REVIEW ARTICLE

PATHOLOGY OF COLLAGEN DEGRADATION

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Pathology of Collagen Degradation

A Review

Ruy Pérez-Tamayo

A FIRST REACTION to the title of this review might be that it refers to pathologic minutiae. Who but the incurably esoteric would be interested in such a remote corner of human pathology as the disorders in collagen degradation? A glance at the list of diseases probably (or possibly) related to that peculiar pathogenetic mechanism, however, should convince the skeptic that this is not an exercise in biologic futility. Rheumatoid arthritis and cirrhosis of the liver are anything but rare, and new knowledge on the pathogenesis of these two diseases should be welcomed by all physicians, especially those concerned with treating the unfortunate thousands afflicted by such progressive and incurable disorders. Pathologists reading slides prepared from rheumatoid joint tissues or from cirrhotic liver biopsies should be conscious that their observations contain data valuable not only for diagnosis but also for the understanding of mechanisms of tissue damage and/or irreversibility. This review has been written by a practicing pathologist who also works in the experimental field of collagen degradation. The reader should be warned that data have been sorted out and arranged to fit a personal view; much information on collagen at large has been omitted, which might have been relevant to other reviewers. This fact is duly acknowledged but not apologetically. What follows is an honest attempt to present a unitary view of the role of collagen degradation in the pathology of some human diseases.

Collagen Structure and Biosynthesis

Collagen is the most abundant protein in mammals. It occurs as extracellular paracrystalline fibers with structural and supportive functions. To the pathologist, collagen represents the more frequently recognized component of intercellular substances, a pink, fibrillary, and conspicuous material which is always there, supporting cells of all types and providing architectural sense to complicated combinations of multicellular tissues.

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Essentially the same chemical substance is revealed by reticulum stains, although it is obvious that there must be some differences with collagen fibers, perhaps in primary structure polymerization or in the associated molecules. Finally, basement membranes also belong to the same family of proteins, and here the differences with collagen fibers are better known. There may be more types of molecular arrangements of the basic collagen monomer, such as the oxytalan fiber or the arcuate fibrils in the spleen, but there is very little biochemical information on them.

Collagen Structure

Collagen is one of the better known mammalian proteins; several general reviews are available.[1-10] A few facts have been selected which seem relevant to the main topic of this review. It has been established that tissue collagens are polymers composed of the repetitious aggregation of monomers; these monomers are known as tropocollagen.[11] Although the designation is not without objection (some would like to call it simply collagen), it serves the useful function of identifying an operationally well-defined molecular species, namely that which can be solubilized undenatured (at low temperatures and by different neutral salt or acid solutions), from the connective tissues of many kinds of animals. Tropocollagen is not the direct product of secretion of fibroblasts and other collagen synthesizing cells, nor is it the final form in which the protein becomes insolubilized in the tissues. Tropocollagen is an intermediate molecular species, preceded by procollagen, the soluble precursor with nonhelical peptide extensions at both N- and C-terminal ends, followed by a range of insoluble aggregates with increasing numbers of intramolecular and intermolecular cross-links, culminating in fibrils that cannot be extracted without denaturation (so-called polymeric insoluble collagen).

Biochemistry

The amino acid composition of a solution of pure tropocollagen obtained from normal human skin shows several interesting features: a) a high content of glycine, alanine, proline, and hydroxyproline; b) absence of tryptophan and cysteine and low content of tyrosine; and c) presence of hydroxylysine.[12] Glycine represents 30% of all amino acids (throughout most of each polypeptide chain every third amino acid is glycine), and collagen can be considered as a polymer of tripeptide units with the formula -Gly-X-Y-. The "X" in the formula is frequently proline, which limits the rotation of the polypeptide chain because of its ring structure. The "Y" in the formula is frequently hydroxyproline, another amino acid

with a ring structure which further limits the rotation of the polypeptide chains.[3] The lack of cysteine precludes the formation of intramolecular and intermolecular disulfide cross-links.[13] The presence of hydroxylysine is interesting not only because of the rarity of this hydroxylated amino acid residue but also because the small amount of carbohydrate in collagen (galactose or glucosylgalactose) is linked only to the hydroxyl group of hydroxylysine.[14,15] Collagen in basement membranes shows some differences from the above description, namely a higher content of hydroxyproline (much of this hydroxyproline is hydroxylated in Position 3 of the proline ring, instead of Position 4, as in other collagens) and hydroxylysine, the presence of 4 to 10 cysteine residues per 1000 amino acid residues, and far more carbohydrate than the other collagens.[16,17]

The tropocollagen monomer consists of three polypeptide chains or subunits that are coiled into a rigid helical structure.[18,19] Each chain contains approximately 1000 amino acids; the three chains are parallel and extend the full length of the monomer, which has a molecular weight of approximately 300,000 daltons.[3,4,20] The tropocollagen molecule is a very long and thin rod, barely visible under the electron microscope; it measures 300 nm in length and 1.5 nm in diameter.[21] In the extracellular spaces it associates into polymers by joining tail to head with other monomers, forming long microfibrils, and by aggregating laterally with other microfibrils in the well-known quarter-stagger arrangement.[22] It has been suggested [23] that fibrillogenesis *in vivo* does not occur by the individual aggregation of tropocollagen molecules onto a growing fibril, but rather by the accretion of intermediate molecular aggregates or subassemblies, in a step-wise morphogenetic process. Under the electron microscope, tissue collagen fibrils show transverse banding with a periodicity of approximately 68 nm due to the concentration of charged amino acid residues in five regions approximately 68 nm apart along the tropocollagen monomer.

Although the quarter-stagger aggregation of tropocollagen monomers is accepted as the mechanism by which normal collagen fibers are formed in the interstitial tissues, tropocollagen monomers can be easily induced *in vitro* (and, perhaps not so easily, *in vivo*) to aggregate in other ways. Dialysis of diluted collagen solutions in acetic acid at low molarity against negatively charged molecules such as ATP will result in precipitation of crystallites in which all molecules aggregate laterally in register in the same direction. Since tropocollagen molecules measure 300 nm in length, the crystallites will have the same length; in addition, when positively stained these structures show approximately 58 dark bands which probably represent the distribution of polar side chains along the length of the

tropocollagen molecule.[24] These crystallites are known as SLS (segment-long-spacings) and are very useful in the detection of early collagenolysis and in the examination of the mechanism of enzyme action. (See section on Direct Tissue Extracts).

The three subunits are held together in the monomer by hydrogen bonding between HN groups of glycyl residues to O=C groups in the peptide bonds of the other chains, especially prolyl residues in the -Gly-Pro-Y- position.[25] Another source of stability in the monomer is the intramolecular cross-links, established first by the enzymatic synthesis of aldehydes at the E- amino groups of several of the lysil and hydroxylysyl residues in the polypeptide chains and then by either aldol condensation between two aldehydes on two adjacent chains or by Schiff-base formation by condensation of one aldehyde with the amino group of lysyl or hydroxylysyl residues in another chain.[26] Reduction of the Schiff bases and dehydration of the aldol condensation contribute to further stabilize the intramolecular cross-links. Similar types of cross-linking exist between different tropocollagen molecules, and it is claimed that they contribute substantially to stabilize collagen fibers and to provide them with some of their more salient physical properties, such as elasticity and tensile strength.[27-29] It has been established that to break a collagen fiber 1 mm in diameter requires a load of 10 to 40 kg.[30]

Heterogeneity

Collagen is not the name of a single specific protein but, rather, is a generic term encompassing several types of molecules which may aggregate in the tissues in various forms and also in association with different amounts and kinds of other molecules. Therefore, collagen heterogeneity may be examined at three different levels of organization: a) primary structure, b) type of extracellular aggregation, and c) the molecules that become associated with them.

Primary Structure. Variations in primary structure of the polypeptide chains composing the tropocollagen monomer are of two different origins, ie, genetic and posttranslational. There are at least five genetic types of polypeptide chains, and there may be more.[19,31-33] The polypeptide chains are known as alpha subunits and they combine to form at least four types of monomers. Collagen Type I is formed by two identical alpha 1 (I) subunits and one alpha 2 subunit; it therefore has the condensed formula (alpha 1 [I])$_2$ alpha 2 and is the major component of skin, tendon, and bone. Collagen Type II is formed by three identical alpha 1(II) subunits, which have a primary structure different from the other alpha subunits. It has the condensed formula (alpha 1[II])$_3$ and is the major component of cartilage. Collagen Type III is also formed by three identical alpha 1(III)

subunits, again different from all others. It has the condensed formula (alpha 1[III])$_3$ and is found in blood vessels, uterine leiomyomas, and fetal skin. Finally, collagen Type IV has three identical alpha 1(IV) subunits. It has the condensed formula (alpha 1[IV])$_3$ and corresponds to basement membranes.

Posttranslational heterogeneity [34] in collagen is due to the degree of hydroxylation of prolyl and lysyl residues in the polypeptide chains, which has been found to be variable in different collagen preparations. Each alpha chain contains approximately 100 triplets of -Gly-X-Hypro-; if in any chain as many as 50 triplets may be either -Gly-X-Hypro- or -Gly-X-Pro-, then there are at least 2^{50} possible kinds of each alpha chain in the trimer in any tissue.[35] To this source of heterogeneity other possibilities should be added, such as the degree of glycosilation of hydroxylysine and the number and types of intermolecular and intramolecular cross-links.

Extracellular Aggregation. There are three major forms in which tropocollagen monomers aggregate in the extracellular space, ie, collagen fibers, reticulum fibers, and basement membranes. Collagen fibers show different sizes under the electron microscope: in cartilage they have an average diameter of 15 to 25 nm; in the cornea they measure 30 nm; in tendons they vary between 30 and 130 nm; and in the skin they average 60 nm.[35] Reticulum fibers are a prominent component of the supportive network of parenchymatous organs, but the reasons for their peculiar histologic and ultrastructural characteristics are yet to be established [36]; it has been suggested that, at least in the liver, they could represent collagen Type III,[37] although some of the evidence is controversial.[38] Basement membranes do not have a fibrillary ultrastructure; they appear as a finely granular homogeneous and variably electron-dense material. This is probably due to the large amount of carbohydrates and glycoproteins with which they are associated [16,17]; the same lack of microfibrils is characteristic of cartilage, in which collagen is associated with mucopolysaccharides.

Associated Molecules. It has been mentioned that another source of heterogeneity in the population of collagen structures in a given tissue is the nature and amount of molecules which associate with them, either covalently linked or simply adsorbed to the fibrils. Although not much is known about the precise kinds of interactions that occur between collagen and the various proteoglycans, they should be kept in mind when discussing the susceptibility of collagen to degradation.

Collagen Biosynthesis

Only a brief summary of the salient features of collagen biosynthesis will be given, with emphasis on those aspects related to the main theme of

this paper. There are several recent reviews of the subject.[6,7,35,39-46] The various reactions leading to complete biosynthesis of collagen molecules may be conveniently separated into intracellular and extracellular steps.

Intracellular Steps

Six steps may be considered in the intracellular biosynthesis of collagen:

Translation of collagen mRNA in polysomes larger than 23 ribosomal aggregates but smaller than 50 to 60 ribosomal aggregates, which theoretically accommodate an mRNA molecule of the appropriate size to code for a single polypeptide chain of approximately 1400 amino acids: This polypeptide chain is not an alpha subunit, but a precursor, known as pro-alpha chain, which contains peptide extensions on both ends of the molecule.[47] The polysomes are bound to the endoplasmic reticulum of the collagen-synthesizing cell, and it is widely believed that the nascent polypeptide chains pass through the membrane and fall into the cisternae of this subcellular organelle.

Hydroxylation of peptidyl proline by a specific enzyme, which starts during assembly of the nascent polypeptide chains and continues after the pro-alpha chains have been released from the polysomes: Completion of this step is essential for the formation of a stable helical conformation of the tropocollagen molecule at 37 C. It is believed that hydroxylation of prolyl residues in the pro-alpha chain occurs within the cisternae of the endoplasmic reticulum.

Hydroxylation of peptidyl lysine by another specific enzyme, simultaneous with hydroxylation of prolyl residues and taking place also in the cisternae of endoplasmic reticulum: This step is necessary for the establishment of intramolecular and intermolecular cross-links and for glycosylation of the collagen molecule.

Synthesis of interchain disulfide bonds, which are probably essential for helix formation, occurs only after translation of the pro-alpha chains is completed but still within the cisternae of the endoplasmic reticulum. It should be recalled that the disulfide bonds are present only in procollagen (they are located in the C-terminal peptide extension) and in collagen Type III.

Formation of the triple helix, which represents the assembly of the three alpha subunits in the appropriate conformation, to constitute a completed procollagen molecule, also occurs within the endoplasmic reticulum.

Glycosylation is also an intracellular step, catalyzed by specific enzymes, which can occur within the cisternae of the endoplasmic reticulum but which is probably continued and completed within the Golgi vacu-

oles. It was believed for some time that glycosylation was a step necessary for the protein to leave the cell, as most proteins synthesized for "export" are glycosylated. The discovery of hydroxylysine-deficient collagen in patients with the Ehlers–Danlos Type IV phenotype,[48] which precludes glycosylation but does not impede collagen secretion, refutes the generalization that a sugar moiety is necessary for the extracellular secretion of proteins.[49]

Extracellular Steps

It is supposed that the protein secreted by the collagen-synthesizing cell is procollagen, the transport form of the molecule.[47] Sometime and somewhere after it has been secreted, procollagen undergoes a series of enzymatic transformations ending in tropocollagen, the insoluble monomer, which precipitates as one more unit component of extracellular collagen fibrils. The number of intermediate molecules and the corresponding enzymes responsible for this transformation are under active investigation. The disease dermatosparaxis, originally described in cattle but which also occurs in sheep, dogs, and humans,[50] led to the initial description of an enzyme, known as procollagen peptidase, responsible for the catalytic severance of the N-terminal peptide extension of procollagen. In dermatosparaxis, or Ehlers–Danlos syndrome Type VI in humans, there is a pronounced decrease in the activity of procollagen peptidase, and this results in highly abnormal extracellular collagen aggregates which, in principle, explain the major clinical features, such as increased skin friability, deficient healing, and prolonged bleeding.[8,51]

Collagen Degradation

This relatively new aspect of collagen metabolism has been the major subject of recent reviews,[51-67] few of which were written with the pathologist in mind.

The existence of collagen degradation under physiologic conditions was ignored for many years, partly because of the impression of collagen stability derived from physicochemical and structural studies and partly because of the results of the classic isotopic studies of Neuburger and Slack, published in 1953.[68] These authors examined the metabolic turnover of collagen from liver, bone, skin, and tendon in the normal rat and concluded that there is essentially no turnover of body collagen. The collagen synthetized during the early growth periods remains with the animal the rest of its life; the half-life of tail tendon collagen was found to be longer than the life span of the rat. In the following year, however, the Harkness'[69] published their pioneer work revealing that in the uterine

horns of pregnant rats, collagen falls from an average of 29.2 mg immediately after delivery to 3.1 mg 5 days post partum. (This was later shown [70-72] to represent a change in the half-life of collagen from approximately 40 days under basal conditions to 24 hours during the first 2 days postpartum.) Further work established that a small but significant fraction of total body collagen is rapidly synthetized and degraded throughout life, that this fraction is quantitatively different in various tissues, and that abnormal collagen degradation, either by excess or deficiency, might play a role in some human diseases.[73] The search for endogenous mechanisms of collagen degradation was started around 1960, but no positive results were forthcoming with the use of techniques that had proved successful for other systems.

Discovery of Animal Collagenases

Several workers (I am sure many more were equally frustrated but remained silent) published their negative results in attempting to extract collagenolytic substances from various tissues with different methods. Then in 1962 a seminal paper by Gross and Lapiére appeared,[74] describing an *in vitro* method to demonstrate the existence of endogenous specific collagenase in animal tissues. A careful study of this paper is rewarding because it contains much of what is accepted today in the field, both conceptually as well as methodologically. Gross and Lapiére reasoned that an enzyme such as collagenase was a potentially dangerous substance, so that a strict regulation of synthesis, activity, and diffusion was essential and should be under close local control. For these same reasons, the actual amount of active enzyme present at a given time in any tissue would be minimal, thus explaining the failure of traditional enzymology techniques to yield positive results. A method for detection of endogenous collagense was devised following the conditions mentioned above: small fragments of tail and other organs of tadpoles undergoing active metamorphosis were explanted on the surface of reconstituted collagen gels. Success was forthcoming with the presence of gel lysis around the tissue fragments some 24 to 48 hours after explantation. Reconstituted collagen gels were prepared with radioactive collagen, so that a quantitative appreciation of lysis was possible, and SLS crystallites of the lysed collagen were prepared and examined under the electron microscope. This simple experiment succeeded despite two misconceptions: a) the idea that little or no active collagenase is normally present in the extracellular tissue compartment was wrong and b) the authors did not add fetal serum to their tadpole tissue explants because they had no expertise in tissue culture techniques,[6] thus eliminating all the multiple and potent serum collagenase inhibitors.

A most useful lesson should be learned from this story: it is wiser to try the experiment once it has been conceived than to search in the literature and ask experts for all possible pros and cons for it. Further tests of the tissue explant on collagen gels technique quickly followed, and it was shown that mammalian bone and uterus gave the same results.[75,77] Tissue explants became (and to this day remain) the basic technique for obtaining working amounts of animal collagenolytic enzymes. Examination of enzyme reaction products by electron microscopy of SLS collagen crystallites is still one of the more elegant and conclusive demonstrations of specific collagenolytic activity.

Methodology

The pathologist looking down the microscope may become suspicious that a connective tissue change, such as extensive necrosis with loss of all structures (as in aneurysms eroding vertebral bones), or malignant cells freely invading fibous tissue (as in melanoma of the skin), or collagenous deposits disturbing the normal tissue architecture (as in cirrhosis of the liver), could involve a disturbance in collagen degradation, either by excess or by defect. If the pathologist is enterprising and wishes to gain more insight, a methodology which is already available should be used to put it to test. Most of the techniques to be described are not complicated and require reagents and equipment usually available in many academic laboratories of pathology. They do, however, demand rigorous adhesion to basic research principles and an adventurous spirit, coupled with sufficient interest in the problem to last longer than the tedious weeks or months devoted to acquiring the indispensable technical expertise and conceptual familiarity with the field. To this extent, collagen degradation is not different from all other areas of investigation into the mechanisms of tissue damage in human disease. Parenthetically, this is one way to transform the pathology of 1878 into the pathology of 1978 and help to bring to a belated end the current (and silly) controversy on the "future" of pathology.

Although the specific field of collagen degradation belongs in the area of protein catabolism, it nevertheless has a number of unique features that prevent uncritical extrapolation of general principles.[59] Some of these features are the extracellular and insoluble nature of the substrate; the lack of any biologic activity of collagen that might be used to follow its breakdown, as in the case of enzyme proteins; the rigorous physiologic conditions that must be observed to prevent collagen from undergoing denaturation in *in vitro* tests; and the inability of many (if not all) pure animal collagenases to split some synthetic substrates, which precludes

their simple use. Such peculiarities have resulted in a series of principles that apply to any technique devised to establish the presence of collagenolytic activity in preparations derived from animal tissues and which may be listed as follows:

1. The substrate should be in a demonstrable native state, which is usually established by its ability to resist hydrolysis by all known nonspecific animal proteases. Operationally, this is usually reduced to less than 10% degradation when exposed to active trypsin at a protein:enzyme ratio of 100:1 and under physiologic conditions of pH, temperature, and ionic strength. Other ways to show the native state of collagen are the high intrinsic viscosity and strong negative optical rotation of solutions kept below 23 C, the transformation of clear cold neutral salt solutions into a homogeneous gel by bringing them to 37 C, or the examination of the electrophoretic pattern of the molecule in acrylamide gel electrophoresis at acid pH (5.5 units), which should reveal no molecular species other than alpha subunits, dimers, trimers, and higher molecular weight polymers.

2. Incubation of reaction mixtures should be carried out under strict physiologic conditions of pH, temperature, and ionic strength to avoid denaturation of the substrate during exposure to the preparation with suspected or known collagenolytic activity.

3. Because collagenolysis is defined as cleavage of peptide bonds in the helical portion of native collagen molecules, it is mandatory that the reaction products should be identified, instead of limiting the assay to the demonstration of one or more changes in some of the physical properties of collagen, such as viscosity, optical rotation, turbidity, gel formation, or release of radioactive material to the supernatant of labeled reconstituted collagen fibrils. This may be accomplished by acrylamide gel electrophoresis at acid pH or with SDS, by measuring the length of SLS crystallites prepared from the reaction products, or by their isolation by means of fractional ammonium sulfate precipitation.

Sources of Collagenase

There are several techniques for obtaining collagenolytic activity from tissue preparations. They are all unsatisfactory with respect to yield and purity, but no better methods have been devised. They may be discussed under three headings:

Tissue Explants. Originally introduced by Gross and Lapiére,[74] this remains the more widely used technique for obtaining working amounts of animal collagenase. It consists of the aseptic preservation of small fragments (1.0 mm) from the desired tissue under conditions prescribed

for cell culture but in the absence of serum. Tissue culture medium is replaced every 24 to 48 hours and is pooled; subsequent treatment of the pooled medium varies according to several techniques, but initial purification usually starts by precipitating the active material with ammonium sulfate at 40 to 60% saturation, recovery of the precipitate in 0.05 M Tris–HCl buffer, pH 7.4, with 0.005 M $CaCl_2$, extensive dialysis against the same buffer, and preservation either at 4 C or below 0 C. Intermittent addition of serum to the cultures will prolong the time that the tissue may survive as an explant, but the active collagenase in the tissue culture medium will disappear during the periods in which serum is present.[78] Although this peculiar behavior of tissue explants has been attributed to various inhibitors in the serum, the explanation may be more complicated. Nevertheless, the pooled, serum-free tissue culture medium supporting the survival of small tissue fragments for approximately 10 days has yet to be superseded by a more efficient technique for obtaining larger amounts of crude collagenase. Culture of pure cell lines has also been successful, especially fibroblasts and sinovial cells,[79,80] but the technique is more demanding and so far no reports are forthcoming to justify their superiority (but see Reference 78).

Tissue culture media that show little or no collagenolytic activity may be activated by limited proteolysis with trypsin,[59,78-86] other proteases,[87-91] or endogenous substances not yet well characterized.[92-100] Treatment with 3.0 M NaSCN, 3.0 M NaI,[101,102] or a thiol-binding reagent, 4-aminophenyl mercuric acetate,[103] will also activate crude collagenase preparations. Tissue explants or cell cultures are stimulated to increased synthesis of collagenase by colchicine,[104] heparin,[105,106] lipopolysaccharides,[107] cytochalasin B,[108] lymphokines,[109] lymphocyte extracts,[110] and phagocytosis.[111] The same effect has been observed in bone explants with parathyroid hormone,[112,113] calcitonin,[114] and in tadpole tissue explants with thyroxin.[115]

Direct Tissue Extracts. Several workers have described collagenolytic activity in extracts prepared by various means. Nagai and Hori [116] were able to extract collagenase directly from tadpole back skin and rat dermis and also from human synovial membrane. Other authors have isolated the enzyme from homogenates of embryonic chick bones,[117] from involuting mouse uterus and mouse skin,[118] and from involuting rat uterus, liver, and kidney.[119] The various methods have in common the use of a 6000*g* pellet and either heating at 60 C for short periods or exposure to denaturing agents such as 5.0 M urea. A recovery of 60 to 70% of the total enzyme activity in the tissue has been claimed.[119] These authors seem to be extracting collagen-bound collagenase, which is discussed next.

Collagen-Bound Collagenase. The well-known instability of cold neutral salt solutions of pure collagen extracted from various animal sources and from different species has been found to be due to collagenase,[120] which is normally bound *in vivo* to all structural forms of collagen [121] and accompanies the substrate throughout the various steps during extraction and purification.[122] Two techniques are available to separate the enzyme from collagen: one method involves acrylamide gel electrophoresis of denatured collagen at pH 8.1 and elution of the gel section containing the single protein band which migrates in the lower half of the gel; the other method is based on the resistance of collagen-bound collagenase to trypsin digestion and is performed by prolonged exposure of denatured collagen to the action of trypsin with continued dialysis. At the end of 5 days, all collagen has been digested and dialyzed away and the dialysis bag contains nothing but trypsin and collagenase.[122]

These two procedures result in highly purified collagenase, as demonstrated by their ability to stimulate the production of monospecific antibodies with enzyme-inhibitory capacity. By immunodiffusion it has been shown that such antibodies reveal identity between a component of crude collagenase preparations obtained by the tissue culture explant technique and collagen-bound collagenase purified by either of the two procedures described above.[122]

It should be emphasized that the yield of purified enzyme protein obtained by all methods described so far is extremely low and has prevented studies of enzyme kinetics, turnover, chemical composition, structure, and many others that would greatly increase the very limited understanding of this interesting protein. High yields have been reported using serum-free cultures of fibroblasts derived from human skin;[78] it is also possible to obtain larger amounts of relatively pure collagenase from polymorphonuclear leukocytes [123] and from human platelets.[124] The relationship of these intracellular (and presumably lysosomal) enzymes to secreted and to collagen-bound collagenases is yet to be determined.

Assay of Collagenolytic Activity

The basic principles and general methods for detection of true collagenolytic activity have been mentioned. A combination of several techniques should always be used, aiming at two goals: the establishment of change in some physicochemical or biologic property of native collagen, as a result of its exposure to the experimental preparation, and the demonstration of the products of peptide bond cleavage occurring in the helical portion of the collagen molecule.[59,125] Some of the methods available for exploration of each of the two events mentioned are summarized in

Table 1—Assays for Collagenolytic Activity

Assay	Significance
Change in physicochemical or biologic properties of native collagen	
Viscometry	Changes in length and helical content of the tropocollagen monomer
Optical rotation	Changes in helical content of the tropocollagen monomer
Melting curve (T_m)	Geometric order along the polypeptide chains
Turbidity	Changes in length and helical content of the tropocollagen monomer
Gel lysis	Clearing and solubilization of collagen fibrils formed at 37 C
Release of hypro-containing or radioactively labeled collagen peptides	Change in insolubility at 37 C
Identification of products of peptide bond cleavage in the helical portion of the collagen molecule	
Disk electrophoresis in polyacrylamide gels	Fragments of molecular weight smaller than alpha subunits
SLS crystallites	Changes in length of the tropocollagen molecule
Filtration through Millipore of appropriate size (<100,000)	Fragments of molecular weight smaller than alpha subunits
Chemical isolation of collagen fragments	Changes in solubility of collagen fragments smaller than alpha subunits

Table 1. In our laboratory we usually assay collagenase by the following combination of techniques:

1. Release of soluble material to the supernatant of a radioactively labeled, native collagen solution, as a consequence of exposure to some potentially collagenolytic preparation. The method developed by Teramoto et al [126] has been particularly useful for this purpose. Controls, including reaction mixtures incubated in the presence of specific collagenase inhibitors such as EDTA, cysteine, or whole serum, as well as in the absence of the enzyme, should always be part of the assay. Interference with the precipitation of intact collagen molecules would give the same results as release of cleaved peptides.

2. Acrylamide gel electrophoresis at pH 5.5 of the supernatant of the above reaction mixture according to a simple modification of the original technique published by Sakai and Gross [127] permits the identification of molecular species of smaller size and/or different charge than beta or alpha subunits. Under appropriate conditions, TC_A and TC_B fragments, the fingerprint of specific animal collagenolytic activity, are identified by this procedure.

3. Whenever possible, an additional or alternative method for the unequivocal detection of reaction products of true collagenolysis should be used, namely measurement of the length of SLS crystallites prepared

from substrate preparations after exposure to potentially active enzyme mixtures.[74,128] As recommended for assays aimed at detecting a change in physicochemical properties of the substrate, controls including the absence of the enzyme preparation and some potent inhibitors such as whole serum, cysteine, or EDTA should never be omitted.

If everything goes well, an increase in radioactive material in the supernatant of a reaction mixture containing labeled substrate and a suspected collagenolytic preparation, which fails to appear in controls containing specific inhibitors, should simultaneously reveal a change in the control pattern of acrylamide gel electrophoresis of the reaction mixture (decrease in the normal collagen fingerprint and the appearance of additional molecular species migrating below the level of dimers and monomers are the expected result). SLS crystallites prepared from the supernatant should have decreased dimensions, conforming to the classic TC_A and, less frequently, TC_B fragments of collagen monomers.

Interpretation

This is a very sensitive problem, mainly because it usually refers to degrees of enzymatic activity established in ignorance of the actual amount of enzymatic protein in the reaction mixture. The basic difference between the interpretation of these two parameters should be underlined since control of collagen degradation could be the result of several different mechanisms, such as variations in enzyme concentration, in enzyme activity, or in substrate susceptibility or different combinations of some or all of these factors. We would like to know exactly what is going on in the living tissues at a given time, usually when damage is occurring. Unfortunately, there is no information available on this very important problem.

Catalogue

Using the techniques outlined above, an imposing catalogue of animal collagenases has been built in the past 15 years.[59,62,67] The impression gained from the field is one of healthy growth, confirming early predictions that collagenases should be universally distributed in animal tissues. The current feeling is that physiologic degradation of collagen occurs everywhere in multicellular organisms, albeit at different rates, and that the specific enzymatic work is performed by collagenases. Information published as late as November 1977 is summarized in Table 2. It has been compiled to give a general overview of the state of the art rather than an exhaustive tabulation. It should be noted that collagenases are synthesized and secreted by many different types of epithelial and mesenchymal cells, both normal and abnormal. Some animal models, such as the

Table 2—Animal Collagenases

Species	Sources
Human	Skin, bone, synovium, synovial fluid, gingiva, cornea, stomach, embryonal skin, cholesteatoma, tumors, granulocytes, macrophages, fibroblasts, synovial cells, platelets
Bovine	Gingiva
Dog	Erupting teeth
Goat	Bone
Pig	Synovium
Rabbit	Skin (wound), cornea, synovium, bone, uterus, colon, cartilage, granuloma, tumors, fibroblasts, macrophages
Guinea pig	Skin, skin wound, bone, granuloma, macrophages
Rat	Skin, uterus, bone, cornea, liver (CCl_4), kidney, granuloma, tumors, Kupffer cells, eosinophils, neutrophils
Mouse	Skin, bone, uterus, liver, macrophages
Chicken	Skin, embryonal skin, bone
Tadpole	Tailfin, skin
Newt	Regenerating limb
Crab	Hepatopancreas
Flat worm	Whole body
Fish	Pylorus
Snake	Poison

metamorphosing tadpole tail, the postpartum rat uterus, and the carrageenin granuloma, as well as some human tissues, especially synovial explants and skin fibroblasts, have been widely used by several groups of investigators. There seem to be two reasons for the preference: a) tissues undergoing massive degradation of collagen amplify phenomena which occur at very slow rates under basal conditions and b) pure cell line cultures simplify the vexing complexity of whole tissues and the almost insurmountable problems of intact animals. The operational advantages of amplification and/or simplification are not enjoyed for nothing, since simultaneously they create the need to validate rigorously any extrapolation of the experimental results to the physiologic and/or pathologic situation in intact animals or humans. However, this is one of the rules of the game.

Molecular Forms

Collagenases have been claimed to occur in animal tissues and fluids in at least three molecular forms: a) latent enzyme, b) free enzyme, and c) collagen-bound enzyme. An unsolved controversy revolves around the nature of latent enzyme; many investigators [78-101] claim that these molecular forms of collagenase are true zymogens or precursors of the active form of the enzyme, which are synthesized and secreted as such by different cells. Such proenzymes would require activation by specific mechanisms, which may be partial proteolysis by other enzymes, so-called autoactiva-

tion, or still other, less well-known factors. Other workers [103,129,130] suggest that all latent collagenases are enzyme-inhibitor complexes and that activation represents dissociation of collagenase from inhibitors. The point is not esoteric since in either case regulation of enzyme activity would operate through entirely different mechanisms. It is certainly possible that both positions are real and either that the two mechanisms will be found to coexist in the same tissue at a given time or that one mechanism will prevail under a given set of circumstances and the other will operate under different conditions. What is urgently needed is that proponents of both theories present evidence in support of the actual existence *in vivo* of the molecular form of collagenase they believe to exist. The burden of proof rests on the investigators suggesting the presence of a given molecular form of collagenase in living tissues, namely those who assert that all latent forms of the enzyme are enzyme-inhibitor complexes.[103,129,130] It is much easier to prove the presence of something than it is to exhaustively document its absence under all circumstances: there will always remain one untried experiment.

Active, free collagenase is an operational entity, but this is far from being a physiologic reality. Much of the early work in this field is based on the demonstration of free collagenolytic activity in various experimental *in vitro* models.[78-101] Unequivocal proof of active collagenase in synovial fluid obtained from patients with rheumatoid and other forms of arthritis [101,102,131] supported the claim that active collagenolytic enzymes may be present *in vivo*. There is no doubt that collagen is degraded under normal conditions, even if the turnover rate of this protein is very slow, and this fact demands the presence of active collagenase in living tissues. The problem is not one of existence but of regulation of free enzyme activity. Is all active tissue collagenase derived from the transformation of proenzyme into catalytic molecules, or is there a reserve of enzyme-inhibitor complexes that is activated by different mechanisms according to local metabolic needs? The question has yet to be answered and will remain as such as long as techniques applicable to whole tissues or intact animals are not developed.

The demonstration of the existence of widely distributed collagen-bound collagenase in normal animal tissues [120-122] has created new problems. Here is an actually or potentially active collagen-degrading enzyme firmly bound to its substrate and capable of specifically cleaving native collagen molecules when placed under conditions optimal for enzymatic activity. Any theoretic scheme devised to explain the regulation of physiologic degradation of collagen should take this observation into account. Accordingly, a hypothesis on the control of collagen breakdown has been developed [132] and is summarized below. (See *Regulation*.)

Mechanisms of Collagenolysis

It is fairly well established that pure preparations of almost all types of animal collagenases cleave native soluble collagen or reconstituted collagen fibrils at a single peptide bond along the helical portion of the molecule when incubation is carried out at physiologic pH and ionic strength and at temperatures below the denaturation point of the reaction products (30 C).[133] In this context, purity of the collagenase preparations means absence of other proteolytic enzymes, which is not easy to achieve. The specific peptide bond cleaved by collagenases occurs between Residues 772 and 773 of the alpha 1 subunit of Type I collagen, which are -Gly-Ala-, and between the presumably corresponding residues in alpha 2 chain, represented by -Gly-Leu-.[134-139] At the electron microscopic level, cleavage occurs between Bands 41 and 42, according to the numbering by Bruns and Gross.[24] The two peptides resulting from collagenolytic cleavage are uneven in size, the larger one accounting for approximately 75% of the collagen molecule and the smaller one for the remaining 25%; the two peptides are known respectively as TC_A and TC_B.[127] Although few systematic studies have been reported on the specificity of collagenases for collagen, the enzymes seem to attack few or no other proteins; the exception is leukocyte collagenase, which in highly purified form will also degrade fibrinogen and proteoglycans.[123] On the other hand, collagenases recognize no substrate species specificity, ie, regardless of their source, collagenases degrade collagens extracted from many different animal species with equal efficiency. Variations in the relative susceptibilities of collagen Types I, II, and III to rheumatoid synovial collagenase have been reported,[140,141] Type III being the more susceptible collagen and Type II the least; it has also been suggested that Type III collagen is resistant to leukocyte collagenase.[142]

When incubation of reaction mixtures containing active collagenase and native collagen as substrate is carried out at temperatures above the denaturation point of the reaction products, especially at 37 C, some animal collagenases will initiate degradation as previously described but will continue to break the two uneven peptides down to dialyzable sizes.[143-148] Because denatured collagen becomes susceptible to nonspecific proteases and also because truly pure preparations of collagenase are so rare, it is unwise to discard the possibility that further breakdown of collagen fragments produced by collagenase is due to "contaminating" proteases. The alternative possibility that some collagenolytic enzymes are capable by themselves to carry collagen degradation beyond the TC_A and TC_B level when incubation is at physiologic temperatures remains to be disproved.

It appears that the kinetics of collagenolytic attack depend a great deal

on the degree of aggregation and cross-linking of the substrate. Under identical conditions of incubation, soluble collagen molecules are more susceptible than reconstituted fibrils [149,150] and the susceptibility decreases with the number of cross-links in the substrate.[149,151-156] This has been shown experimentally by measuring the relative rates of degradation of collagens cross-linked with different numbers of methylene bridges introduced with formaldehyde.[151] The significance of this observation cannot be overemphasized, since it throws light on one of the more vexing questions in this field: collagen degradation as it occurs in the tissues of multicellular organisms can hardly be compared with what is observed in test tubes containing mixtures of purified enzymes and soluble substrate. Understanding of complex problems must begin by analysis and simplification, but after enjoying the neat and clear-cut insight gained from the examination of such artificial (and artifactual) models, the climb back to the hazy and complex heights of real living tissues is both difficult and treacherous. Test tube enzyme kinetics are fine as a first approach to the understanding of basic molecular mechanisms, but they are not an end in themselves; they are just operational means to penetrate the darkness represented by normal and/or pathologic conditions in tissues or whole animals.

The effect of collagenases on collagen not extracted and purified but present in the tissues in its normal state of aggregation has been studied in various experimental models. When a 0.02% solution of human skin collagenase is incubated with approximately 2 to 3 mg of whole fresh human skin dermis at 37 C for 14 hours, approximately 25% of all hydroxyproline in the tissue is released into the medium, compared with no liberation of hydroxyproline in the absence of the enzyme.[156] A similar experiment conducted with human synovial collagenase and ^{14}C-labeled guinea pig skin after extraction of salt soluble collagen gave similar results.[157] Rat tail tendons, suspended by a glass weight in buffer and incubated at 37 C, were digested to the point of rupture by the same enzyme. Canine patella cartilage was also degraded by human synovial collagenase in the absence of enzymes that degrade protein-polysaccharide;[158] all that remained after incubation was a soft, transparent film, and electron microscopic study of the cartilage revealed depletion of collagen fibers. The preceding observations support the view that collagenases are capable of degrading a certain fraction of the collagen in organized tissues, but they fail to disclose which fraction is the susceptible one. The experimental concentrations of the added enzyme and the incubation times are completely arbitrary; there must be something very artifactual in an experiment in which 25% of all collagen in a

skin sample is solubilized (degraded?) in 14 hours. The result in the control (incubated in the absence of exogenous collagenase) is physiologically closer to what could be anticipated: no detectable collagen degradation in that period. Leibovich and Weiss [152] reported that purified rheumatoid synovial collagenase at concentrations capable of degrading collagen in solution at neutral pH in characteristic fashion did not degrade polymeric (insoluble) collagen from human synovial tissues. They suggested that an essential prelude to collagenolytic attack was depolymerization by other proteinases, presumably derived from lysosomes. Although Harper and Gross [63] were able to show 50% degradation of the same insoluble substrate when exposed to semipurified tadpole collagenase, the original observation of Leibovich and Weiss remains unchallenged since they did not claim that polymeric collagen was resistant to human rheumatoid synovial collagenase but that, at enzyme concentrations at which soluble collagen is degraded, the insoluble fibers remain untouched. By raising the amount of enzyme to which the substrate is exposed, it is possible that all kinds of collagen will be degraded; however, when collagenase concentration is the limiting factor, the state of aggregation and other properties of the substrate become major determinants of susceptibility.

The preceding discussion may be summarized by stating that there appear to be two possible mechanisms of collagenolysis *in vivo:* a) a single enzyme, collagenase, is capable of initiating collagen degradation by cleaving a single peptide bond on the helical portion of the three subunit chains of the native substrate and continuing the further breakdown of the reaction products unaided by other enzymes but helped by their denaturation at physiologic temperature;[60,63] and b) the multiple enzyme mechanism suggests that several enzymatic steps are necessary to degrade native insoluble collagen, starting with depolymerization (catalyzed by nonspecific proteolytic enzymes), followed by specific cleavage by collagenases, and continued by digestion of denatured reaction products, first by specific endopeptidases and exopeptidases, then by peptidases of low specificity, and finally by nonspecific exopeptidases.[54,133]

Immunology

Despite the difficulties encountered by most investigators in attempts to obtain sufficient amounts of purified collagenases by the use of many techniques, some workers have succeeded in preparing monospecific antibodies against the enzyme.[121,123,159-160] Rigorous observance of several criteria of enzyme purity are, of course, indispensable in studies of this type. Impurities at concentrations below the sensitivity of many biochem-

ical techniques are still detected by the rabbit, which has the exasperating habit of producing antibodies directed against all kinds of contaminants in response to the injection of an antigen giving a single protein band in SDS–acrylamide electrophoresis! On the other hand, an occasional rabbit has endeared itself to the laboratory by giving a monospecific anti-collagenase antibody *d'emblé*, even when the antigen was obviously an impure enzyme preparation.

From the limited number of immunologic studies on collagenases, certain facts have emerged, which may be summarized as follows:

1. Collagenases obtained from different tissue sources from the same individual reveal immunologic identity.[122-159,163,167-171]

2. Collagenases derived from different individuals of the same species are also immunologically identical.[167-171]

3. There is some degree of immunologic cross-reactivity among collagenases of closely related species,[170] but the only demonstrated interspecies identity is between rat and mouse skin collagenases.[171]

4. Anticollagenase antibodies fail to distinguish between latent and active forms of the enzyme.

5. Fibroblasts in tissue culture derived from human skin are the only cells in which collagenase has been shown to be present in the cytoplasm by means of fluorescent-labeled anticollagenase antibodies.[172] The enzyme was present in a finely granular form throughout the cytoplasm.

6. Also by immunohistochemical techniques, several authors [121,164,165,172-174] have demonstrated that collagenases are present in the extracellular structures of normal animals. In one study [121] many different tissues of the normal rat were surveyed and collagenase was detected bound to the three major histologic types of collagen, ie, collagen bundles, reticulum fibers, and basement membranes.

7. A detailed examination of collagenase distribution in the rat uterus during postpartum involution by immunohistochemical methods [174] revealed that detectable collagenase decreases at the time of greater collagen degradation (24 to 72 hours post partum). This finding suggested that the enzyme bound to insoluble intact collagen is also insoluble and, therefore, visible with the immunofluorescent antibody.

8. Many anticollagenase antibodies specifically inhibit the enzyme activity, and in some instances the inhibition has been shown to depend on the concentration of the antibody. On the other hand, it is not known if the antigenic determinants on the collagenase molecule include, or are in any way related to, the active site, since all studies have tested the collagenolytic activity remaining in the supernatant of the incubation mixture and have ignored the precipitate.

The potential of immunologic studies of collagenases is still in its infancy. Immunoabsorbent purification techniques, immunofluorescence surveys of collagenase presence and distribution in many diseases and general pathologic processes, enzyme turnover studies, discrimination of proenzymes from enzyme inhibitor complexes and perhaps other molecular forms of collagenase, isolation of polysomes with attached nascent enzyme polypeptide chains are only a few of the many different avenues for research opened by the availability and judicious use of specific anticollagenase antibodies. The major problem has been the preparation of sufficient amounts of pure enzyme to use as antigen. Nevertheless, the current state of development of this aspect of research in animal collagenases leaves the reviewer with a sense of hopeful anticipation.

Regulation

One of the major aspirations in the field of animal collagenases is to understand their regulation in living tissues. This goal holds the key to many phenomena, both normal and abnormal, many of which are standing still, waiting for clarification of the mechanisms that will explain the complex relations of cells with their supporting structures. Starting with morphogenesis and ending with chronic degenerative diseases, disclosure of the regulation of collagenolytic activity within tissue microenvironments will surely represent a breakthrough in biology. There seem to be two major candidates for the primary role of the rate-limiting factor in physiologic collagen degradation: variations in enzyme activity and/or concentration and changes in substrate susceptibility.[63] Of course, the two mechanisms mentioned are not mutually exclusive, and it seems quite likely that both will be found to be involved to various relative degrees in different situations. This is in recognition of Murphy's famous II law: "Things are more complicated than they seem."

Variations in enzyme activity and/or concentration could result from two circumstances, namely changes in active enzyme synthesis or modulation of enzyme activity through activation of proenzymes or local fluctuations in inhibitors. It is possible to envisage regulation of local collagenolytic activity by changes in the total amount of active enzyme synthesized and secreted by local cells, responding perhaps to messages delivered by some soluble extracellular tissue component. The evidence for this, however, is almost entirely based on *in vitro* experiments, indicating that factors such as colchicine, heparin, cytochalasin B, lipopolysaccharides, lymphokines, and phagocytosis increase the amount of collagenolytic activity associated with various types of cells; hormones such as thyroxin and parathormone have also been implicated (see *Tissue*

Explants.) Little is known of the mechanisms involved and even less of the physiologic significance of such observations. Increased collagenase production has been claimed in situations in which massive collagen degradation is clearly documented, such as postpartum involution of the uterus,[71,72,147] carrageenin granuloma,[175–178] alkali-burn corneal ulcerations,[179–182] periodontal disease,[183–185] rheumatoid and other destructive forms of arthritis,[141,158,186–188] epidermolysis bullosa,[189,190] wound healing,[191,192] tumor invasion,[193–196] and several other pathologic processes. Decreased collagenase production has been implied in cirrhosis of the liver,[59,197,198] scleroderma,[59,199] osteopetrosis,[51,200] and other human diseases. Although a more detailed analysis of each of the above pathologic conditions is presented below, here it should be said that in no single instance is there convincing demonstration of an actual change in the total amount of enzyme protein per milligram of tissue. All that is available deals with changes in enzyme activity, which is an entirely different matter, and could be equally explained by variations in activation of proenzymes and/or local fluctuation of inhibitors. Such regulating mechanisms are also fully supported by a host of *in vitro* observations, some pertinent to the requirements for enzyme activity (chelating and denaturing inhibitors) and others revealing the existence of biologic activators and inhibitors of potentially physiologic significance. The physiologic and pathologic meaning of these observations cannot be ascertained, but it would be astonishing if all were to represent mere *in vitro* artifacts.

Regulation of collagenase activity by local modifications in the susceptibility of the substrate is also supported by many data, but again mostly based on *in vitro* models. The major factors involved have been mentioned, ie, prevailing genetic type of collagen molecules (Types I, II, III, or IV), state of aggregation (determined by time of synthesis and site on the collagen bundle), degree of cross-linking, and associated molecules. Perhaps the more dramatic demonstration of the influence of the genetic type of collagen on its susceptibility to specific collagenase attack is the difference in the rate of degradation of Types I and III by polymorphonuclear leukocyte collagenase [142] (other studies were previously mentioned; see *Mechanisms of Collagenolysis*). It has been established that recently synthesized collagen is more susceptible to specific degradation,[201,202] and there is visual evidence that collagen-bound collagenase is present on the periphery of collagen bundles, presumably occupied by younger tropocollagen molecules.[121] The higher the degree of cross-linking in collagen, either artificially created [151] or resulting from natural aging processes,[203] the greater their resistance to collagenase. Finally, it has been suggested that corneal mucopolysaccharides may protect collagen from breakdown

by corneal collagenase either by inhibiting the enzyme or by presenting a physical barrier which excludes the collagenase from its substrate.[204-206] The issue, however, remains controversial since recent work[207] demonstrated that porcine or human cartilage proteoglycans failed to significantly inhibit the effect of synovial collagenase on reconstituted collagen fibrils. It has been mentioned that collagen Type II (with a high carbohydrate component, characteristic of cartilage) is more resistant to specific collagenases.[140,141] It is difficult to ignore the weight of accumulated data supporting a regulating role for the substrate in the rate of collagenolysis, but more evidence is obviously needed before a decision is reached on its significance in each of the experimental and human models mentioned. By simple analogy (a weak consideration, to say the least) with intracellular soluble protein catabolism, substrate susceptibility should emerge as one of the major determining factors of the regulation of extracellular collagen degradation.[132,208] This is not an attractive proposition, since it creates new problems in a field already crowded with them. But there is enough evidence to warrant at least one or a few crucial experiments before it can be confidently relegated to the background of the stage of the *in vivo* regulation of collagenolysis.

Other Enzymes

The issue of enzymes other than specific collagenases participating in collagen degradation seems to have more lives than a cat. It keeps emerging in the literature,[133,209] at times openly disregarding the orthodox definition of a true collagenase, at other times disguised as aiding or complementary enzyme activities, necessary to either initiate or complete the dismantling of the macromolecular substrate, carried out primarily by a specific collagenase. Believers in the need for depolymerization of insoluble collagen fibrils prior to specific cleavage of the helical portion of native collagen have documented both the existence of neutral or acid depolymerizing enzymes[210-218] and the inability of partially purified collagenase to attack insoluble collagen.[152] Imaginative investigators[219-221] have interpreted the presence of *bona fide* collagen fibrils (or similar structures) within cytoplasmic single membrane-bound vesicles in mesenchymatous cells as evidence of intracellular degradation of collagen by lysosomal enzymes.[52,59,62]

Native collagen is attacked by monospecific proteases such as trypsin, pronase, and chymotrypsin but only at the nonhelical portion of the molecule, the so-called telopeptides, and the reaction products preserve practically all the physical and chemical properties of intact collagen, such as viscosity, high negative optical rotation, and the ability to gel at 37 C

and to form SLS crystallites of normal length and configuration.[133] Once collagen is denatured, it becomes susceptible to nonspecific proteases, which break it down to small, dialyzable peptides.[54] Degradation of native collagen by noncollagenolytic enzymes, cleaving the helical portion of the molecule under physiologic conditions of pH, temperature, and ionic strength, has been documented only for solutions of collagen Type III incubated with trypsin.[222] This is a very intriguing observation, suggesting perhaps that the heterogeneity of collagen extends to the susceptibility to various proteases, although the possible presence of collagen-bound collagenase, which perhaps can be activated by trypsin, was not rigorously excluded in this study. Solutions of purified collagen Type III and trypsin are not likely to occur as such in living tissues, so the extrapolation of this finding to physiologic situations remains to be explored.

A simplified but not unfair summary of views on the role of noncollagenolytic enzymes in collagen degradation would consider two different positions: a) all types of tissue collagens, if degraded at all, are broken down by specific collagenases without participation of any other proteolytic enzymes and b) collagen breakdown in living tissues is a multienzyme process in which specific and nonspecific catalytic molecules play various roles at different levels of substrate breakdown. Because of the stubborn tendency of Nature to become more complicated with each new finding, it can be confidently expected that the future picture will first turn out to be more complex before it becomes simpler.

Pathology of Collagen Degradation

Impatient readers, who first turned to this section of the paper looking for information quickly applicable to histologic diagnosis, should be warned that the remainder of this review represents an attempt to find pathologic significance in all the preceding data. Free speculation about mechanisms is mingled with solid observational facts, a practice not unfamiliar with, but not often recognized by, tissue diagnosticians. Day-to-day practice of pathology is rewarding on two different accounts, ie, the feeling that a proper label for tissue changes observed under the microscope is mandatory for selection of adequate therapy of individual patients and the conviction that the advance of medicine can only flourish by the combination of hard facts and original thoughts.

Classification

In view of the limited extent of present knowledge, the simplest way to classify disorders of collagen degradation is to separate them into two groups: a) those due to a pathologic excess in collagen breakdown and b)

Table 3—Diseases of Collagen Degradation

Excessive	Deficient
Corneal ulcers	Fibrosis
Rheumatoid arthritis	Cirrhosis of the liver
Other arthritis	Scleroderma
Epidermolysis bullosa	Osteopetrosis
Periodontal disease	Pulmonary fibrosis
Cholesteatoma	Diabetes mellitus
Tumor invasion	
Paget's disease of bone	
Pulmonary emphysema	
Wound healing	

those secondary to abnormal deficiency in the mechanism of collagen degradation. Table 3 lists not only probable but also possible disease entities and general pathologic processes in which abnormal catabolism of collagen may play a pathogenetic role. It is not implied that such mechanism is the sole, or even the major, factor in tissue damage in these conditions. Although that may be the case in some of them, such as alkali-burn corneal ulcerations or carrageenin granuloma, it is almost certain that conditions such as rheumatoid arthritis, periodontal disease, or cirrhosis of the liver will be found to result from the additive effects of several pathologic mechanisms producing either direct tissue damage or superimposed changes responsible for amplification, perpetuation, or irreversibility of the lesions. No distinction has been attempted into primary and secondary disorders of collagen degradation, although it is conceivable that such classification will be possible in the near future: primary disturbances in collagen degradation would be due to changes in the relevant enzymatic mechanisms, while diseases caused by secondary abnormalities in collagen breakdown would result from pathologic modifications in the susceptibility of the substrate. Nevertheless, such information as is available on this aspect of the molecular pathology of collagen will be mentioned at the appropriate places in the following discussion.

Excessive Collagen Degradation

Corneal Wounds

Although few practicing pathologists routinely examine eye tissues, either as surgical specimens or as part of postmortem studies, corneal ulcers have been chosen to open this discussion of the pathology of collagen degradation because of the success-defeat-success or Cinderella character of the story. The cornea is formed by orthogonally arranged layers of collagen fibers covered on the outer surface by an epithelium of

ectodermal origin. The perfect regularity of the alternating layers of collagen fibers creates a quasi-crystalline structure which accounts for the translucence of the organ. There is overwhelming evidence supporting the epithelial origin of the corneal stroma. Injury to the surface corneal epithelium results in late destruction of underlying collagen, with eventual ulceration and loss of vision. This sequence of events has been known for years and is the unhappy outcome of conditions such as alkali burns, radiation or rosacea keratitis, ocular pemphigoid, scleroderma, and Steven–Johnson syndrome.[179-182,204-206] Another condition associated with destruction of the cornea is vitamin A deficiency; clinically, this is characterized by dryness of the epithelium, followed by edema of the stroma, keratinization of the epithelium, ulceration, and, finally, perforation of the cornea. This severe form is found mainly in children who are seriously malnourished, but the syndrome may be reproduced in rats deficient only in vitamin A.[223] Whatever the mechanism of epithelial damage, the destructive nature of the process and the almost pure collagenous structure of the tissue undergoing necrosis and dissolution first suggested that a collagenase might be at work.[224] Although early tests of this hypothesis were carried out with an artificial substrate (PBZ–peptide, 4-phenylazobenzylcarbonyl-L-prolyl-L-leucyl-L-glycyl-L-prolyl-D-arginine) which is *not* degraded by purified animal collagenases but by nonspecific proteases, a typical mammalian collagenase was later isolated from *in vitro* cultures of corneal epithelium.[225-227] The enzyme was shown to degrade collagen molecules in solution and to degrade corneal collagen fibrils as well. In alkali-burned cornea, exposure to the injurious agent results in immediate death of all epithelial cells and loss of most of the proteoglycans of the organ. After approximately 1 week, the epithelium, accompanied by the vascularized stroma at the margin of the wound, begins a slow central progression. A very interesting feature of these lesions is that ulcerations are seldom seen before the end of the first week. The localization of the ulcer relative to the advancing edge of regenerating epithelium and underlying vascular stroma is also very suggestive since tissue loss is usually central to them while the peripheral area is intact. It was suggested that corneal ulcerations occurred in the central area of the lesion, surrounded by regenerating epithelium but still devoid of granulation (and highly vascularized) tissue. This requirement was pertinent in view of the belief at that time that collagenase inhibitors were mainly circulating proteins of high molecular weight, which could easily filter across the highly permeable barrier of newly formed capillaries. In the case of the vitamin-A–deficient rat, corneal ulceration is not preceded by loss and regeneration of epithelium but occurs when there is invasion

of the corneal stroma by polymorphonuclear leukocytes and capillaries. Additional studies, designed to bring the corneal collagenase into conformity with all other known animal collagenases, have been published,[228-236] and the effect of topical applications of enzyme inhibitors (such as EDTA, cysteine, and others) to experimentally injured eyes in rabbits, and also in humans, was quickly explored.[237-246] Results of these tests showed that continuing application begun shortly after burning the cornea with alkali would prevent the development of deep ulceration and perforation in a statistically significant number of animals, and the same measures were shown to be effective in humans. Some failures, however, were recorded, together with the need for frequent applications of collagenase inhibitors and also some evidence of the toxicity of these compounds.[247] A new approach to these problems has been developed by Newsome and Gross,[248] who reasoned that the inhibitory effect of progesterone on collagenase synthesis in postpartum uteri of rats[249] and rabbits[250] could be used to block the tissue destructive action of the enzyme in alkali-burn corneal ulcerations. These authors used medroxyprogesterone (Provera) in three therapeutic modes, ie, a single or twice daily instillation of one drop of the hormone solution in the subconjunctival sac, weekly subconjuntival depots of hormone and vehicle, and weekly intramuscular injections. Quantitative measurement of collagenolytic activity of treated and control corneas was carried out *in vitro*. All three modes of administration of medroxyprogesterone were strikingly effective, since 49 of 85 control corneas perforated, compared with 8 of 87 treated eyes, and only 4 of 85 control corneas healed without serious damage, compared with 51 of 87 hormone-treated corneas that healed. Collagenolytic activity of alkali-burned corneas treated with medroxyprogesterone was reduced to less than one half that of untreated controls. This careful study supports the causal role of collagenase in corneal ulceration secondary to alkali burns and also opens new therapeutic possibilities for the ulcerative corneal diseases.

The source of the damaging collagenase in corneal ulcerations has not been clearly established, and it may be different according to the etiologic factors of the process. Some data suggest that in alkali-burns collagenase is produced by the regenerating epithelium,[230,248] since ulcers rarely appear before 7 days after alkali exposure and no new lesions develop later than 30 days after the burn. Ulcers bear no specific relationship to the blood vessel ingrowth, and *in vitro* collagenolytic activity of burned corneas is inhibited by cycloheximide or freeze-thawing, thus eliminating the possibility that polymorphonuclear leukocyte collagenase (a lysosomal enzyme) is involved. On the other hand, corneal fibroblasts have been shown

to produce specific collagenase *in vitro*,[225-227,237] and they remain a possible source for the enzyme. In vitamin A deficiency, corneal ulceration occurs when the stroma shows rich capillary ingrowth and massive infiltration by polymorphonuclear leukocytes;[223] it has been suggested that conjunctival collagenase [251] may also play a role in the destruction of corneal structures in this experimental model.

Although somewhat remote from the daily practice of the pathologist, corneal ulcerations have been reviewed with some detail because they teach a very important lesson: the molecular mechanisms of dramatic tissue changes may be subtle, as inconspicuous morphologically as their consequences may be overwhelming. The primary role of the cornea is to be transparent, and this is accomplished by preservation of its almost perfect orthogonal distribution of paracrystalline fibers. Interference with this distribution may be brought about by different mechanisms, one of them being excessive degradation of the collagenous framework. To the unsuspecting eye, broken down collagen fibers or "increased eosinophilia with loss of fiber continuity, scarce cellular infiltration, and some capillary proliferation" will mean very little. An entire new field, however, is to be found by those interested in molecular pathology.

Rheumatoid (and Other) Arthritis

Here is a more familiar field for pathologists. Rheumatoid nodules, proliferated synovial tissue, partially destroyed joint structures, skin, striated muscle, kidney, and salivary gland biopsies are generated by many active and forward-looking groups of rheumatologists. Of course, lupus erythematosus, rheumatoid arthritis, dermatomyositis, scleroderma, mixed connective tissue disease, vasculitis, and several other so-called collagen diseases will be high in their diagnostic agenda. Microscopic examination of this wealth of material may be thought uninspiring, unless it is performed against a solid background of current information on infectious, immunologic, enzymatic, and other possible mechanisms of tissue damage. Abnormal collagen degradation has been implicated in at least two of the previously mentioned diseases, ie, rheumatoid arthritis and scleroderma; the latter disease is discussed later.

Chronic active rheumatoid arthritis is a destructive disease, often resulting in joint erosion, deformity, and partial or complete loss of function.[252] One of the first events in the development of articular damage is the proliferation of synovial cells, together with inflammation and vascular neoformation in the stroma of synovial tissue. Such inflammatory reaction, or pannus, is apparently capable of eroding collagenous structures such as cartilage, bone, tendons, and ligaments.[253] As the disease pro-

gresses, the proliferating synovial tissue extends over the articular cartilage and erodes from the joint surface down to and including the subchondral bone. Neighboring chondrocyte lacunae appear enlarged, and the cells may have two or more nuclei. In advanced lesions, only small islands of articular cartilage remain and there is extensive resorption of subchondral bone; synovial granulation tissue may reveal areas of proliferated synovial cells with epithelioid character.[254] Polymorphonuclear leukocytes are infrequent; most inflammatory cells are mononuclear, with a predominance of lymphocytes and plasma cells. Electron microscopic study of the interface between mesenchymal cells and articular cartilage revealed a narrow band several microns wide separating cartilage from invading cells. In this zone, presumably partially degraded collagen fibers, amorphous material, and thin cytoplasmic processes of macrophage-like cells containing dilated vesicles of rough endoplasmic reticulum were seen.[254]

Thus, the joint lesion in chronic active rheumatoid arthritis would seem to involve two different processes: a primary, apparently inflammatory and proliferative reaction of synovial tissue to an unknown stimulus and a secondary, degenerative, and destructive lesion, which is probably a consequence of the invasive capabilities of the rheumatoid synovium.[255-257] Although loss of proteoglycans[258] and other connective tissue components may decrease the natural resistance of cartilage to mechanical breakdown, it appears unlikely that such mechanism is responsible for all deformities and erosions of rheumatoid joints.

Collagenases have been demonstrated using different preparations derived from human rheumatoid synovial tissue or fluid.[60,67,90,91,101,102,116,131,140,141,150,157,158,168,169,186-188] The first report established the release of collagenase by explants of rheumatoid synovium *in vitro*, and it was soon followed by a more complete characterization of the enzyme.[158] Further studies have presented some conflicting evidence regarding the number of active collagenolytic components produced by the *in vitro* system, with one group claiming that there are two[169] and another that there is only one.[150] Using synovium obtained by closed needle biopsy, it was also shown that rheumatoid arthritis is not the only joint disease with excessive production of collagenase but that a similar situation holds for scleroderma, Reiter's syndrome, pseudogout, and degenerative arthritis.[259] Another source of collagenase in rheumatoid arthritis is synovial fluid;[101,102,131,141,158] in approximately 10% of the patients studied, a free active collagenase was demonstrated, despite the presence of potent serum inhibitors, such as α_2-macroglobulin.[131] In this system two distinct collagenases were separated by gel-filtration chromatography. The larger

one (enzyme "A," 50,000 daltons) was resistant to the inhibitory action of serum, whereas the smaller component (enzyme "B," 30,000 daltons) was inhibited by serum and seemed identical to the collagenase found in cultures of synovial tissue. More recently it has been recognized that enzyme "A" is probably derived from polymorphonuclear leukocytes, although the evidence is circumstantial.[60] Latent collagenase is present in large amounts in rheumatoid synovial fluid.[101,102] Finally, another source of synovial collagenase is isolated adherent rheumatoid synovial cells of obscure identity but without macrophagic or fibroblastic markers. These cells have been found to produce not only high quantities of collagenase for prolonged culture periods but also prostaglandin E_2, which has been shown to accelerate bone resorption by osteoblasts.[260] In this *in vitro* system, collagenase production may be stimulated up to 400-fold by a human lymphocyte soluble factor with an apparent molecular weight of approximately 12,000; the *in vitro* production of such lymphocyte factor is enhanced by phytohemagglutinin or concanavalin A and is inhibited by incubation at 4 C.[100] Collagenase has been identified by immunofluorescence at the cartilage-pannus junction in rheumatoid arthritis.[260a]

In addition to the collagenases previously mentioned, at least two other neutral peptidases have been described in relation to synovium. One, an endopeptidase capable of degrading gelatin to small fragments, has been found in the mediums from cultures of rheumatoid synovium.[261] This endopeptidase, which does not degrade undenatured collagen, is inhibited by EDTA, by serum proteins, and by cysteine. The other neutral protease has been identified in synovial fluid, was present in sonicates of polymorphonuclear leukocytes, and attacked the telopeptides of fluorescein-labeled polymeric collagen fibrils in a fashion similar to trypsin, ie, without causing depolymerization of the substrate.[211] The possible role of these two enzymes in collagen degradation is still uncertain, but it has been suggested that the endopeptidase from rheumatoid synovial tissue is responsible for at least part of the subsequent breakdown of the primary products of collagenolysis. Although fibroblasts derived from human synovial tissue have not been found to secrete collagenase (in difference with skin fibroblasts, see *Epidermolysis Bullosa*), rabbit synovial fibroblasts in culture release both a collagenase and a neutral proteinase,[262] and the rate of secretion of these two enzymes is increased after the ingestion and storage of latex particles within the vacuolar system of the cells.[111]

There is abundant evidence that the various collagenases described in relation to human rheumatoid synovium are capable of degrading articular cartilage, used as substrate either in slices or as whole patella or as purified $(\alpha\ 1[II])_3$ soluble molecules in fibril form.[140,141,149,150,153]

In summary, then, collagen degradation is one of the major features of chronic active rheumatoid and other forms of arthritis, collagenases are produced by rheumatoid synovial tissue and cells *in vitro*, latent and active collagenases are present in rheumatoid synovial fluid, and such collagenases are capable of degrading articular cartilage. In addition, other neutral proteases that could continue breaking down the initial products of collagenolysis are also present. Much of the research summarized in this section was stimulated by a fruitful hypothesis which not only attempted to explain the mechanism of articular tissue damage and destruction in chronic rheumatoid arthritis but also carried significant therapeutic implications. The hypothesis suggested that locally increased collagenase and endopeptidase activities are responsible for collagen breakdown and resorption, once they manage to sidestep the blocking action of powerful inhibitors. This may be accomplished by saturation of all inhibitor molecules present or by the secretion of an inhibitor-resistant enzyme. A useful test of the hypothesis would be the prevention of joint damage by therapeutically decreasing the amount of collagenase production, as in alkali-burn corneal ulcers,[248] by some agent that will act in a chronic and sustained way. This would not only add support to the hypothetic mechanism of tissue damage but would also contribute greatly to alleviate the misery of many patients.

Epidermolysis Bullosa

This generic term designates a heterogeneous group of hereditary diseases characterized by the formation of blisters on minor mechanical trauma to the skin.[263-265] Three genetic types of dystrophic epidermolysis bullosa are recognized, which are also distinguished by several clinical parameters but have in common the dermolytic type of blister characterized by a sharp separation of the entire epithelium from the dermis below the basement membrane on electron microscopy. In addition, anchoring fibrils are usually absent and [266] there is attenuation of elastic fibers, vascular neoformation and round cell infiltration in the dermis, and phagocytosis of collagen fibrils by macrophages.

The dominantly inherited form of the disease is less severe than the recessive, and there is a localized recessive type which may not be present at birth but develops at a later age, usually in infancy or childhood. Another recessive type of the disease is epidermolysis bullosa letalis. This is a very widespread affection, present at birth, with severe involvement of all the skin but often sparing distal extremities, characterized by cleavage between the basement membrane and the plasma membrane of the basal cells of the epidermis.[267] Histologic differentiation between the various

types of epidermolysis bullosa is not possible; genetic and clinical evaluation and electron microscopy of very early blisters, usually produced by friction a few minutes before biopsy, are necessary to properly classify the disease.[268,269]

The similarity of dermal ultrastructural changes on the skin of patients with the recessive form of dystrophic epidermolysis bullosa to those observed in the metamorphosing tadpole tailfin when increased collagenolytic activity is present in the tailfin suggested that the enzyme might play a role in the human disease.[189] Five patients with dystrophic epidermolysis bullosa were studied (it was not possible to establish the type of inheritance since no relatives were affected, but clinically they appeared to represent the recessive form) by preparing blister and uninvolved skin explants on radioactive collagen gels. Results were expressed in specific activity, ie, cpm of ^{14}C-collagen solubilized per milligram of tissue cultured. Both types of explants, blister and uninvolved skin, revealed increased enzyme activity, the former approximately sixfold and the latter twofold, compared with normal skin controls. Interestingly, in a specimen in which separation of epidermis from dermis occurred, both tissues showed increased collagenolytic activity. On the basis of these results it was suggested that dystrophic epidermolysis bullosa is a generalized cutaneous abnormality characterized by the enhanced production of collagenase, although the possibility that there might be an associated deficiency of collagenase inhibitors in the serum was not discarded.

Two other bullous skin diseases, pemphigus vulgaris and bullous pemphigoid, were examined by the same technique but collagenolytic activity was not significantly elevated. In addition, Eisen [189] stated that no enzyme activity was detected in blister fluid, which was examined in all 5 cases. In our experience with a limited number of cases of dystrophic epidermolysis bullosa, blister fluid did contain collagenolytic activity, demonstrated by both increased solubilization of radioactive collagen and the presence of collagen breakdown products by acrylamide gel electrophoresis.[270]

The problem was restudied in 19 patients with various genetic types of the disease,[271] but this time the technique was not direct tissue explants on radioactive collagen gels but on culture medium, which was replaced daily for 7 days and assayed separately by both radioactive collagen and acrylamide gel electrophoresis methods. With these materials and techniques it was shown that uninvolved skin did not produce increased amounts of collagenase. Blistering lesions did show a twofold to threefold increase in collagenolytic activity, but this was considered secondary to tissue damage. The general conclusion of this work was that the primary pathophysiologic event in epidermolysis bullosa is not increased collage-

nase activity but is probably a genetically determined defect in the structure of the papillary dermis.

An uncontrolled study on the effect of vitamin E therapy in 2 patients with dystrophic epidermolysis bullosa[272] revealed that uninvolved skin had no significant increase in collagenolytic activity (3 and 6%, respectively, of collagen solubilized during a 4-day incubation period at 35 C) and that the higher enzymatic activity detected in blistered skin (15 and 21%, in the same conditions) returned to basal levels after 30 days of vitamin E therapy. This decrease in collagenolytic activity coincided with marked clinical improvement of both patients. It is clear that this study requires both controls and increased number of patients, but as it stands, it reflects some support for the lack of pathogenetic role of collagenase in epidermolysis bullosa.

More recently, a study of 40 patients with various forms of epidermolysis bullosa has been published,[187] this time by means of a radioimmunoassay for human skin collagenase. It is hoped that this study reflects what is going on in the tissue at a given moment more clearly than the *in vitro* techniques used so far. Results of this study indicate that there is a significant increase (fourfold) of collagenase in the uninvolved skin of patients with the generalized form of recessive dystrophic epidermolysis bullosa, an almost comparable increase (3- to 3.5-fold) in the localized form of the disease and in recessive epidermolysis bullosa letalis, and a slight but not statistically significant increase in patients with the dominant dystrophic variety of the disease. It should be emphasized that, since the antibody does not distinguish between latent and active molecular forms of the collagenase, it measures the amount of enzyme protein. Participation of increased collagenolytic activity in the pathogenesis of *in vivo* changes still requires an activating mechanism, which might be triggered by trauma and/or explantation of the skin. The possibility that the collagenase itself might be abnormal in recessive dystrophic epidermolysis bullosa has been briefly suggested in an abstract,[273] and the publication *in extenso* of the data is awaited with interest.

To summarize, a heterogeneous group of genetic diseases characterized by the formation of blisters after minor mechanical trauma to the skin has been studied in search of the possible pathogenetic role of collagenase. Although the evidence appears inconclusive, the uninvolved skin of patients with some forms of the disease contains higher levels of enzyme protein than do controls. The possibility that increased collagenolytic activity is involved in pathologic blister formation seems good, but final judgment must be deferred until more knowledge is available on this group of rare and interesting diseases.

Chronic Periodontal Disease

The major structural protein of the soft and hard periodontal tissues is collagen. The attachment of the marginal gingival tissue to the calcified tooth surface is through a basement membrane and hemidesmosomes. In addition, strong bundles of collagen fibers, originating from the cementum, just apical to the edge of the epithelium, pass through the gingival chorium and terminate in the free marginal gingiva. Finally, there are also the circular and alveologingival fiber groups, completing the bulk of the connective tissue of the gingiva. Disorganization of such supporting structure, which provides tone to the tissue and a framework for the rich capillary plexus subjacent to the junctional epithelium, results in increased pocket depth, loss of gum tissue, and loosening and, finally, loss of teeth.[184] Because chronic inflammation is the unfailing background of periodontal disease and also because the crevice region represents an interface between bacteria and connective tissue (a normal adult with 28 teeth may harbor approximately 16 mg of bacterial debris), it has been accepted for some time that bacteria initiate and perpetuate the sequence of events leading from acute inflammation to dental loss.[274-276]

Connective tissue changes in chronic periodontal disease have been documented by several techniques.[277-279] Actual decrease in the total collagen content of inflamed gingiva was established by microdissection (to separate infiltrated and noninfiltrated fractions of human biopsy specimens) followed by biochemical analysis. In two separate studies of this type [280-281] collagen reduction was found to be 60 and 70% of normal, respectively. There is also the suggestion that the size of the acid-soluble collagen compartment is more reduced than that of the other collagens. A pronounced decrease in hexosamine content has also been documented in inflamed gingivas.[282]

The heterogeneous bacteria forming the so-called microbial plaque have been held ultimately responsible for all the connective tissue changes characteristic of chronic inflammatory gingival and periodontal disease.[275,276,283] The bacterial debris in the gingival crevice fluid of patients with these disorders may increase from 2- to 20-fold above normal. There are innumerable potentially toxic substances in the plaque, many of them capable of producing disastrous tissue damage.[275] There are some collagenase-producing bacteria, such as *Bacteroides melaninogenicus* and *Clostridium histolyticum*, which could easily account for the gingival collagen loss. Nevertheless, there is some evidence that this is not the whole story and that perhaps in this condition there are some endogenous mechanisms that may be causing some of the tissue changes:

1. In contrast to acute necrotizing ulcerative gingivitis,[284] micro-

organisms do not appear to invade the gingival tissue in commonly encountered chronic inflammatory gingival and periodontal disease.

2. Explants of gingival tissue are capable of degrading collagen gels,[285-287] and this property is apparently independent of bacteria since gingiva from germ-free rats is also capable of producing collagenase.[288]

3. Both epithelium and fibroblasts derived from gingival tissue have been shown to be the source of collagenase.[286] The properties of the enzymes have been studied and the reaction products characterized, and both have been shown to be identical with all other animal collagenases.[289] Of special interest is that while gingival collagenase is inhibited by cysteine, the enzyme produced by *B melanogenicus*[290] is stimulated by this reducing substance.

4. Gingival fibroblasts produce a latent collagenase[291-295] which is activated by trypsin,[81,86] by a mast cell factor,[89,296,297] by a lymphocyte factor,[298] and by a thermolabile, nondialyzable microbial plaque factor.[98]

5. Crevicular fluid contains a collagenolytic activity positively correlated with the severity of gingival inflammation and pocket depth.[299,300] The electrophoretic pattern of the collagen breakdown products indicated its origin from tissues and not from bacteria.

In addition, it has been suggested on the basis of ultrastructural and morphometric studies[184] that decrease in collagen content in chronic inflammatory gingival disease may also be due to inhibition in collagen synthesis, perhaps by cytopathic alterations of fibroblasts brought about by sensitized lymphoid cells.

Tumors

The invasive nature of malignant tumors makes them natural candidates for collagenolytic enzymes. "Histolytic" substances have been postulated for a long time to explain the ability of malignant cells to invade the host tissues. The temptation would be high, in studies attempting to document the existence and nature of such substance, to prefer those neoplasms which are more aggressive and appear to overwhelm more easily the natural tissue barriers. One of the major uncertainties with the study of tumors is that unless pure neoplastic cells are studied, there is always a certain amount of nonneoplastic stroma which could be the source of the enzyme. In fact, there is some suggestive evidence that this may be the case, at least with some tumors. Of course, such findings would not negate *a priori* the possible significance of collagenase in the dissemination of cancer cells; it would turn the phenomenon from an aggression into a collaboration. On the other hand, pure tumor cells preserved in culture are several steps removed from their native habitat, and results

obtained may be more relevant to the potentiality of their physiologic repertoire than to their actual behavior *in vivo*.

A large number of human tumors have been examined for collagenolytic activity by a variety of techniques.[193-196,301-303] Although positive results are encouraging, and there is even a report of positive correlation between enzyme content and clinical outcome,[304] only a few of these studies have attempted to characterize the enzyme, and none offers definitive proof of its actual origin from the neoplastic cells. Malignant epithelial tumors, especially squamous and basal cell carcinomas of the skin and adenocarcinomas of the colon,[193,305-313] have a high frequency of collagenolytic activity, whereas sarcomas only rarely produce the enzyme. The study by Hashimoto et al[306] is interesting because they were able to detect collagenolytic activity in homogenates of squamous cell carcinoma, a feat that is very difficult to achieve with normal skin. A similar finding has been reported in one melanoma.[308] The well-known resistance of cartilage to invasion by neoplastic cells has been attributed to an inhibitor of collagenase; in this experiment collagenase was obtained from cultures of human osteosarcoma and mammary carcinoma.[314]

Others

It is tempting to include in this section several other conditions in which excessive collagen degradation may play a pathogenetic role, but unfortunately there is little or no evidence that this is the case. Pulmonary emphysema, especially in patients with deficiency of α_1-antitrypsin, Paget's disease of bone, osteoporosis, and pressure atrophy as in bedsores or destruction of bone by a growing arterial aneurysm have been suggested as possible results of excessive collagen degradation. In cholesteatoma, a nonneoplastic, proliferative disease involving both epithelium and connective tissue in the middle ear, the presence of neutral collagenases produced by both elements of the lesion has been established.[88,315,316] This makes it very likely that bone resorption, which is common in this disorder, may be the result of enzymatic degradation of collagen. A similar finding has been claimed for keratocysts or epithelial inclusion cysts, although the collagenase has not been well characterized.[317]

There is ample evidence that collagenase production increases during experimental wound healing.[191,192,318-320] Of course, one hesitates to call wound healing a "pathologic" process, although traditionally it occupies a prominent place in textbooks of general pathology. It is not normal, as witnessed by its absence from physiology treatises; perhaps it would be better to consider it a "natural" process. Of interest here is that wound healing involves not just deposition of collagen but extensive remodelling

of both old and new collagen fibers, and for this reason, at least in theory, there may be instances in which collagen degradation is overdone.

Experiments with colon anastomosis in rabbits suggested that between 4 and 10 days after surgery there is decreased collagen content in the tissues surrounding the healing wound, increased collagenase production, and a resulting decrease in the mean bursting wall pressure of the large intestine.[321]

Deficient Collagen Degradation

The ultimate goal of regenerative and restitutive processes in the diseased organism is the complete recovery of all structures damaged and all functions altered during destructive and incapacitating conditions. The degree to which this goal is achieved is, of course, extremely variable and depends on a constellation of factors. Regeneration is limited in many epithelial organs, and wound healing may be able to fill anatomic gaps but at the price of incomplete recovery of specialized functions. The resulting revised architecture is variously named scarring, fibrosis, or sclerosis. The visibility (or reality) of the initiating injury permits the distinction of two types of fibrosis, ie, those in which the stimulus is easily identifiable and, therefore, are considered as secondary or reactive fibrosis, and those without a recognizable cause or preceding damage, which then become primary fibrosis. A healed myocardial infarction and the pulmonary scar of a cured tuberculous cavity are examples of the first or reactive type of fibrosis; scleroderma and retroperitoneal fibrosis are instances of the second or primary type of fibrosis.

Tradition has created a rather pessimistic attitude in relation to fibrosis. A scar is uniformly considered as an end stage, and for good reasons (tradition is seldom empty of facts). Pathologists have contributed in no small measure to generate and support that tradition, since nothing can look more inert and permanent than a good, solid scar replacing epithelial tissue elements. But collagen is neither inert nor permanent; it has been shown to have a metabolic turnover, and the normal organism possesses the information necessary to regulate the amount and distribution of its own supporting structures. Irreversible loss of the mechanisms responsible for such fine regulation is a possibility in many diseases characterized by either reactive or primary fibrosis, but there is yet another possibility to be considered: increased knowledge of the mechanisms involved in collagen degradation may open the road to reversibility of at least some of the pathologic conditions currently considered end stages. It is likely that each disease with a fibrotic component, be it reactive or primary, will have

to be considered by itself, as a problem *sui generis*, but at the present state of knowledge some generalizations may be useful.

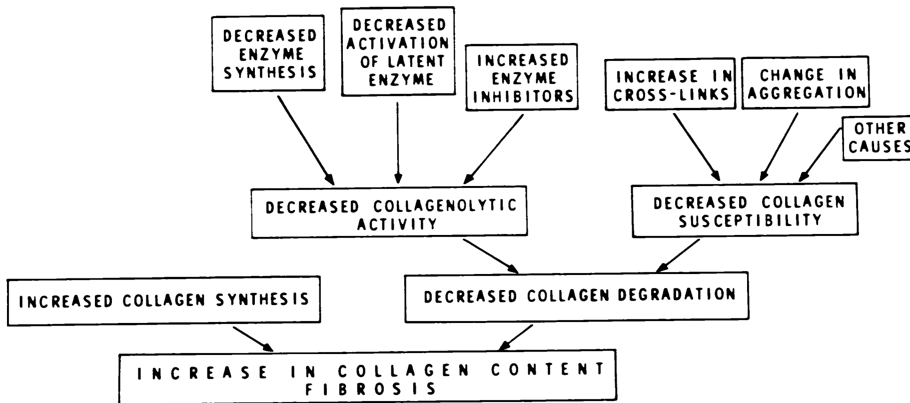
An abnormal increase in collagen content in a given tissue may be the result of one of three processes: a) increased collagen synthesis and deposition, with preservation of normal levels of collagen degradation; b) decreased collagen breakdown, with maintenance of collagen synthesis and deposition at physiologic levels; and c) a combination of both factors, independent of the levels at which they occur, provided that synthesis is always in excess of degradation. It has been mentioned that the level of collagen degradation may be due to two factors, ie, variations in the amount of active collagenolytic enzymes present or change in the susceptibility of the substrate. It was also mentioned that the amount of active collagenolytic enzymes present at a given moment is theoretically the result of a balance between three variables, which are activators, inhibitors, and active enzyme. If the reader feels confused, let me add that the reviewer is keeping him company. Perhaps reference to Text-figure 1 may help to integrate the various factors mentioned.

Fibrosis and Cirrhosis of the Liver

A wealth of information is available on different aspects of fibrosis and cirrhosis of the liver;[197,318-325] an international symposium devoted to the subject has been published.[326] Although excessive collagen deposition in the liver is not synonymous with cirrhosis, the irreversibility of the disease is probably linked to it. A simple way to look at the problem suggests that if excessive fibrous tissue could be eliminated, adequate dietary and perhaps other therapeutic measures would give better results in these unfortunate patients. At first view, this way of looking at cirrhosis of the liver disregards the mechanisms by which the disease is initiated and becomes established. Most concerned investigators subcribe to the concept that in cirrhosis of the liver, fibrosis represents a good example of reactive or secondary deposition of connective tissue.[325] It is equally possible, however, that at least in some cases the increased collagen content characteristic of cirrhosis of the liver is the result of a primary disturbance in the regulation of this extracellular macromolecule and, more specifically, of collagen degradation. The following data would support such heterodox position:

1. Normal rat liver is capable of reabsorbing most or all collagen deposited as a consequence of an acute localized parenchymal injury. On the other hand, CCl_4 cirrhotic rat livers preserve local scars as long as the disease is present.[198]

2. The experimental work mentioned above was prompted by the rarity



TEXT-FIGURE 1—Scheme of collagen synthesis and degradation.

and small size of liver scars in patients with adequately documented cured amebic abscesses of the liver in a large autopsy series.[327]

3. Collagenolytic activity is present in liver explants during the early stages of CCl_4-induced cirrhosis of the liver in rats.[328] In more advanced disease, such enzymatic activity has been observed to decrease. Collagenase has been detected in preparations of Kupffer cells of the rat liver,[329] in rat liver homogenates,[330] and in experimental mouse liver schistosomiasis [331]; an early paper suggested the presence of the enzyme in CCl_4-damaged rat liver.[332] Immunofluorescence studies have shown that collagenase is present both on collagen fibers in portal spaces and on reticulum fibers [121] in the normal rat liver.

4. Experimental CCl_4-cirrhosis of the rat liver has been shown to go through two stages: an early one, in which the process is reversible provided that the toxic is discontinued, and a late one, irreversible despite discontinuation of CCl_4 administration.[333-337] In these studies it was established that the presence of a metabolically inert collagen fraction in the liver parallels the onset of irreversibility.

5. Unpublished observations in our laboratory [338] have shown that in early, reversible CCl_4-cirrhosis in the rat liver the collagenous septums reveal collagen-bound collagenase by immunofluorescence methods. In the late, irreversible stage of the experimental liver disease, collagen-bound collagenase is absent from the fibrous tissue.

Thus, it appears that experimental CCl_4-induced liver cirrhosis is a reversible process as long as collagen-bound collagenase is detectable in the fibrous tissue septums. It is possible that this phenomenon is not limited to the specific CCl_4-type of experimental cirrhosis, since Henley et al [339] have suggested, on the basis of indirect data, that the collagen-

increasing effect of ethanol on the livers of animals receiving a cirrhogenic diet was due to an inhibition of collagen breakdown.

All the data summarized above are derived from experimental models of liver cirrhosis. Their relevance to human disease is yet to be established. No claim is made here to the validity of any extrapolations, as humble as they may be, from experimental toxic or dietary liver cirrhosis, to their human counterpart. But it seems inescapable that some lessons, either positive or negative, should be learned from animal models of human disease. Since the substance of this review has been openly speculative, it appears justified to suggest that at least some part of the irreversibility of established human cirrhosis of the liver might be accounted for by a disturbance in the normal mechanisms of collagen breakdown in this organ.

Scleroderma

Progressive systemic sclerosis is almost exclusively viewed as the result of excessive deposition of collagen.[340] The outstanding pathologic feature of this disease is the increased amount of collagen in skin, gastrointestinal tract, lungs, heart, and several other organs. Other anatomic changes, of course, are probably due to different mechanisms, such as the renal lesions[341] or the frequent association with Sjögren's syndrome.[342-344] The wide range of diameters displayed by the dermal collagen fibers has been mentioned as evidence of a long-persisting synthesis.[345,346] Enhanced prolyl hydroxylase activity in the skin of some patients[347-349] and increased number of reducible cross-links in the extractable collagen[350] also support the hypothesis of increased collagen synthesis. The ultrastructural aspect of fibroblasts is compatible with hyperactivity,[345] and it has been demonstrated that cultures of these cells obtained from the skin of patients with active scleroderma synthesize larger amounts of collagen than normal controls.[351,352] Contradictory data[353,354] could be explained by the longstanding nature of the disease, with alternating periods of quiescence and activity. In chronic cases the histologic aspect of the skin does not suggest an active process of collagen deposition: cells are very few and far between and collagen bundles are thick, hyaline, and densely arranged.[340,355] The impression is that very little is going on there, at least in the sense of metabolic turnover. The urinary excretion of hydroxyproline in these patients has shown wide variations. The possibility that the disease is due at least in part to decreased collagen degradation has been mentioned,[59] and there is some evidence indicating that this may be the case. Using the homogenate pellet technique of Ryan and Woessner,[356] skin biopsy specimens from 12 patients with scleroderma and from 9 controls were stud-

ied.[199] In 7 of those cases in which extensive involvement of the forearm and trunk skin existed, collagenase activity of the involved skin was minimal or absent. Moreover, in the same patient, regions of marked skin involvement showed no collagenase activity, whereas clinically uninvolved skin areas exhibited normal or nearly normal levels of enzyme activity. In other patients in whom clinical symptoms were systemic and not associated significantly with the skin, collagenase activity approximated normal levels. The results clearly indicate decreased specific collagenolytic activity in the scleroderma cases. These results may represent actual decrease of the enzyme, presence and/or increase of inhibitors, or lower susceptibility of the substrate. Whatever the mechanism, the observation is of sufficient interest to warrant a more extensive study.

Osteopetrosis

This heritable disorder occurs in many animal species, such as mouse, rat, fowl, rabbit, and humans. The mode of hereditary transmission of the human disease is autosomal recessive in the malignant form and autosomal dominant in the benign form.[357] Osteopetrosis is a systemic disorder of bone in which resorption occurs at a slower rate than deposition. Curiously enough, bone explants of mice affected by the disease produce an increased amount of collagenase.[200] This observation has been interpreted as indicating activation or release of a stock of latent enzyme but at the same time suggests that results of *in vitro* experiments need not reflect what is going on *in vivo*. Nevertheless, using the same animal model, it has been observed that parathormone fails to release lysosomal enzymes,[358] which it does in normal bones;[112,113] therefore, the current view of the mechanisms of this animal type of osteopetrosis is of defective activation in the release of proteases necessary for bone resorption. An interesting experiment on the therapeutic effect of parabiosis in mouse osteopetrosis should be mentioned. After temporarily joining a normal mouse to one affected by the heritable bone disease, and presumably allowing the passage of competent osteolytic cells from the normal to the diseased mouse, clinical and radiologic signs of osteopetrosis disappeared from the latter.[359]

Others

In human pathology there is no dearth of conditions in which deficient collagen degradation could play a role. In addition to the three already mentioned, which affect primarily collagen bundles, others may be mentioned which probably represent disturbances in the resorption of the other two major forms of structural collagen aggregates, ie, reticulum

fibers and basement membranes. Pulmonary fibrosis [360,361] is a broad morphologic term that, translated to the molecular level, means an increase in collagen. In cases of the so-called Hamman and Rich syndrome, or primary (idiopathic) pulmonary interstitial fibrosis, silver stains usually reveal a phenomenal increase in the amount of reticulum fibers.[363] The correlation with biochemical studies has been poor, to say the least. Experimental models of pulmonary fibrosis include radiation, O_2, paraquat, N-nitroso-N-methylurethane, bleomycin, viral infections, silica, asbestos, cadmium sulfate, ozone, antilung antibodies, and others. (See Reference 9 for review.) In some studies, total lung collagen has been shown to increase after radiation;[362] fewer reports contain observations relevant to the rate of collagen degradation, which remains a potential candidate to explain at least some of the increased reticulum fibers. The best example of excessive deposition of basement membrane material is diabetes. In this disease there is overwhelming evidence that, regardless of the many other metabolic disturbances, there is something wrong with the turnover of basement membranes. Reduplication of capillary basement membranes throughout the body has been extensively documented [363-370] and has even given support to one of the current and more intriguing theories on the pathogenesis of the disease.[371] One of the major features of renal involvement in diabetes is increased deposition of basement membrane in the glomerulus.[372,373] Examination of the ultrastructure of the glomerulus in chronic cases of diabetes with renal involvement will convince any skeptic that there is something wrong with the turnover of basement membrane material. Increased deposition may be a first approximation to the mechanism responsible for the abnormality, but perhaps imbalance of the relative rates of synthesis and degradation is better, and this may involve defective collagen degradation. Other instances may be mentioned: increased thickness of bronchial epithelium basement membrane in asthma, in atrophic testicular or renal tubules, and in inflammatory conditions of the salivary or mammary glands. The list could be easily extended but would become repetitive of the same principle, which is that a histologic increase in basement membrane material may be revealing of a local disturbance in collagen degradation.

Final Remarks

This review was written with a single major objective: to stimulate practicing pathologists to look at some of the usually less remarkable features of their slides with a more inquiring eye. Another objective was to recruit into the field those few pathologists who are willing to add to their diagnostic efforts a little spice and flavor. A basic requirement to join the

group is an adventurous spirit; souls enslaved by traditional methodology and established concepts are not eligible. If a single independent and courageous pathologist is willing to master the techniques, the ideas, and, above all, the rigor and objectivity needed to enter the field, this review will not have been written in vain.

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