils, *Proc. Natl. Acad. Sci. USA*, 46, 187, 1960.
53. **Petruska, J. A. and Hodge, A. J.,** A subunit model for the tropocollagen macromolecule, *Proc. Natl. Acad. Sci. USA*, 51, 871, 1964.
54. **Gallop, P. M.,** Concerning some special structural features of the collagen molecule, *J. Biophys.*, 4, 79, 1964.
55. **Blumenfeld, O. O., Rojkind, M., and Gallop, P. M.,** Subunits of hydroxylamine-treated tropocollagen, *Biochemistry*, 4, 1780, 1965.
56. **Bornstein, P.,** The nature of a hydroxylamine-sensitive bond in collagen, *Biochem. Biophys. Res. Commun.*, 36, 957, 1969.
57. **Butler, W. T.,** The identity of a hydroxylamine-sensitive bond in the α1 chain of rat skin collagen, *J. Biol. Chem.*, 244, 3415, 1969.
58. **Vuust, J. and Piez, K. A.,** Biosynthesis of the chains of collagen studied by pulse-labeling in culture, *J. Biol. Chem.*, 245, 6201, 1970.
59. **Naughton, M. A. and Dintzis, H.,** Sequential biosynthesis of the peptide chains of hemoglobin, *Proc. Natl. Acad. Sci. USA*, 48, 1822, 1962.
60. **Goldberg, B. and Green, H.,** Collagen synthesis on polyribosomes of cultured mammalian fibroblasts, *J. Mol. Biol.*, 26, 1, 1967.
61. **Lazarides, E. and Lukens, L. N.,** Collagen polypeptides: Normal release from polysomes in the absence of proline hydroxylation, *Science*, 173, 723, 1971.
62. **Layman, D. L., McGoodwin, E. B., and Martin, G. R.,** The nature of the collagen synthesized by cultured human fibroblasts, *Proc. Natl. Acad. Sci. USA*, 68, 454, 1971.
63. **Lane, J. M. and Miller, E. J.,** Isolation and characterization of the peptides derived from the α chain of chick bone collagen after cyanogen bromide cleavage, *Biochemistry*, 8, 2134, 1969.
64. **Miller, R. L.,** Chromatographic separation of the enzymes required for hydroxylation of lysine and proline residues of protocollagen, *Arch. Biochem. Biophys.*, 147, 339, 1971.

65. Kivirikko, K. I., Kishida, Y., Sakakibara, S., and Prockop, D. J., Hydroxylation of $(X\text{-Pro-Gly})_n$ by protocollagen proline hydroxylase: Effect of chain length, helical conformation and amino acid sequence in the substrate, *Biochim. Biophys. Acta*, 271, 347, 1972.
66. Bornstein, P., Comparative sequence studies of rat skin and tendon collagen. I. Evidence for incomplete hydroxylation of individual prolyl residues in the normal proteins, *Biochemistry*, 6, 3082, 1967.
67. Rhoads, R. E., Undenfriend, S., and Bornstein, P., *In vitro* enzymatic hydroxylation of prolyl residues in the α1-CB2 fragment of rat collagen, *J. Biol. Chem.*, 246, 4138, 1971.
68. Pinnell, S. R., Fox, R., and Krane, S. M., Human collagens: Differences in glycosylated hydroxylysines in skin and bone, *Biochim. Biophys. Acta*, 229, 119, 1971.
69. Spiro, R. G., Characterization and quantitative determination of the hydroxylysine-linked carbohydrate units of several collagens, *J. Biol. Chem.*, 244, 602, 1969.
70. Katzman, R. L. and Kang, A. H., The presence of fucose, mannose, and glucosamine-containing heteropolysaccharide in collagen from the sea anemone *Metridium dianthus*, *J. Biol. Chem.*, 247, 5486, 1972.
71. Muir, L. and Lee, Y. C., Glycopeptides from earthworm cuticle collagen, *J. Biol. Chem.*, 245, 502, 1970.
72. Butler, W. T., Piez, K. A., and Bornstein, P., Isolation and characterization of the cyanogen bromide peptides from the α1 chain of rat skin collagen, *Biochemistry*, 6, 3771, 1967.
73. Kimura, S., Determination of the glycosylated hydroxylysines in several invertebrate collagens, *J. Biochem.*, 71, 367, 1972.
74. Prockop, D. J. and Juva, K., Synthesis of hydroxyproline *in vitro* by the hydroxylation of proline in a precursor of collagen, *Proc. Natl. Acad. Sci. USA*, 53, 661, 1965.
75. Bhatnagar, R. S., Kivirikko, K. I., and Prockop, D. J., Studies on the synthesis and intracellular accumulation of protocollagen in organ culture of embryonic cartilage, *Biochim. Biophys. Acta*, 154, 196, 1968.
76. Cooper, G. W. and Prockop, D. J., Intracellular accumulation of protocollagen and extrusion of collagen by embryonic cartilage cells, *J. Cell. Biol.*, 38, 523, 1968.
77. Margolis, R. L. and Lukens, L. N., The role of hydroxylation in the secretion of collagen by mouse fibroblasts in culture, *Arch. Biochem. Biophys.*, 147, 612, 1971.
78. Ramaley, P. B. and Rosenbloom, J., Inhibition of proline and lysine hydroxylation prevents normal extrusion of collagen by 3T6 fibroblasts in culture, *FEBS Lett.*, 15, 59, 1971.
79. Müller, P. K., McDoodwin, E., and Martin, R., Studies on protocollagen: Identification of a precursor of proto α1, *Biochem. Biophys. Res. Commun.*, 44, 110, 1971.
80. Kerwar, S. S., Kohn, L. D., Lapière, C. M., and Weissbach, H., *In vitro* synthesis of procollagen on polysomes, *Proc. Natl. Acad. Sci. USA*, 69, 2727, 1972.
81. Bhatnagar, R. S., Prockop, D. J., and Rosenbloom, J., Intracellular pool of unhydroxylated polypeptide precursors of collagen, *Science*, 158, 492, 1967.
81a. Lukens, L. N., The formation of collagen α chains in the absence of proline hydroxylation, *J. Biol. Chem.*, 245, 453, 1970.
82. Eylar, E. H., On the biological role of glycoproteins, *J. Theor. Biol.*, 10, 89, 1966.
83. Winterburn, P. J. and Phelps, C. F., The significance of glycosylated proteins, *Nature (Lond.)*, 236, 147, 1972.
84. Kikuchi, Y., Fujimoto, D., and Tamiya, N., The enzymic hydroxylation of protocollagen models, *Biochem. J.*, 115, 569, 1969.
85. Trelstad, R. L., Vacuoles in the embryonic chick corneal epithelium, an epithelium which produces collagen, *J. Cell. Biol.*, 48, 689, 1971.
86. Coulombre, A. J. and Coulombre, J. L., Corneal development. IV. Interruption of collagen excretion into the primary stroma of the cornea with L-azetidine-2-carboxylic acid, *Dev. Biol.*, 28, 183, 1972.
87. Dehm, P. and Prockop, D. J., Synthesis and extrusion of collagen by freshly isolated cells from chick embryo tendon, *Biochim. Biophys. Acta*, 240, 358, 1971.
88. Harsch, M., Murphy, L., and Rosenbloom, J., Metabolism by isolated fibroblasts of abnormal collagens containing analogues of proline or lysine, *FEBS Lett.*, 26, 48, 1972.
89. Christner, P. J. and Rosenbloom, J., Effects of incorporation of *trans*-4,5-dehydrolysine on collagen biosynthesis and extrusion in embryonic chick tibiae, *J. Biol. Chem.*, 246, 7551, 1971.
90. Grant, M. E., Kefalides, N. A., and Prockop, D. J., The biosynthesis of basement membrane collagen in embryonic chick lens. I. Delay between the synthesis of polypeptide chains and the secretion of collagen by matrix-free cells, *J. Biol. Chem.*, 247, 3539, 1972.
91. Bosmann, H. B. and Eylar, E. H., Glycoprotein biosynthesis: The biosynthesis of the hydroxylysine-galactose linkage in collagen, *Biochem. Biophys. Res. Commun.*, 33, 340, 1968.
92. Bosmann, H. B. and Eylar, E. H., Attachment of carbohydrate to collagen. Isolation, purification and properties of the glucosyl transferase, *Biochem. Biophys. Res. Commun.*, 30, 89, 1968.
93. Isemura, M., Ikenaka, T., and Matsushima, Y., Evidence for occurrence of a characteristic amino acid sequence of glycopeptides in the linkage region between peptide and carbohydrate, *Biochem. Biophys. Res. Commun.*, 46, 457, 1972.
94. Ehrlich, H. P. and Bornstein, P., Microtubules in transcellular movement of procollagen, *Nat. New Biol.*, 238, 257, 1972.

95. Dehm, P. and Prockop, D. J., Time lag in the secretion of collagen by matrix-free tendon cells and inhibition of the secretory process by colchicine and vinblastine, *Biochim. Biophys. Acta*, 264, 375, 1972.
96. Krane, S. M., Pinnell, S. R., and Erbe, R. W.,Lysyl-protocollagen hydroxylase deficiency in fibroblasts from siblings with hydroxylysine-deficient collagen, *Proc. Natl. Acad. Sci. USA*, 69, 2899, 1972.
97. Bellamy, G. and Bornstein, P., Evidence for procollagen, a biosynthetic precursor of collagen, *Proc. Natl. Acad. Sci. USA*, 68, 1138, 1971.
98. Ehrlich, H. P. and Bornstein, P., Further characterization of procollagen: The identification of a pro-2 chain, *Biochem. Biophys. Res. Commun.*, 46, 1750, 1972.
99. Bornstein, P., von der Mark, K., Wyke, A. W., Ehrlich, H. P., and Monson, J. M., Characterization of the pro-α1 chain of procollagen, *J. Biol. Chem.*, 247, 2808, 1972.
100. Church, R. L., Pfeiffer, S. E., and Tanzer, M. L., Collagen biosynthesis: Synthesis and secretion of a high molecular weight collagen precursor (procollagen), *Proc. Natl. Acad. Sci. USA*, 68, 2638, 1971.
101. Jimenez, S. A., Dehm, P., and Prockop, D. J., Further evidence for a transport form of collagen. Its extrusion and extracellular conversion to tropocollagen in embryonic tendon, *FEBS Lett.*, 17, 245, 1971.
102. Uitto, J., Jimenez, S. A., Dehm, P., and Prockop, D. J., Characterization of the precursor forms of the α1 and α2 chains of collagen from matrix-free tendon cells, *Biochim. Biophys. Acta*, 278, 198, 1972.
103. Bornstein, P., Ehrlich, J. P., and Wyke, A. W., Procollagen: Conversion of the precursor to collagen by a neutral protease, *Science*, 175, 544, 1972.
104. Lapière, C. M., Lenaers, A., and Kohn, L. D., Procollagen peptidase: An enzyme excising the coordination peptides of procollagen, *Proc. Natl. Acad. Sci. USA*, 68, 3054, 1971.
105. Dehm, P., Jimenez, S. A., Olsen, B. R., and Prockop, D. J., A transport form of collagen from embryonic tendon: Electron microscopic demonstration of an NH_2-terminal extension and evidence suggesting the presence of cystine in the molecule, *Proc. Natl. Acad. Sci. USA*, 69, 60, 1972.
106. Speakman, P. T., Proposed mechanism for the biological assembly of collagen triple helix, *Nature (Lond.)*, 229, 241, 1971.
107. Ottesen, M., Induction of biological activity by limited proteolysis, *Annu. Rev. Biochem.*, 36, 55, 1967.
108. Kitabchi, A. E., Duckworth, W. C., Stentz, F. B., and Yu, S., Properties of pro-insulin and related polypeptides, *CRC Crit. Rev. Biochem.*, 1, 59, 1972.
109. Milstein, C., Brownlee, G. G., Harrison, T. M., and Mathews, M. B., Post-synthetic cleavage of immunoglobulin light chains, *Nat. New Biol.*, 239, 117, 1972.
110. Burgeson, R. E., Wyke, A. W., and Fessler, J. H., Collagen synthesis by cells. II. Secretion of a disulfide linked material, *Biochem. Biophys. Res. Commun.*, 48, 892, 1972.
111. Grant, M. E., Kefalides, N. A., and Prockop, D. J., The biosynthesis of basement membrane collagen in embryonic chick lens. II. Synthesis of a precursor form by matrix-free cells and a time-dependent conversion to α chains in intact lens, *J. Biol. Chem.*, 247, 3545, 1972.
112. Ehrlich, H. P. and Bornstein, P., Further characterization of procollagen: The identification of a pro-α2 chain, *Biochem. Biophys. Res. Commun.*, 46, 1750, 1972.
113. Clark, C. C. and Veis, A., High molecular weight α chains in acid-soluble collagen and their role in fibrillogenesis, *Biochemistry*, 11, 494, 1972.
114. Veis, A., Anesey, J. R., Garvin, J. E., and Dimuzio, M. T., High molecular weight collagen: A long-lived intermediate in the biogenesis of collagen fibrils, *Biochem. Biophys. Res. Commun.*, 48, 1404, 1972.
115. Hodge, A. J., Highberger, J. H., Deffner, G. G., and Schmitt, F. O., The effects of proteases on the tropocollagen macromolecule and on its aggregation properties, *Proc. Natl. Acad. Sci. USA*, 46, 199, 1960.
116. Leibovich, S. J. and Weiss, J. B., Electron microscope studies of the effects of endo- and exopeptidase digestion on tropocollagen, *Biochim. Biophys. Acta*, 214, 445, 1970.
117. Steven, F. S., Grant, M. E., Ayad, S., Jackson, D. S., Weiss, J. B., and Leibovitch, S. J., The action of crude bacterial α-amylase on tropocollagen. A case of strictly limited proteolysis, *Biochim. Biophys. Acta*, 236, 309, 1971.
118. Hodge, A. J. and Petruska, J. A., Recent studies with the electron microscope on ordered aggregates of the tropocollagen macromolecule, in *Aspects of Protein Structure*, Ramachandran, G. N., Ed., Academic Press, New York, 1963, 289.
119. Smith, J. W., Molecular pattern in native collagen, *Nature (Lond.)*, 219, 157, 1968.
120. Veis, A., Bhatnagar, R. S., Shuttleworth, C. A., and Mussell, S., The solubilization of mature, polymeric collagen fibrils by lyotropic relaxation, *Biochim. Biophys. Acta*, 200, 97, 1970.
121. Miller, A. and Wray, J. S., Molecular packing in collagen, *Nature (Lond.)*, 230, 437, 1971.
122. Bouteille, M. and Pease, D. C., The tridimensional structure of native collagenous fibrils, their proteinaceous filaments, *J. Ultrastruct. Res.*, 35, 314, 1971.
123. Pinnell, S. R. and Martin, G. R., The cross-linking of collagen and elastin: Enzymatic conversion of lysine in peptide linkage to α-aminoadipic-δ-semialdehyde (allysine) by an extract from bone, *Biochemistry*, 61, 708, 1968.
124. Fowler, L. J., Peach, C. M., and Bailey, A. J., *In vitro* studies on the enzymic biosynthesis of the collagen cross-links, *Biochem. Biophys. Res. Commun.*, 41, 251, 1970.
125. Fairweather, R. B., Tanzer, M. L., and Gallop, P. M., Aldol-histidine, a new trifunctional collagen crosslink, *Biochem. Biophys. Res. Commun.*, 48, 1311, 1972.

126. Robins, S. P. and Bailey, A. J., Age-related changes in collagen: The identification of reducible lysine-carbohydrate condensation products, *Biochem. Biophys. Res. Commun.*, 48, 76, 1972.
127. Tanzer, M. L., Fairweather, R., and Gallop, P. M., Collagen cross-links: Isolation of reduced N-hexosylhydroxylysine from borohydride-reduced calf skin insoluble collagen, *Arch. Biochem. Biophys.*, 151, 137, 1972.
128. Deshmukh, K. and Nimni, M. E., Chemical changes associated with aging of collagen *in vivo* and *in vitro*, *Biochem. J.*, 112, 397, 1969.
129. Franzblau, C., Kang, A. H., and Faris, B., *In vitro* formation of intermolecular cross-links in chick skin collagen, *Biochem. Biophys. Res. Commun.*, 40, 437, 1970.
130. Tanzer, M. L., Collagen crosslinks: Stabilization by borohydride reduction, *Biochim. Biophys. Acta*, 133, 584, 1967.
131. Blumenfeld, O. O. and Gallop, P. M., Amino aldehydes in tropocollagen: The nature of a probable cross-link, *Proc. Natl. Acad. Sci. USA.*, 56, 1260, 1966.
131a. Piez, K. A., Cross-linking of collagen and elastin, *Annu. Rev. Biochem.*, 37, 547, 1971.
132. Kang, A. H. and Gross, J., Relationship between the *intra* and *inter*molecular cross-links of collagen, *Proc. Natl. Acad. Sci. USA*, 67, 1307, 1970.
133. Ayad, D. and Wynn, C. H., The effect of semicarbazide on the nature and stability of collagen fibrils, *Biochem. J.*, 118, 61, 1970.
134. Tanzer, M. L., Monroe, D., and Gross, J., Inhibition of collagen intermolecular cross-linking by thiosemicarbazide, *Biochemistry*, 5, 1919, 1966.
135. Rojkind, M. and Gutierrez, A. M., The binding of thiosemicarbazide to collagen *in vitro*, *Arch. Biochem. Biophys.*, 131, 116, 1969.
136. Davis, N. R. and Bailey, A. J., Chemical synthesis of the reduced form of an intermolecular cross-link of collagen: A re-evaluation of the structure of syndesine, *Biochem. Biophys. Res. Commun.*, 45, 1416, 1971.
137. Tanzer, M. L., Intermolecular cross-links in reconstituted collagen fibrils, *J. Biol. Chem.*, 15, 4045, 1968.
138. Bruns, R. and Gross, J., *Biochemistry*, 12, 808, 1973.
139. Siegel, R. C., Pinnell, S. R., and Martin, G. R., Cross-linking and elastin. Properties of lysyl oxidase, *Biochemistry*, 9, 4486, 1970.
140. Layman, D. L., Narayanan, A. S., and Martin, G. R., The production of lysyl oxidase by human fibroblasts in culture, *Arch. Biochem. Biophys.*, 149, 97, 1972.
141. Cannon, D. J. and Davison, P. F., Cross-linking and aging in rat tendon collagen, *Exp. Gerontol.*, in press, 1973.
142. Butzow, J. J. and Eichhorn, G. L., Physical chemical studies on the age changes in rat tail tendon collagen, *Biochim. Biophys. Acta*, 154, 208, 1968.
142a. Jackson, D. S. and Steven, F. S., Age changes in the cross-linking of human collagen, *Gerontologia*, 15, 77, 1969.
143. Everitt, A. V., Gal, A., and Steele, M. G., Age changes in the solubility of tail tendon collagen throughout the lifespan of the rat, *Gerontologia*, 16, 30, 1970.
144. Gal, A. and Everitt, A. V., Age changes in the polymer composition of acid soluble collagen prepared from rat tail tendon, *Exp. Gerontol.*, 5, 1, 1970.
145. Maekawa, T., Rathinasamy, T. K., Altman, K. I., and Forbes, W. F., Changes in collagen with age. I. The extraction of acid soluble collagens from the skin of mice, *Exp. Gerontol.*, 5, 177, 1970.
146. Mechanic, G. and Tanzer, M. L., Biochemistry of collagen cross-linking isolation of a new cross-link, hydroxylysinohydroxynorleucine, and its reduced precursor, dihydroxynorleucine, from bovine tendon, *Biochem. Biophys. Res. Commun.*, 41, 1597, 1970.
147. Kang, A. H., Studies on the location of intermolecular cross-links in collagen. Isolation of a CNBr peptide containing S-hdyroxylysinonorleucine, *Biochemistry*, 11, 1828, 1972.
148. Eyre, D. R. and Glimcher, M. J., The distribution of cross-linking aldehdyes in $\alpha 1$ and $\alpha 2$ chains of chicken bone collagen, *Biochim. Biophys. Acta*, 278, 206, 1972.
149. Stark, M., Rauterberg, J., and Kühn, K., Evidence for a non-helical region at the carboxyl terminus of the collagen molecule, *FEBS Lett.*, 13, 101, 1971.
150. Deshmukh, K. and Nimni, E., *In vitro* formation of cross-links in mature rat skin collagen in the absence of N-terminal peptides, *Biochem. Biophys. Res. Commun.*, 43, 416, 1971.
151. Deshmukh, K. and Nimni, M. E., Characterization of the aldehydes present on the cyanogen bromide from mature rat skin collagen, *Biochemistry*, 10, 1640, 1971.
152. Shuttleworth, A. and Glimcher, M. J., The time-dependent solubility of cyanogen bromide and chymotrypsin-treated collagen gels *in vitro*, *Biochim. Biophys. Acta*, 200, 332, 1970.
153. Zimmermann, B. K., Pikkarainen, J., Fietzek, P. P., and Kühn, K., Cross-linkages in collagen. Demonstration of three different intermolecular bonds, *Eur. J. Biochem.*, 16, 217, 1970.
154. Leibovich, S. J. and Weiss, J. B., Failure of human rheumatoid synovial collagenase to degrade either normal or rheumatoid arthritic polymeric collagen, *Biochim. Biophys. Acta*, 251, 109, 1971.
155. Volpin, D. and Veis, A., Differences between CNBr peptides of soluble and insoluble bovine collagens, *Biochem. Biophys. Res. Commun.*, 44, 804, 1971.
156. Miller, E. J., Collagen cross-linking: Identification of two cyanogen bromide peptides containing sites of intermolecular cross-link formation in cartilage collagen, *Biochem. Biophys. Res. Commun.*, 45, 444, 1971.

157. **Miller, E. J., Woodall, D. L., and Vail, M. S.,** Biosynthesis of cartilage collagen: Use of pulse-labeling to order the cyanogen bromide peptides in the α1(II) chain, *J. Biol. Chem.*, in press, 1973.
158. **Davison, P. F., Cannon, D. J., and Andersson, L. P.,** The effects of acetic acid on collagen cross-links, *Connect. Tiss. Res.*, 1, 205, 1972.
159. **Schiffmann, E. and Martin, G. R.,** Spontaneous generation of cross-links in aldehyde-containing collagen, *Arch. Biochem. Biophys.*, 138, 226, 1970.
160. **Page, R. C. and Benditt, E. P.,** The restricted reactivity of the side-chain amino groups of collagen toward formaldehyde, *FEBS Lett.*, 9, 49, 1970.
161. **Bensusan, H. B.,** Protein cross-linkages. I. Effect of iodination on the time-dependent solubility changes of reconstituted collagen fibrils, *Arch. Biochem. Biophys.*, 115, 77, 1966.
162. **Dabbous, M. K.,** Inter- and intramolecular cross-linking in tyrosinase-treated tropocollagen, *J. Biol. Chem.*, 241, 5307, 1966.
163. **LaBella, F., Waykole, P., and Queen, G.,** Formation of insoluble gels and dityrosine by the action of peroxidase on soluble collagens, *Biochem. Biophys. Res. Commun.*, 30, 333, 1968.
164. **Dabbous, M. K., Seif, J., and Brinkley, E. C.,** The action of tetranitromethane on acid-soluble tropocollagen, *Biochem. Biophys. Res. Commun.*, 48, 1586, 1972.
165. **Fujimori, E.,** Ultraviolet light-induced change in collagen macromolecules, *Biopolymers*, 3, 115, 1965.
166. **Kelley, F. W. and LaBella, F. S.,** Isolation of dityrosine from an alkali-soluble connective tissue protein, *Biochim. Biophys. Acta*, 263, 52, 1972.
167. **Deyl, Z., Praus, R., Sulcova, H., and Goldman, J. N.,** Fluorescence of collagen – properties of tyrosine residues and another fluorescent element in calf skin collagen, *FEBS Lett.*, 5, 187, 1969.
168. **Armstrong, W. G. and Horsley, H. J.,** Isolation and properties of fluorescent components associated with calcified tissue collagen, *Calcif. Tiss. Res.*, 8, 197, 1972.
169. **Puleo, L. E. and Sobel, H. H.,** Oxygen-modified collagen and its possible pathological significance, *Aerosp. Med.*, 43, 429, 1972.
170. **Gerber, G., Gerber, G., and Altman, K. I.,** Studies on the metabolism of tissue proteins, *J. Biol. Chem.*, 235, 2653, 1960.
171. **Gross, J. and Lapière, C.,** Collagenolytic activity in amphibian tissues: A tissue culture assay, *Proc. Natl. Acad. Sci. USA*, 48, 1014, 1962.
171a. **Gross, J. and Nagai, Y.,** Specific degradation of the collagen molecule by tadpole collagenolytic enzyme, *Proc. Natl. Acad. Sci. USA*, 54, 1197, 1965.
172. **Harris, E. D., Jr. and Farrell, M. E.,** Resistance to collagenase: A characteristic of collagen fibrils cross-linked by formaldehyde, *Biochim. Biophys. Acta*, 278, 133, 1972.
173. **Leibovich, S. J., Weiss, J. B., and Woolley, D. E.,** Sites of cleavage of tropocollagens by rheumatoid synovial collagenase, *Scand. J. Clin. Lab. Invest.*, 29, 22, 1972.
174. **Woessner, J. F.,** Biological mechanisms of collagen resorption, in *Treatise on Collagen*, Vol. 2, Part B, Gould, B. S., Ed., Academic Press, New York, 1968, 253.
175. **Jeffrey, J. J., Coffey, R. J., and Eisen, A. Z.,** Studies on uterine collagenase in tissue culture. I. Relationship of enzyme production to collagen metabolism, *Biochim. Biophys. Acta*, 252, 136, 1971.
176. **Slansky, H. H. and Dohlman, C. H.,** Collagenase and the cornea, *Surv. Ophthal.*, 14, 402, 1970.
177. **Harris, E. D., DiBona, D. R., and Krane, S. M.,** A mechanism for cartilage destruction in rheumatoid arthritis, *Clin. Res.*, 18, 534, 1970.
178. **Bauer, E. A., Eisen, A. Z., and Jeffrey, J. J.,** Studies on purified rheumatoid synovial collagenase *in vitro* and *in vivo*, *J. Clin. Invest.*, 50, 2056, 1971.
179. **Bauer, E. A., Jeffrey, J. J., and Eisen, A. Z.,** Preparation of three vertebrate collagenases in pure form, *Biochem. Biophys. Res. Commun.*, 44, 813, 1971.
180. **Bauer, E. A., Eisen, A. Z., and Jeffrey, J. J.,** Radioimmunoassay of human collagenase: Specificity of the assay and quantitative determination of *in vivo* and *in vitro* human skin collagenase, *J. Biol. Chem.*, 247, 6679, 1972.
181. **Tokoro, Y., Eisen, A. Z., and Jeffrey, J. J.,** Characterization of a collagenase from rat skin, *Biochim. Biophys. Acta*, 258, 289, 1972.
182. **Donoff, R. B., McLennan, J. E., and Grillo, H. C.,** Preparation and properties of collagenases from epithelium and mesenchyme of healing mammalian wounds, *Biochim. Biophys. Acta*, 227, 639, 1971.
183. **Eisen, A. Z., Bauer, E. A., and Jeffrey, J. J.,** Animal and human collagenases, *J. Invest. Dermatol.*, 55, 359, 1970.
183a. **Bauer, E. A., Eisen, A. Z., and Jeffrey, J. J.,** Immunologic relationship of a purified human skin collagenase to other human and animal collagenases, *Biochim. Biophys. Acta*, 206, 152, 1970.
184. **Harper, E., Bloch, K. J., and Gross, J.,** The zymogen of tadpole collagenase, *Biochemistry*, 10, 3035, 1971.
185. **Abe, S. and Nagai, Y.,** Evidence for the presence of a latent form of collagenase in human rheumatoid synovial fluid, *J. Biochem.*, 71, 919, 1972.
186. **Vaes, G.,** The release of collagenase as an inactive proenzyme by bone explants in culture, *Biochem. J.*, 126, 275, 1972.
187. **Ryan, J. N. and Woessner, J. F., Jr.,** Mammalian collagenase: Direct demonstration in homogenates of involuting rat uterus, *Biochem. Biophys. Res. Commun.*, 44, 144, 1971.
188. **Abe, S. and Nagai, Y.,** Interaction between tadpole collagenase and human α2-macroglobulin, *Biochim. Biophys. Acta*, 278, 125, 1972.

189. **Eisen, A. Z., Bauer, E. A., and Jeffrey, J. J.,** Human skin collagenase. The role of serum alpha-globulins in the control of activity *in vivo* and *in vitro*, *Proc. Natl. Acad. Sci. USA*, 68, 248, 1971.
190. **Bauer, E. A., Eisen, A. Z., and Jeffrey, J. J.,** Regulation of vertebrate collagenase activity *in vivo* and *in vitro*, *J. Invest. Dermatol.*, 59, 50, 1972.
191. **Robertson, P. B. and Miller, E. J.,** Cartilage collagen: Inability to serve as a substrate for collagenases active against skin and bone collagen, *Biochim. Biophys. Acta*, 289, 247, 1972.
192. **Berman, M., Dohlman, C. H., Gnadinger, M., and Davison, P. F.,** Characterization of collagenolytic activity in the ulcerating cornea, *Exp. Eye Res.*, 11, 255, 1971.
193. **Strawich, E. and Nimni, M. E.,** Properties of a collagen molecule containing three identical components extracted from bovine articular cartilage, *Biochemistry*, 10, 3905, 1971.
194. **Bailey, A. J.,** Comparative studies on the nature of the cross-links stabilizing the collagen fibres of invertebrates, cyclostomes and elasmobranchs, *FEBS Lett.*, 18, 154, 1971.
195. **von der Mark, K., Wendt, P., Rexrodt, F., and Kühn, K.,** Direct evidence for a correlation between amino acid sequence and cross striation pattern of collagen, *FEBS Lett.*, 11, 105, 1970.
196. **Berman, M., Kerza-Kwiatecki, A. P., and Davison, P. F.,** Characterization of human collagenase, *Exp. Eye Res.*, in press, 1973.
197. **Eisen, A. Z. and Gross, J.,** The role of epithelium and mesenchyme in the production of a collagenolytic enzyme and a hyalurinidase in the anuran tadpole, *Dev. Biol.*, 12, 408, 1965.
198. **Eisen, A. Z.,** Human skin collagenase: Localization and distribution in normal human skin, *J. Invest. Dermatol.*, 52, 442, 1969.
199. **Seifter, S. and Harper, E.,** Collagenases, in *Methods in Enzymology*, Vol. 19, Perlmann, G. E. and Lorand, L., Eds., Academic Press, New York, 1970, 613.
200. **Layman, D. L., Sokoloff, L., and Miller, E. J.,** Collagen synthesis by articular chondrocytes in monolayer culture, *Exp. Cell. Res.*, 73, 107, 1972.
201. **Holtzer, H., Chacko, S., Abbott, J., Holtzer, S., and Anderson, H.,** Variable behavior of chondrocytes *in vitro*, in *Chemistry and Molecular Biology of the Intercellular Matrix*, Vol. 3, Balazs, E. A., Ed., Academic Press, New York, 1970, 1471.
202. **Blumenkrantz, N. and Sondergaard, J.,** Effect of prostaglandins E_1 and $F_{1\alpha}$ on biosynthesis of collagen, *Nat. New Biol.*, 239, 246, 1972.
203. **Weiss, P. A.,** *Dynamics of Development: Experiments and Inferences*, Romano, J., Ed., Academic Press, 1968.
204. **Hay, E. D. and Revel, J-P.,** Fine structure of the developing avian cornea, in *Monographs in Developmental Biology*, Vol. 1, Wolsky, A. and Chen, P. S., Eds., S. Karger, New York, 1969.
205. **Fitton-Jackson, S.,** The morphogenesis of collagen, in *Treatise on Collagen*, Vol. 2, Gould, B. S., Ed., Academic Press, London and New York, 1968, 1.
206. **Jeffrey, J. J., Coffey, R. J., and Eisen, A. Z.,** Studies on uterine collagenase in tissue culture. II. Effect of steroid hormones on enzyme production, *Biochim. Biophys. Acta*, 252, 143, 1971.
207. **Gribble, T. J., Comstock, J. P., and Udenfriend, S.,** Collagen chain formation and peptidyl proline hydroxylation in monolayer tissue cultures of L-929 fibroblasts, *Arch. Biochem. Biophys.*, 129, 308, 1969.
208. **Bates, C. J., Bailey, A. J., Prynne, C. J., and Levene, C. I.,** The effect of ascorbic acid on the synthesis of collagen precursor secreted by T6 mouse fibroblasts in culture, *Biochim. Biophys. Acta*, 278, 372, 1972.
209. **Bates, C. J., Prynne, C. J., and Levene, C. I.,** The synthesis of underhydroxylated collagen by T6 mouse fibroblasts in culture, *Biochim. Biophys. Acta*, 263, 397, 1972.
210. **Peterkofsky, B.,** The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts, *Arch. Biochem. Biophys.*, 152, 318, 1972.
211. **Butler, W. T.,** The structure of α1-CB3, a cyanogen bromide fragment from the central portion of the α1 chain of rat collagen. The tryptic peptides from skin and dentin collagens, *Biochem. Biophys. Res. Commun.*, 48, 1540, 1972.
212. **Miller, E. J., Lane, J. M., and Piez, K. A.,** Isolation and characterization of the peptides from the α1 chain of chick bone collagen after cyanogen bromide cleavage, *Biochemistry*, 8, 30, 1969.
213. **Kang, A. H., Piez, K. A., and Gross, J.,** Characterization of the cyanogen bromide peptides from the α1 chain of chick skin collagen, *Biochemistry*, 8, 1506, 1969.
214. **Rauterberg, J. and Kühn, K.,** Acid soluble calf skin collagen. Characterization of the peptides obtained by cyanogen bromide cleavage of its α1-chain, *Eur. J. Biochem.*, 19, 398, 1971.
215. **Barnes, M. J., Constable, B. J., Morton, L. F., and Kodicek, E.,** Hydroxylysine in the N-terminal regions of the α_1- and α_2-chains of various collagens, *Biochem. J.*, 125, 433, 1971.
216. **Barnes, M. J., Constable, F. J., Morton, L. F., and Kodicek, E.,** Hydroxylysine in the N-terminal telopeptides of skin collagen chick embryo and newborn rat, *Biochem. J.*, 125, 925, 1971.
217. **Levene, C. I., Shoshan, S., and Bates, C. J.,** The effect of ascorbic acid on the cross-linking of collagen during its synthesis by cultured 3T6 fibroblasts, *Biochim. Biophys. Acta*, 257, 384, 1972.
217a. **Levene, C. I., Bates, C. J., and Bailey, A. J.,** Biosynthesis of collagen cross-links in cultured 3T6 fibroblasts: effect of lathyrogens and ascorbic acid, *Biochim. Biophys. Acta*, 263, 574, 1972.
218. **Katz, E. P. and Li, S.,** The molecular packing of collagen in mineralized and non-mineralized tissues, *Biochem. Biophys. Res. Commun.*, 46, 1368, 1972.

218a. **Morgan, P. H., Jacobs, H. G., Segrest, J. P., and Cunningham, L. W.,** A comparative study of glycopeptides derived from selected vertebrate collagens, *J. Biol. Chem.*, 245, 5042, 1970.
219. **Schofield, J. D., Freeman, I. L., and Jackson, D. S.,** The isolation, and amino acid and carbohydrate composition, of polymeric collagens prepared from various human tissues, *Biochem. J.*, 124, 467, 1971.
220. **Kawasaki, T. and Bernardi, G.,** Heterogeneity of tropocollagen as investigated by chromatography on hydroxyapatite columns, *Biopolymers*, 9, 269, 1970.
221. **Trelstad, R. L., Kang, A. H., Toole, B. P., and Gross, J.,** Collagen heterogeneity, *J. Biol. Chem.*, 247, 6469, 1972.
222. **Trelstad, R. L. and Coulombre, A. J.,** Morphogenesis of the collagenous stroma in the chick cornea, *J. Cell Biol.*, 50, 840, 1971.
223. **Coulombre, A. J.,** Problems in corneal morphogenesis, *Adv. Morphog.*, 4, 81, 1965.
224. **Schwarz, W. and Keyserlingk, D. G.,** Electron microscopy of normal and opaque human cornea, in *The Cornea*, Langham, M. E., Ed., The Johns Hopkins Press, Baltimore, 1969, 123.
225. **Nakao, K. and Bashey, R. I.,** Fine structure of collagen fibrils as revealed by ruthenium red, *Exp. Mol. Pathol.*, 17, 6, 1972.
226. **Rambourg, A.,** Morphological and histochemical aspects of glycoproteins at the surface of animal cells, *Int. Rev. Cytol.*, 31, 57, 1971.
226a. **Butler, W. T. and Ponds, S. L.,** Chemical studies on the cyanogen bromide peptides of rat skin collagen. Amino acid sequence of α1-CB4, *Biochemistry*, 10, 2076, 1971.
227. **Mathews, M. B.,** Molecular organization of connective tissue matrix, in *The Cornea*, Langham, M. E., Ed., The Johns Hopkins Press, Baltimore, 1969, 107.
228. **Shuttleworth, A. and Veis, A.,** The isolation of anionic phosphoproteins from bovine cortical bone via the periodate solubilization of bone collagen, *Biochim. Biophys. Acta*, 257, 414, 1972.
228a. **Langham, M. E., Hart, R. W., and Cox, J.,** The interaction of collagen and mucopolysaccharides, in *The Cornea*, Langham, M. E., Ed., The Johns Hopkins Press, Baltimore, 1969, 157.
229. **Toole, B. P., Kang, A. H., Trelstad, R. L., and Gross, J.,** Collagen heterogeneity within different growth regions of long bones of rachitic and non-rachitic chicks, *Biochem. J.*, 127, 715, 1972.
230. **Kohn, R. R.,** *Principles of Mammalian Aging*, Prentice Hall, Englewood Cliffs, New Jersey, 1971.
231. **Nordwig, A. and Hayduk, U.,** A contribution to the evolution of collagen, *J. Mol. Biol.*, 26, 351, 1967.
232. **Nordwig, A. and Hayduk, U.,** Invertebrate collagens: Isolation, characterization and phylogenetic aspects, *J. Mol. Biol.*, 44, 161, 1969.
233. **Clark, C. C. and Bornstein, P.,** Cyanogen bromide cleavage of guinea pig skin collagen. Isolation and characterization of peptides from the α1 and α2 chains, *Biochemistry*, 11, 1468, 1972.
234. **Piez, K. A., Balian, G., Click, E. M., and Bornstein, P.,** Homology between the α1 and α2 chains of collagen, *Biochem. Biophys. Res. Commun.*, 48, 990, 1972.
235. **Lewis, M. S. and Piez, K. A.,** The characterization of collagen from the skin of the dogfish shark, *Squalus acanthias*, *J. Biol. Chem.*, 229, 3336, 1964.
236. **Trelstad, R. L., Kang, A. H., Igarashi, S., and Gross, J.,** Isolation of two distinct collagens from chick cartilage, *Biochemistry*, 9, 4993, 1970.
237. **Stark, M., Miller, E. J., and Kühn, K.,** Comparative electron-microscope studies on the collagens extracted from cartilage, bone, and skin, *Eur. J. Biochem.*, 27, 192, 1972.
238. **Matalon, R., Kennedy, J. P., Jr., and Dorfman, A.,** Hurler's syndrome, an a-L-iduronidase deficiency, *Biochem. Biophys. Res. Commun.*, 47, 959, 1972.
239. **Waldenstrom, J.,** Inborn metabolic errors of connective tissue, in *Aging of Connective and Skeletal Tissue*, Engel, A. and Larsson, T., Eds., Nordiska Bokhandelns Förlag, Stockholm, 1968, 245.
240. **Narayanan, A. S., Siegel, R. C., and Martin, G. R.,** On the inhibition of lysyl oxidase by β-aminopropionitrile, *Biochem. Biophys. Res. Commun.*, 46, 746, 1972.
241. **Pinnell, S. R., Krane, S. M., Kinzora, J. E., and Glimcher, M. J.,** A heritable disorder of connective tissue. Hydroxylysine-deficient collagen disease, *N. Engl. J. Med.*, 286, 1013, 1972.
241a. **Eyre, D. R. and Glimcher, M. J.,** Reducible cross-links in hydroxylysine-deficient collagens of a heritable disorder of connective tissue, *Proc. Natl. Acad. Sci. USA*, 69, 5994, 1972.
242. **Mechanic, G.,** Cross-linking of collagen in a heritable disorder of connective tissue: Ehlers-Danlos syndrome, *Biochem. Biophys. Res. Commun.*, 47, 267, 1972.
243. **Eisen, A. Z.,** Human skin collagenase: Relationship to the pathogenesis of epidermolysis bullosa dystrophica, *J. Invest. Dermatol.*, 52, 449, 1969.
244. **Abramson, M.,** Collagenolytic activity in middle ear cholesteatoma, *Ann. Otol. Rhinol. Laryngol.*, 78, 112, 1969.
245. **Laitinen, O., Uitto, J., Hannuksela, M., and Mustakallio, K. K.,** Solubility and turnover of collagen in collagen diseases, *Ann. Clin. Res.*, 1, 64, 1969.
245a. **Leroy, E. C.,** Connective tissue synthesis by scleroderma skin fibroblasts in cell culture, *J. Exp. Med.*, 135, 1351, 1972.
246. **Lenaers, A., Ansay, M., Nusgens, B. V., and Lapière, C. M.,** Collagen made of extended α-chains, procollagen, in genetically-defective dermatosparaxic calves, *Biochem. J.*, 23, 533, 1971.
247. **Mulvihill, J. J.,** Congenital and genetic disease in domestic animals, *Science*, 176, 132, 1972.
248. **Kefalides, N. A. and Denduchis, B.,** Structural components of epithelial and endothelial basement membranes, *Biochemistry*, 8, 4613, 1969.