COLLAGEN BIOSYNTHESIS

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INTRODUCTION

Collagen is a protein the role of which is basically that of a passive architectural structure member. It forms protective boundaries defining the size and shape of tissues, organs, and whole organisms. In vertebrates it provides the framework for the development of skeletal members and acts in the transduction of the energy of muscular contraction into mechanical work performed in skeletal movement. The collagen fiber system is large and extensive, occupying a volume much larger than the cells engaged in assembling the structure. Clearly, the principal physiological functions are expressed in extracellular rather than intracellular compartments.

The unique mechanical properties of the fibers – high tensile strength, structural stability, and inextensibility – stem directly from the fact that each molecule of collagen is itself a rigid, essentially fully extended compound rope of three helically entwined polypeptide chains. The compound helix is resistant to most proteolytic enzymes and, in fibrous form, is stable at body temperature. Additional stabilization is achieved by the extracellular imposition of covalent cross-linkages between reactive groups on neighboring molecules in their fibrous array. Collagen-synthesizing cells face the problem of preparing this extended, marginally stable, highly reactive structural element and depositing it in the proper extracellular location without either premature degradation or aggregation. This is accomplished in a series of both intracellular and extracellular events.

Until quite recently, a discussion of collagen biosynthesis would have focused primarily on hydroxylation reactions. The unique appearance of hydroxyproline and hydroxylysine in collagen, and the ease of following the presence of collagen via hydroxyproline analyses, led early investigators to study these reactions almost exclusively even after it was realized that the hydroxylation was a secondary reaction occurring after peptide chain synthesis. A few striking observations have turned this situation around, and the flow of events in intracellular assembly of collagen is becoming somewhat more clear: synthesis of the precursor polypeptide chains, hydroxylation of proline and lysine, chain registration and assembly of three chains into molecular units, triple-helix formation, glycosylation, and, finally, secretion as completed procollagen.

It had been postulated[1,2] that collagen was synthesized in a precursor form different from that found in tissue extracts, but very little experimental evidence existed to support this view until recently. Perhaps one reason for the difficulty in establishing the presence of a precursor can be found in the methodology utilized in early biosynthetic studies. For the most part, whole animal studies or isolated tissue fragments were used. After relatively long labeling times (measured in hours), the tissues were extracted using the classical salt and acetic acid techniques. We now know that the time required for the entire intracellular processing is measured in minutes. Thus, the early investigations were following turnover rather than biosynthesis. In addition, in normal, uninhibited tissues, the biosynthesis and processing of the precursor molecule is quite rapid so that very little of the precursor is present at any one time. The precursor extracted in the classical procedures was highly diluted with molecules already subjected to further extracellular modification.

Studies utilizing cell culture,[3,4] isolated intact cells,[5,6] and intact calvaria,[7,8] combined with shorter labeling times, finally provided the experimental evidence for a collagen precursor. The picture is still not completely clear; indeed, recent work[9-11] has furnished some evidence that many of the components described as "precursors" may in reality be artifacts of the preparative techniques or, more importantly, intermediate products in the sequential processing of the primary precursor. The collagen assembly system still lacks the complexity of the blood clotting system, but it may not lag too far behind as further details are uncovered.

Once outside the cell, several processes are linked. The collagen precursors are modified in one or more enzymic degradation steps, and the modified molecules are organized into fibrils and fibers. These structural elements are stabilized by cross-linking. Finally, the fiber system must be degraded and turned over to allow for normal growth and tissue remodeling. This last stage requires a system for the depolymerization of the cross-linked network and degradation of the individual molecules.

In this review we will deal with the intracellular aspects of procollagen production and the extracellular processing of the collagen up to the formation of collagen fiber. We shall not consider the details of fibril structure, cross-linking, turnover, or tissue degradation.

INTRACELLULAR EVENTS IN COLLAGEN BIOSYNTHESIS

A. Nature of the Precursor Form of Collagen

The exact composition and structure of the complete collagen precursor molecule is as yet unknown. Indeed, as we shall see, it may be very difficult to specify a point in time where the completed collagen precursor molecule exists as a well-defined entity. The current best evidence indicates that the molecule is assembled from three separate polypeptide chains. The molecular weight of these chains is not yet known; however, their molecular weights have a minimum value of more than 150,000. Each chain is comprised of three sections. The first synthesized amino-terminal region may be as large as 50,000 daltons in weight and is noncollagenous in the sense that it does not contain the sequences typical of the finished collagen molecule. The next section, comprising approximately 1,000 amino acid residues, is the "collagen" portion. This region is characterized by triplet amino acid sequences in which glycine appears regularly as the first residue of each triplet. In all interstitial mammalian collagens, the sequence GLY-PRO-PRO-(HYPRO) occurs frequently. Postsynthetic hydroxylation occurs exclusively (except in the earthworm cuticle collagen) on the proline in the third position. The one distinguishing feature, however, in the collagen region sequences is the regular appearance of glycine at every third residue. The peptide chain is completed with a second noncollagenous polypeptide sequence.[12] Much less is known about this carboxyl-terminal sequence than about any other part of the collagen molecule. Tanzer et al.[13] have recently indicated that the carboxyl-terminal extension of nascent chains is on the order of several hundred angstroms, which would indicate a molecular weight per chain segment of close to 20,000. The collagen molecule is formed from three such chains, arranged so that the three amino termini are all at the same end of the finished, hence highly asymmetric or polarized, structure.

In the collagen molecule only the collagen

portion is in a triple-helical conformation. Each of the end regions has some other as yet unknown conformation. Francis O. Schmitt[1] was the first to suggest that collagen molecules were synthesized in a form containing extrahelical peptide regions. His work was severly challenged, but gradually the evidence became overwhelming in favor of this idea. Speakman[2] hypothesized that the extrahelical extension and the amino-terminal region in particular served two purposes. He proposed that the information in the amino acid sequence of this peptide served to bring together or register the separately synthesized procollagen chains in the correct alignment for subsequent triple-helix formation. The tertiary structure of the complex, that is, the triple-helical collagen plus the "registration peptide," would keep the molecule from interacting and precipitating within the cell. Once secreted, the extrahelical peptides would have to be cleaved so that the residual collagen rods could aggregate in appropriate form. Speakman's hypothesis gave expression to thoughts current in many laboratories at the time. Although unsupported by data, the paper had the important effect of directing attention to the intracellular processing of the collagen molecule and explicitly providing a function for the extrahelical amino-terminal region. Much of the subsequent work used the language suggested in this very stimulating paper.

B. Synthesis on Membrane-bound Polysomes. Size of the m-RNA

All proteins destined for export are thought to be synthesized by membrane-bound ribosomes and transported through the membrane to the cisternae of the endoplasmic reticulum where they are readied for export.[14-16] In the collagen system, Diegelmann et al.[17] have shown that for synthesis at least this is also likely to be the case. From 70 to 90% of the collagen-synthesizing activity of chick embryo fibroblasts and osteoblasts is localized within membrane-bound polysomes rather than free polysomes.

Protein synthesis, however, may begin before the ribosomes attach themselves to the endoplasmic reticulum. In the model of Blobel and Sabatini,[18] the nascent amino-terminal region contains the information which provides for the attachment of the polysome to some particular site on the endoplasmic reticulum. The binding factor may be either a soluble protein present in the cytoplasm or a protein on the endoplasmic reticulum surface. In the case of the immunoglobulin light chains, the attachment peptide is cleaved prior to secretion of the protein into the cisternae.[19] This model suggests that the first role of the amino-terminal peptide region of nascent procollagen chains may be in recognizing the appropriate binding site so that three chains attach to the membrane in the appropriate region and with the geometry necessary to direct their interaction, hence their chain registration in the very act of binding to the endoplasmic reticulum surface.[8,9,20-26] A second aspect of the amino-terminal region function may be in facilitating the penetration of the endoplasmic reticulum as the first step in extrusion of the nascent chain into the endoplasmic reticulum cisternae.

The size of the m-RNA for collagen was first thought to be extremely large,[27,28] although it now seems that these conclusions were due to the artifactual aggregation of extracellular collagen during preparation of the polysomes after lysis of the cells.[29,30] These findings led to the speculation that all three chains of the completed collagen molecule could be synthesized on a single m-RNA. That is, the m-RNA for collagen was thought to be polycistronic, carrying three messages: one for the α2-chain and two redundant messages for the α1-chain. Church et al.[4] supported this concept with the finding that the procollagen molecule had a molecular weight between 500,000 and 600,000. The noncollagenous portion of their procollagen comprised almost one half of their procollagen molecule as judged by collagenase digestion. In these experiments using confluent 3T6 fibroblasts the high molecular weight procollagen was secreted without modification into the cell medium, indicating that the proteolytic degradation of procollagen occurs extracellularly. They suggested that the entire procollagen molecule could exist initially as one continuous polypeptide strand.[4,31,32]

It appears more likely that the m-RNA synthesizing collagen is of smaller size.[30] Lazarides and Lukens[33] developed a cell-free system in which isolated polysomes could complete and release nascent collagen chains. Polysomes were isolated from 8-day-old chick embryo fibroblasts by sucrose gradient centrifugation and shown to be capable of collagen synthesis in vitro. Sea urchin polysomes obtained during early embryogenesis and myosin-synthesizing polysomes from chick embryo muscle were used to calibrate the

sucrose gradients. The collagen-synthesizing polysomes sedimented at a position intermediate between these two polysome preparations and appeared to have a sedimentation coefficient of approximately 330 S. The sea urchin polysomes are an aggregate of 23 ribosomes and a m-RNA complex, while the myosin polysomes contain 50 to 60 ribosomes. The collagen-synthesizing polysomes are thus intermediate in size between these two well-defined systems. If an α-chain is on the order of 95,000 daltons, then this polysome size is in good agreement with that calculated for a monocistronic m-RNA molecule. The polysome is too small to be consistent with a di- or polycistronic message. This is particularly true if one takes into account the fact that the nascent procollagen chain has a molecular weight well above 95,000.

Burgeson et al.[34] isolated an unusually high molecular weight component during the CM-cellulose chromatography of chick embryo fibroblast culture medium. This substance was converted to α-1- and α2-like components upon digestion with pepsin, indicating the presence of a noncollagenous portion in these molecules. However, this substance also was converted to a component co-chromatographing with α1-chains and CM-cellulose merely by reduction with mercaptoethanol or sodium borohydride followed by carboxymethylation. Thus, these procollagen α-chains may be linked to noncollagenous material not connected directly through a peptide bond. Smith et al.[20] came to a similar conclusion from examinations of the collagen in the medium of cultured human fibroblasts by DEAE-cellulose chromatography. The most positively charged component eluting in their system had a much lower hydroxyproline to proline ratio than would be expected for pro α-chains alone. This material was of very high molecular weight as it eluted in the exclusion volume of an 8% agarose column. Denaturation of this component in 8 *M* urea in the presence of a reducing agent yielded a peak identical to pro α-chains on molecular sieve chromatography.

A similar nonpeptide bond-mediated interaction between procollagen and another noncollagenous protein may explain the high molecular weight component isolated by Church et al.[4,31,32] Thus, while a polycistronic m-RNA for collagen has not been absolutely ruled out, the evidence concerning the size of the polysomal complex and the uncertainty over the nature of the noncollagenous protein interaction argue against this hypothesis. Still stronger evidence against a polycistronic m-RNA comes from kinetic studies by Vuust and Piez.[35] Calvaria from newborn rats were pulse-labeled with ^{14}C-glycine, and in a Dintz's type experiment, the CNBr peptides of both the α1- and α2-chains were analyzed as a function of time after labeling. The translation time was found to be about 209 residues per minute for both α1- and α2-chains, corresponding to a total time of 6 min for synthesis of a completed pro α-chain of about 1,250 residues. This estimate assumes a constant m-RNA collagen translation rate through both helical and extrahelical regions. Since the synthesis time is the same for both α1- and α2-chains, they must be synthesized concurrently, not consecutively, as would be the case if the m-RNA were polycistronic.

C. Post-ribosomal Modifications

After the amino acids of the procollagen α-chain peptide backbones are linked together as directed by the code of the specific procollagen m-RNAs, extensive modifications of the polypeptide chains occur. These modifications have generally been considered to be post-ribosomal events. However, this may well be a mistaken notion in that the hydroxylation of proline, for example, may occur on chains still in the active process of elongation. The term post-ribosomal as used here is intended to describe the series of enzyme-mediated events necessary for the formation of collagen but which are under only indirect genetic control in contrast to the specific ordering of the amino acids into the polypeptide chains of specific types of collagen.

The pioneering metabolism study of Stetten and his colleagues[36,37] opened the way to the modern era in understanding the biosynthesis of collagen. They showed, using ^{15}N-labeled proline, that ^{15}N-labeled hydroxyproline was produced. At the same time, they demonstrated that free hydroxyproline was not incorporated directly into collagen. Similar studies following lysine incorporation showed that hydroxylysine is not incorporated directly into collagen.[38-41] In both cases, the precursor proline and lysine residues were acted upon and hydroxylated after their assembly into polypeptide chain sequences.

Many biological hydroxylation reactions have been observed, but, as usual with the collagen

system, this set of reactions has some unusual features. Fujimoto and Tamiya[42] found that the oxygen molecule, O_2, was the source of the oxygen for the hydroxyl group rather than the hydroxyl ion of the aqueous medium. Prockop et al.[43] confirmed these results. Furthermore, Peterkofsky and Udenfriend[44] showed that an unhydroxylated form of collagen could be produced in tissues under anaerobic conditions. The accumulated unhydroxylated collagen was readily converted to the normal form upon incubating the tissue slices with oxygen. Succeeding studies[45,46] showed that in addition to molecular oxygen, several other cofactors are required for normal hydroxylation: ferrous ions, α-ketoglutarate, and ascorbic acid. The α-ketoglutarate is directly involved in the reaction. The production of 1 mol of hydroxyproline involves the stoichiometric conversion of 1 mol of α-ketoglutarate to equimolar quantities of succinate and free carbon dioxide.[47] A more convenient way of modulating the hydroxylation reaction than resorting to anoxia or anaerobic conditions is removal of the ferrous ion. The iron chelator α,α′-dipyridyl is the most commonly used means for the inhibition of hydroxylation. Early studies by Bhatnagar et al.[48] and Cooper and Prockop[49] showed that when hydroxylation was inhibited, secretion of collagen was also inhibited. There was a concomitant accumulation of an unhydroxylated material, called protocollagen, within the cells. When more ferrous ion was added to these cells, the preformed protocollagen was hydroxylated and secreted as collagen.[48] Lukens[50] subsequently showed that the protocollagen, extracted from α,α′-dipyridyl-inhibited chick embryo feet slices in culture, chromatogramed on CM-cellulose in a position identical to collagen α-chains. Gel filtration chromatography also showed the secreted protocollagen to have the same molecular weight as collagen α-chains. Thus, inhibition of hydroxylation does not effect polypeptide chain elongation or release of the nascent chains from the ribosomes.

D. Nature of the Hydroxylating Enzyme

The enzyme catalyzing the hydroxylation of proline residues in protocollagen and homologous peptides has been given the trivial names protocollagen proline hydroxylase or peptidyl proline hydroxylase, both abbreviated PPH. The crude enzyme can be extracted from homogenized connective tissues with 0.01 *M* KCl and retained in solution at 30% saturation with ammonium sulfate. First stage purification is achieved by precipitation of the enzyme from 65% saturated ammonium sulfate solution. Highly purified PPH was isolated from such a crude preparation by affinity chromatography[51] in which a very good substrate, reduced and carboxymetholated *Ascaris lumbricoides* cuticle collagen, was coupled to 6% agarose. The enzyme was eluted with the soluble substrate $(PRO\text{-}GLY\text{-}PRO)_n$ and finally separated by chromatography on unmodified 6% agarose. The molecular weight of the intact PPH was determined to be 230,000 by equilibrium ultracentrifugation.

At low ionic strength the PPH dissociates into two identical subunits with weights about 115,000 daltons.[51-53] Further treatment with 6 *M* urea and 0.1 *M* mercaptoethanol divides these subunits into two further subunits of 60,000 and 64,000 daltons in weight, respectively.[51] The PPH thus appears to be an oligomer with a molecular weight of about 230,000 and consisting of two identical protomers. The PPH protomers in turn are comprised of two nonidentical subunits. The complete PPH isolated by the affinity chromatography procedure had neither any detectable activity leading to the formation of 3-hydroxyproline nor any appreciable lysine hydroxylating activity. Only the intact PPH had a high proline hydroxylating activity. The dimer was slightly active; the monomers were completely inactive.

Electron microscopy[54] of the monomers of PPH indicated them to be rodlike structures 33 × 69.5 Å. Dimers of the subunits appear to be v-shaped pairs of the monomer rods. The complete enzyme is built from interlocked pairs of the v-shaped dimers.

Gribble et al.[55] opened up a completely new line of study with their observation that L-929 fibroblasts produced an inactive form of PPH. During early log phase of growth, these cells synthesized a collagenlike but proline-rich component and no appreciable amount of peptide-bound hydroxyproline. However, the proline-rich protein was readily utilized as a substrate by PPH isolated from other sources. When the cells reached early stationary phase, they began to produce peptide-bound hydroxyproline rather than the proline-rich collagenlike PPH substrate. Furthermore, PPH activity was detectable in the culture. Cell concentration was important in stimulating the appearance of PPH activity. PPH activity could also be stimulated in whole cells

with exogeneous lactate. In general, higher concentration or cell crowding stimulated PPH activation. This stimulation of enzyme activity was not blocked by the addition of cycloheximide, puromycin, or actinomycin D, indicating that neither *de novo* protein nor RNA synthesis was necessary for the activation of the enzyme.[56,57] Thus, activation of a previously synthesized inactive precursor seemed to be the most likely method by which PPH activity became detectable. McGee et al.[58] and McGee and Udenfriend[59] utilized immunological cross-reactivity as a probe for the presence of an inactive precursor. Antibodies were prepared to a partially purified peptidyl proline hydroxylase and checked for specificity against highly purified PPH using immunodiffusion and immunoelectrophoresis techniques. No reactivity to proteins other than the enzyme was detected. Using this specific antiserum, a cross-reacting protein to PPH could be demonstrated in the lysate from cultured cells in the earliest phases of growth many hours before PPH activity could be detected. Measurement of the amount of cross-reacting protein during the entire culture period demonstrated that it remained constant even after the appearance of enzymic activity.[58] The cross-reacting protein also was capable of blocking antibody binding sites for PPH. Indeed, the enzyme could be displaced from the antibody by the addition of excess cross-reacting proteins.[59] These data all argue in favor of the hypothesis that PPH is first produced in an inactive precursor form and that the activation process is one means of controlling PPH activity.

The mechanisms by which increased cell density or increased lactate concentration stimulate PPH activity in cell culture are unknown. It has been demonstrated that lactate has no direct effect on the enzyme in in vitro assays in the absence of whole cells.[56] Ascorbic acid, which stimulates the synthesis of peptide-bound hydroxyproline as well as PPH,[60] also functions directly to activate PPH in vitro.[61]

Using whole cells in culture, Stassen et al.[60] found that the addition of ascorbic acid to the culture medium stimulated PPH activity within 1 hr even when protein and RNA synthesis inhibitors had been added prior to the addition of the ascorbic acid. Further, ascorbic acid stimulated PPH in early log phase cells where no activity would normally be detectable. The sigmoid shape of a plot of PPH activity versus ascorbic acid concentration strongly suggests some cooperative binding of ascorbic acid, possibly related to assembly of the active oligomer from the protomers. The cross-reacting protein has a lower molecular weight than active PPH and elutes in a different chromatographic position on DEAE-cellulose than active PPH. Another strong indication that one role of ascorbate may be to facilitate assembly of protomers was the fact that PPH activity was destroyed, and only cross-reacting protein was present, when cells were incubated with dithiothrietol (DTT) prior to lysis and fractionation. Further incubation with ascorbic acid, in the presence of cycloheximide, restored PPH activity. McGee and Udenfriend[59] had demonstrated that DTT (like mercaptoethanol[51]) would reduce the size of the PPH by about half. Thus, in addition to its role as a cofactor in the hydroxylation reaction, it appears that ascorbate plays a second role in the activation of PPH by favoring aggregation of the protomers into the active oligomeric form.

The subunit structure of PPH and the fact that it is comprised of two separate types of subunits suggest that this enzyme may be susceptible to regulation by ligand or cofactor interactions. Bhatnagar et al.,[62] for example, have evidence that Fe^{+2} is bound at two different sites in PPH. One Fe^{+2} is tightly bound at the catalytic site of the enzyme where α-ketoglutarate and the proline-containing substrate are also found. The second Fe^{+2} forms a complex with the enzyme at some other site and appears to be involved in generating superoxide from O_2. The superoxide is then transferred to the active or catalytic site. Bhatnagar et al. suggest that ascorbic acid acts at the noncatalytic site in the superoxide production and transport.

E. Physiological Role of Hydroxylation

Hydroxylation has a profound effect on many aspects of the collagen synthetic system, although, as we have seen, inhibition of hydroxylation does not seem to have an effect on polypeptide chain elongation or on the release of nascent chains from the ribosomes. Early studies by Prockop and his colleagues[48,49,63] indicated that in the absence of hydroxylation the secretion of collagen was drastically inhibited, and they concluded that hydroxylation was an essential event for collagen secretion. This proposition was unambiguously confirmed by Margolis and Lukens.[64] They com-

pared the rate of secretion of protocollagen from 3T6 cells inhibited by α,α′-dipyridyl with that from uninhibited cells. They found that the protocollagen was secreted at only 27% of the normal rate. This value was in good agreement with similar studies on human skin fibroblasts in culture by Switzer and Summer.[65] The degree of inhibition of hydroxylation by α,α′-dipyridyl was dose-dependent; α,α′-dipyridyl at a level of $10^{-4}M$ completely inhibited hydroxylation in 3T6 cells, while a concentration of $10^{-5}M$ had no inhibitory effect.[66] The addition of α,α′-dipyridyl to a cell culture, however, affects more than the hydroxylation reactions. The chelator also has a profound effect on the incorporation of radiolabeled precursors into both noncollagenous and collagenous proteins,[64,66,67] although there is a differential effect. Noncollagenous proteins were synthesized at about 50% of their rate in uninhibited cell cultures, while collagen chain synthesis was reduced to about one third of the uninhibited rate.[64] Secretion of collagen formed within the cell prior to inhibition of hydroxylation with α,α′-dipyridyl is not inhibited. Thus, the accumulation of protocollagen within the cell appears to be due directly to the underhydroxylated state of the newly synthesized material.[65] The secretory mechanism is intact. The underhydroxylated protocollagen is retained within the cell in an undegraded state during at least 12 hr of inhibition in human skin fibroblasts. The accumulated protocollagen molecules are extruded when the hydroxylating system is restored to activity.[65]

While the mechanism by which α,α′-dipyridyl inhibits proline hydroxylation can be readily understood in terms of its abstraction of Fe^{+2} from PPH, it is not so clear why ascorbic acid deficiencies also lead to the inhibition of hydroxylation. Ascorbate has been shown to act as an electron donor in hydroxylation. In this capacity, it is not a specific cofactor and can be replaced by a number of other reducing agents such as low molecular weight thiol compounds.[61] This role is in addition to the previously mentioned effect of ascorbate in intracellular activation of PPH. It has been substantiated that a deficiency of ascorbic acid in cell culture does lead to the synthesis of a proline-rich hydroxyproline-poor polypeptide.[68-74] Whereas the α,α′-dipyridyl-inhibited cells produce protocollagen in which less than 0.3% of the potentially hydroxylated proline residues are actually hydroxylated,[67] ascorbate deficient cells still carry out 10 to 20% of the normal level of hydroxylation.[71,72]

One could explain the lack of complete inhibition of proline hydroxylation by either the presence of low levels of ascorbic acid in the fetal calf serum used in the cell culture medium, by the *de novo* synthesis of ascorbic acid by the cultured cells, or by the presence of some other reducing agents in the culture medium. The least likely explanation is that of the level of ascorbic acid; fetal calf serum contains less than 0.2 μg/ml,[69] an amount much less than that required for even partial stimulation of hydroxylation (5 μg/ml). Thus, the synthesis of partially hydroxylated collagen chains in ascorbate-free or deficient systems may reflect the relative nonspecificity of ascorbic acid as an electron donating cofactor. The absence of ascorbic acid in a culture medium seems to have little effect on cell growth or division. Further, in contrast to the inhibition of protein synthesis in the presence of α,α′-dipyridyl, ascorbic acid deficiency seems to have no effect on the amount of proline incorporated into either collagen or noncollagenous protein.[69-73] Using a protease-free collagenase to specifically assay for the presence of collagenlike polypeptides, Peterkofsky[72,73] and Levene and his colleagues[70,71] showed that at least in the established cell lines 3T3, 3T6, and L929, collagen polypeptide concentration was not reduced due to ascorbic acid deficiency. However, the danger in extrapolating results garnered from in vitro studies is evident in Peterkofsky's observation that collagen synthesis was reduced to 60% of its normal value in fresh chick embryo fibroblasts under the condition of ascorbic acid deficiency.[72] Ramaley et al. also found that ^{14}C-proline incorporation was substantially reduced during an 8 hr incubation of 3T6 fibroblasts under conditions of ascorbic acid deficiency. In these experiments, incorporation was measured in terms of the total contents of the cell layer and medium fractions; thus, one cannot be sure whether or not collagen synthesis was specifically decreased.[74]

The reduction in synthesis of collagen seen in fresh chick embryo fibroblasts during ascorbic acid deficiency under conditions where no reduction was observed in established cell lines[72] may reflect, in part, the histories of the cell types. The fresh chick fibroblasts are much closer to an in vivo system in which all of the fine regulatory controls on protein and especially cell-specific

protein synthesis are operative. It has been known for some time that cell lines which have become established in culture systems tend to lose some of their more differentiated functions. Since the established lines such as 3T6 have been in culture for many years, they may have lost some of the regulatory controls relating to ascorbic acid concentration and collagen polypeptide chain synthesis. The established cell lines make underhydroxylated collagen, under conditions of ascorbic acid deficiency, at the same net rate as they produce fully hydroxylated collagen in the presence of ascorbic acid. Studies of collagen synthesis using these established cell lines cannot, therefore, throw any light on at least this one ascorbic acid-sensitive control mechanism.

Even with the established cell lines, the data relating to synthesis in the absence of ascorbate are not entirely in agreement. As indicated above, Bates et al.[70,71] found the total incorporation of both proline and lysine into protein, including collagen, to be unaffected by ascorbate deficiency, whereas Ramaley et al.[74] found there was a moderate but significant decrease in the incorporation of ^{14}C-proline into the medium of ascorbate-deficient 3T6 cultures. The chief difference between the two studies was that Bates et al. took special precautions to inhibit protease activity by adding inhibitors[70] or by inactivating proteases in the medium by heating it to 100°C immediately upon harvesting.[71] Several studies have shown that underhydroxylated collagen has a lower denaturation temperature than the fully hydroxylated molecule.[67,74-78] Specifically, Rosenbloom et al. demonstrated that the denaturation temperature was directly proportional to the hydroxyproline content of the protocollagen.[67] Under the conditions of tissue culture, 37°C, the collagen synthesized by ascorbic acid-deficient cells may not be in the triple helical conformation and therefore might be susceptible to nonspecific proteases in the culture medium. This could account for the observations of Ramaley et al., who found a lower concentration of proline incorporated into proteins of the culture medium.

In marked contrast with the situation with α,α′-dipyridyl-inhibited hydroxylation, the partially hydroxylated product formed in the ascorbate-deficient systems appears to be secreted at the normal rate. The minimum amount of hydroxylation required for secretion is presently unknown, but it is obvious that the 10 to 20% level of hydroxylation seen in collagen produced under the conditions of ascorbate deficiency is in excess of this minimum amount. Underhydroxylated collagen is found in the medium of ascorbic acid-deficient cells.[69-74] The kinetics of the secretion process have many interesting facets. Levene and his colleagues used a standard period of 24 hr of incubation with labeled amino acids before determining the amount of label incorporated.[61,68-71] In these long-term experiments, the ascorbic acid-deficient and ascorbic acid-supplemented cells synthesized the same amounts of deoxycollagen and collagen, respectively. Peterkofsky, however, in short-term studies, noted that there was a lag period before deoxycollagen was secreted by ascorbic acid-deficient cells.[73] Normal cells, supplemented with ascorbic acid, secreted labeled collagen after a lag period of about 45 min. The ascorbic acid-deficient cells did not secrete their underhydroxylated collagen until 1.5 to 2 hr after the introduction of ^{14}C-proline. Once secretion had begun, it proceeded at a slower rate in the deficient cells than in the supplemented cells. During the period when collagen secretion was reduced by the ascorbate deprivation, newly synthesized but underhydroxylated collagen accumulated in the cell layer. Collagen synthesis proceeded unabated. This effectively argues, at least in the 3T3 cell system, against a regulatory mechanism which postulates that an accumulation of underhydroxylated collagen within the cells can shut off further polypeptide synthesis.

Early studies with α,α′-dipyridyl-inhibited cells indicated that the intracellular proteins were degraded and then released into the medium.[66] Gel filtration chromatography of the culture medium from 3T6 cells which had been labeled for 8 hr with ^{14}C-proline or ^{14}C-lysine in the presence of α,α′-dipyridyl showed labeled polypeptides in the 10,000 to 50,000 molecular weight range. These peptides were susceptible to degradation by purified collagenase. Other investigators who have examined the size of the peptide chains made during α,α′-dipyridyl inhibition[7,50] and ascorbate acid deficiency[70,73] have found no evidence for degradation to small peptides. The experiments of Switzer and Summer are particularly striking.[65] After pulse-labeling human skin fibroblasts for 2 hr with ^{14}C-proline in the presence of both ascorbic acid and α,α′-dipyridyl, these labeled cells were kept inhibited for up to 12

hr. No hydroxy-proline could be detected in either the medium or the cell layer, although ^{14}C-proline was incorporated. Upon release of the cells from inhibition of hydroxylation after the 12-hr period, the cells hydroxylated and secreted the retained deoxycollagen. Thus, the intracellular unhydroxylated collagen is not degraded and is still available for hydroxylation. The collagens synthesized under all methods of inhibition of hydroxylation – (1) ascorbic acid deficiency, (2) α,α'-dipyridyl, and (3) anaerobic incubation – all have molecular weights comparable to that of the α-chain when tissue proteases are inhibited.[74]

It is evident that the post-transcriptional hydroxylation of proline has an important physiological role directly related to the regulation of secretion of collagen. Investigations of the basis for the regulatory effect of hydroxylation have taken two directions: direct examination of the effect of hydroxylation on collagen structure and stabilization, and the minimal requirements for secretion. Both problems have been addressed in an elegant paper by Rosenbloom et al.[67] Fibroblasts were cultured from chick embryo tendons and incubated for 4 hr with ^{14}C-proline. The distribution of collagen between the cell layer and the medium was determined. When α,α'-dipyridyl was added to the system, no change in this distribution was noted until a concentration of $4 \times 10^{-5} M$ was reached. Above this concentration of α,α'-dipyridyl, the distribution began to change in favor of the accumulation of collagen in the cell layer. At concentrations greater than $2 \times 10^{-4} M$, all the collagen was retained, as expected.[5] The collagen was extracted from the cells, the procollagen isolated on SDS-agarose columns, and the degree of hydroxylation determined. The degree of hydroxylation was also determined for the secreted medium collagen in the same way. The results are shown in Table 1, and it is evident that when the average degree of hydroxylation is less than 14%, secretion is inhibited. There is, in addition, a direct relation between denaturation temperature and hydroxyproline content of the procollagen.

A large number of investigators have considered the role of hydroxyproline in stabilizing the collagen triple helix. Gustavson[79] compared the melting temperature, T_M, of collagen from a variety of sources and found a relationship between T_M and hydroxyproline content. Studies of this type were difficult because collagens of different origin also differ in total imino acid content. Such studies led to a more general conclusion that T_M depended better on the total imino acid content than on the hydroxyproline content.[80,81] In the system of Rosenbloom et al.[67] in which the total imino acid content of the collagen polypeptide was constant and dictated by the transcriptional process, there is a clear-cut dependence of the denaturation temperating, T_D, on the degree of hydroxylation (Figure 1). This does not mean to imply that total imino acid content is not a contributing factor when comparing T_M or T_D for a series of collagens of varied origin. It does mean that the stability of a particular type of collagen depends on the completeness of the hydroxylation reaction. Other

TABLE 1

Relationship Between Hydroxyproline Content and Denaturation Temperatures of Procollagen Preparations

	Chromatographically purified procollagen[b]	
α,α'-Dipyridyl concentration during labeling (M)[a]	Degree of hydroxylation (%)[c]	Denaturation temperature (°C)[d]
Medium proteins[e]		
0	44.0	37.9
3.5×10^{-5}	35.5	35.2
4.0×10^{-5}	31.2	33.4
4.5×10^{-5}	30.0	33.6
5.0×10^{-5}	24.8	33.0
Cell proteins[f]		
4.5×10^{-5}	14.5	29.4
5.0×10^{-5}	8.2	27.5
5.5×10^{-5}	4.7	25.6
5.0×10^{-4}	<0.3	23.5

[a]Isolated fibroblasts (10^7/ml) were incubated in Krebs medium at 37°C containing the concentrations of α,α'-dipyridyl listed in the table. After 20 min, [^{14}C] proline was added to a final concentration of 5 μCi/ml, and the incubation continued for 4 hr. The cell suspensions were rapidly chilled and centrifuged for 2 min at 1,500 × g.

[b]Procollagen obtained gel filtration.

[c]Values are 100 times [^{14}C] hydroxyproline per total ^{14}C.

[d]Midpoint of temperature, % pepsin resistance plot.

[e]Labeled proteins secreted by the cells into the medium.

[f]Proteins extracted by 0.5 *M* acetic acid.

Adapted from Rosenbloom, J., Harsch, M., and Jimenez, S., *Arch. Biochem. Biophys.*, 158, 478, 1973.

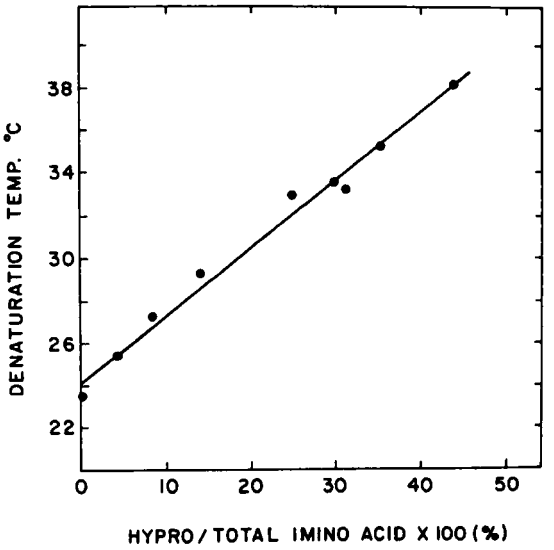


FIGURE 1. Plot of the denaturation temperatures of procollagen as a function of the degree of hydroxylation. The denaturation temperatures were obtained from measurement of midpoints of temperature-pepsin resistance plots. (From Rosenbloom, J., Harsch, M., and Jimenez, S., *Arch. Biochem. Biophys.*, 158, 478, 1973. With permission.)

studies on theoretical[82,83] as well as experimental grounds[74-78] amply confirm this conclusion.

The probe used to determine collagen stability was pepsin degradation. It has been known for some time that collagen in native triple-helical form was resistant to proteolytic enzymes such as pepsin[84] and trypsin and chymotrypsin,[85] whereas separated collagen chains in a less ordered form were readily digested. These data on the pepsin sensitivity of underhydroxylated procollagen demonstrated that it is unlikely that nascent collagen chains assume stable H-bonded structures intracellularly prior to their hydroxylation. It is possible for underhydroxylated or unhydroxylated collagen to be secreted into the medium in culture systems.[66] Hence, if the conclusion just reached is correct, that is, that unhydroxylated collagen chains are not in ordered triple-helical form, then two other factors must be considered. Once secreted, these underhydroxylated collagen chains are readily susceptible to proteolytic degradation in the medium.[66] However, unhydroxylated chains are not degraded when they are held intracellularly[65] for long periods. The content of active proteases intracellularly must be very low. On the basis that hydroxylation does enhance stability of the triple-helix form, it has been argued that the triple-helix structure is required for secretion.[76] This is far from proven, however, since partly hydroxylated collagen, with T_D less than 37°C, has been secreted into the medium from cells in culture.

Extractions of collagen, following good laboratory practice, are generally carried out at cold room temperatures. Under such in vitro conditions, collagen-fold formation[86] takes place leading to chains which fold back on themselves or, if properly registered, to completely native three stranded triple-helical molecules. It is likely that the isolation of triple-helical protocollagen is an artifact of the preparation procedures.[87,88] However, Darnell and Rosenbloom have demonstrated that refolded unhydroxylated protocollagen is in the extended rodlike triple-helical conformation after isolation.[78] The rapid renaturation or folding of the protocollagen suggests that the chains are ordered or registered before any triple-helix formation takes place and that the unhydroxylated procollagen chains become linked together at a very early stage of synthesis.

F. The Effect of Analogs of Proline on Collagen Biosynthetic Processes

A number of studies have been carried out, mainly by Prockop and his colleagues, in which analogs of proline which are incapable of hydroxylation have been added to cells or tissues. These studies have been only of limited success in shedding light on the hydroxylation requirements necessary for secretion of the collagen molecule. It does appear, however, that collagens containing proline analogs are secreted at a slower rate than normal. This correlates with the observation that these collagens may not be as stable as fully hydroxylated collagen and indeed may not even form triple-helical structures in some cases.[89-91]

Four proline analogs can be incorporated reasonably well into collagen peptide chains: 3,4-dehydro-L-proline,[92] *cis*-4-hydroxy-L-proline,[90-94] L-azetidine-2-carboxylic acid,[95] and *cis*-4-fluoro-L-proline.[96] There is also suggestive evidence that *cis*-4-bromo-L-proline can be incorporated.[91] All analogs are not incorporated to the same extent. Azetidine-2-carboxylic acid is incorporated only to the extent of about 4 residues per 1,000 amino acid residues,[96] while *cis*-4-hydroxyproline is incorporated to the extent of 14 residues per α-chain.[94] None of the four analogs successfully incorporated into collagen inhibit synthesis of the peptide chains to an appreciable extent, at

least over a 3-hr period of incubation with intact embryonic tissue[93] or isolated embryonic fibroblasts.[93,97]

A question of different character from that of the effect of the analogs on peptide chain synthesis is that of their effect on the hydroxylation of the remaining proline residues since it is evident from the fact that only a few analog residues are incorporated into each chain that the majority of prolyl residues occupy their normal position. The compound 3,4-dehydro-L-proline affects the hydroxylation of both proline and lysine residues which have been incorporated.[92] This effect was not caused by a decrease in the activity of the protocollagen hydroxylase present in the tissue, and the protocollagen form was the appropriate procollagen size. The protocollagen containing the 3,4-dehydro-proline, however, could not be further hydroxylated by incubating it with excess protocollagen proline hydroxylase. These data were rationalized on the basis that the 3,4-dehydro-proline residue at one position in a substrate-polypeptide chain might sterically hinder the hydroxylation of adjacent prolyl or lysyl residues in the same molecule. It was also found that the PPH formed very stable complexes with collagen containing the analog.

When the effects of the other analogs, *cis*-4-hydroxy-L-proline,[89,90,93] azetidine-2-carboxylic acid,[96] and *cis*-4-floro-L-proline, were examined, their effect on the hydroxylation of the pro-α-chains of collagen was very small and quite variable from experiment to experiment. Thus, these analogs have no significant effect on the hydroxylation of normal proline or lysine residues in the protocollagen molecule. In every case, it was found that the analog-containing collagens, whether or not they were hydroxylated or underhydroxylated, were not secreted at the normal rate. When isolated cells were studied,[90] the secretion of collagen was measured by the presence of hydroxy-^{14}C-L-proline in the medium. In such experiments, it was shown conclusively that the three proline analogs had no effect on either the proline hydroxylase activity or on the secretory process. The inhibition of secretion rate must therefore be related to the conformation or structural stability of the protocollagen or procollagen containing the unhydroxylated analog.

Although analog-containing molecules are retained intracellularly for a longer period of time than the normal uninhibited procollagen molecules, some analog-containing peptides are found in the medium of isolated fibroblasts.[89] When these analog-containing chains were examined by disc gel electrophoresis, only 10% of the radiolabeled molecules in the case of *cis*-4-hydroxy-L-proline and 3,4-dehydro-L-proline were found in a molecular weight peak corresponding to pro-α-chains. The remainder of the incorporated radioactivity was in smaller molecular weight peptides. These data suggest that the proline analogs probably do not form stable triple helices and are susceptible to proteolytic attack just as secreted, underhydroxylated α,α'-dipyridyl-inhibited protocollagen is degraded in the medium cell culture.

The physiological role of hydroxylation of proline thus appears to be directly linked to the process of secretion of newly formed collagen from the cells which manufacture it. The presence of hydroxyproline per se does not, however, seem to be the direct factor involved. Rather, it would appear that secretion is more directly related to the triple-helical character of the collagen molecule; that is, in those cases where hydroxylation of proline is inhibited either due to inhibition by α,α'-dipyridyl, by deficiency of ascorbic acid, or by restriction of the O_2 content, or in those cases where hydroxylation is blocked by incorporation of proline analogs, the intracellular underhydroxylated collagen remains in non-triple-helical form, at least under culture conditions. In this form, the rate of secretion is severely reduced.

G. Substrate Requirements of Protocollagen Proline Hydroxylase

In all collagens except those originating from the earthworm cuticle, the hydroxylation of proline takes place at that residue in the third position of the GLY-X-PRO triplet. No hydroxylation occurs at a proline in position two. Studies on synthetic peptides provide a substantial degree of insight into the substrate requirements of the enzyme. No synthesis of hydroxyproline was observed when the tripeptide GLY-PRO-PRO was used as the substrate,[98] but some hydroxylation was obtained with the tripeptide PRO-PRO-GLY.[99] Larger polypeptides of the type $(X\text{-PRO-GLY})_n$ and $(\text{PRO-PRO-GLY})_n$ were reasonably good substrates, with larger peptides being more readily hydroxylated. On the other hand, the compound poly-L-proline was not a substrate for the enzyme and instead was a competitive inhibitor. These data point to the probability that the

enzyme requires a glycine or some residue with a comparably large conformational freedom in the position following the proline residue to be hydroxylated. Kivirikko et al.[100] obtained data that indicated that the presence of the triple-helical conformation did not prevent hydroxylation; however, in their experiments the conformation of the polytripeptides was not examined directly. Longer polytripeptides in the denatured form have a lower K_m value than shorter peptides, indicating that they are better substrates for the enzyme. The consequence of this is that during the biosynthesis of collagen a certain chain length is probably necessary before the enzyme will recognize the nascent procollagen chain as a substrate. It is not known how many residues of a procollagen chain must be synthesized before PPH begins to act.[100] A number of early experiments using ^{14}C-labeled but underhydroxylated protocollagen as the substrate suggested that both native and denatured molecules were equally good substrates for PPH.[101-103]

More recently[104] it was shown that while native rat tail tendon collagen could not be further hydroxylated by proline hydroxylase prepared from rat skins, denatured rat tail tendon was a substrate for additional hydroxylation. As indicated earlier, it has been demonstrated that the protocollagen is in the denatured, or at least not in triple-helical, form at the temperature of the tissue culture and does not become triple-helical until after hydroxylation occurs under culture conditions. On the other hand, protocollagen can be made into triple-helical form by cooling solutions below about 28°C.[75-77,87,88] Berg and Prockop[88] made use of this property in studying the hydroxylation of ^{14}C-labeled protocollagen as a function of temperature between 15 to 37°C. Very little hydroxylation occurred at temperatures below the melting temperature of the protocollagen, when the protocollagen was in triple-helical form, even when a 22 X molar excess of PPH was added. However, at the melting temperature, 28°C, a sharp increase in extent of hydroxylation was noted. In a similar study, Murphy and Rosenbloom[77] also found that PPH hydrozylated only molecules of unhydroxylated procollagen that were in the denatured state. Molecules of different degrees of hydroxylation could be readily produced by adding PPH to solutions which had been tempered at different temperatures but close to the transition temperature for the formation of the triple helix. The more triple helix induced before addition of PPH, that is, at lower temperatures, the less hydroxylation was observed.

Studies of the sequences of cyanogen bromide peptides of collagen have shown[105] the interesting result that not every proline in the third position of a triplet is hydroxylated to the same extent in every molecule in a given preparation. There is indeed a built-in microheterogeneity with regard to hydroxylation. One cause of this microheterogeneity might be the fact that hydroxylation, in fact, is shut off by collagen-fold formation. The physiologic role of hydroxylation can then be seen in two contexts. First, the extent of hydroxylation clearly relates to the stability of the triple helices of the collagen and the degree of hydroxylation matches the environmental temperature which the animal must encounter. Cold water fishes have low stabilities and low degrees of hydroxylation. Warm-blooded animals living at elevated environmental temperatures have higher degrees of hydroxylation and comparably higher collagen triple-helix stabilities. Second, the degree of hydroxylation is again related to the environmental temperature from the point of view that triple-helix formation appears to be necessary for secretion of collagen from the cells. One may speculate that the nascent protocollagen chains in their disordered form continue to be hydroxylated until the degree of hydroxylation reaches a level, dependent on the temperature of the cells, at which triple-helix formation will occur. Hydroxylation then ceases and the collagen is ready for entering into the later stages of the secretory process.

H. Intracellular Localization of PPH

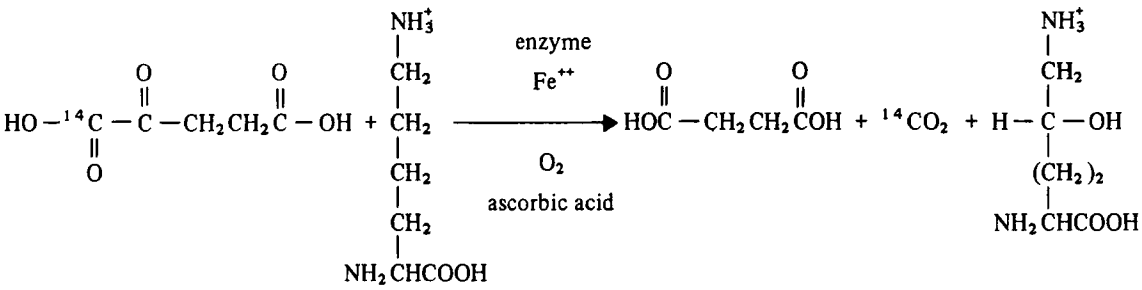
If peptidyl proline hydroxylase acts best on disordered nascent procollagen chains, then it would appear that intracellularly the site of action of PPH must be very closely related to the protein synthesizing apparatus in the cell. As discussed in Section B, the majority of the collagen-synthesizing activity in fibroblasts is localized within membrane-bound polysomes attached to the endoplasmic reticulum. Collagen chains still attached to polysomes have been shown to possess some hydroxyproline.[33,106,107] Diegelman et al.[17] have demonstrated that polyribosomes isolated from chick embryo connective tissue without detergents and, hence, still attached to endo-

plasmic reticular membrane retain the ability to synthesize hydroxyproline in an in vitro protein synthesizing system. This and similar evidence[108,109] demonstrate that the enzyme is either bound to the membrane of the rough endoplasmic reticulum or is closely associated with it. An early investigation by Lane et al.[110] of the kinetics of the incorporation of ^{14}C-proline into collagen and its subsequent hydroxylation showed that there was a lag of about 1.5 min between the incorporation of the ^{14}C-proline and the appearance of ^{14}C-hydroxyproline. They assumed a synthesis time of 1 min for the completion of a collagen chain and thus concluded that hydroxylation did not begin until the polypeptide chain was completed. The studies of Vuust and Piez on the biosynthesis time for the pro-α-chain,[35] however, show that the synthesis time is approximately 6 min for a completed chain, and, therefore, the data of Lane et al. suggest that a considerable amount of hydroxylation must occur before the polypeptide chain is completed. Similarly, Lazarides and Lukens [33] have also found that nascent collagen chains were hydroxylated to considerable extent.

Olsen et al.[108,111] have obtained direct evidence for the localization of PPH on the rough endoplasmic reticulum. Antibodies were prepared to PPH purified on an affinity column. In electron microscopic investigations, ferritin-labeled antibodies were localized on the rough endoplasmic reticulum, and indeed most of the stains appeared to be localized on the cisternal side of the membrane instead of on the ribosomal side. This suggests that as the nascent chains are extruded into the cisternae of the endoplasmic reticulum, the enzyme is positioned so that it can see the nascent, separate, disordered polypeptide chains and that hydroxylation commences almost immediately.

I. Peptidyl Lysine Hydroxylase

It was initially thought that only one enzyme participated in the hydroxylation of both the prolyl and lysyl residues after they were bound in peptide linkage in the nascent collagen chain. The two enzymes, however, have been separated chromatographically.[112–114] The purification is sufficiently good so that all peptidyl lysine hydroxylase activity can be separated free from residual peptidyl proline hydroxylase activity. Even before the enzyme was highly purified, it was known that the same cofactors, ferrous ion, α-ketoglutarate, a reducing agent such as ascorbic acid, and molecular oxygen, were all involved in the hydroxylation of both proline and lysine residues. In the lysine hydroxylation reaction, one atom of the oxygen molecule is incorporated into the C5 position of the lysine to form hydroxylysine, while the other oxygen atom is transferred to the carboxyl group of the succinate formed during the decarboxylation of α-ketoglutarate. This explains the cofactor role of α-ketoglutarate and the 1:1 stoichiometry observed between lysine hydroxylation and decarboxylation of α-ketoglutarate.[115,116] If the number one carbon of the α-ketoglutarate is made radioactive, that is,



lysine hydroxylation can be measured quantitatively by the amount of radioactive carbon dioxide released from the reaction mixture.[115,116]

Two groups have isolated and attempted the characterization of the peptidyl lysine hydroxylase with some conflicting results. Popenoe and Aronson[114] determined a molecular weight for the enzyme of 350,000 daltons, a pH optimum between 8.0 and 8.4, and the fact that the enzyme was stable in dithiothreitol at concentrations of 100 μ*M*, which is the concentration used in the buffer solutions from which the enzyme is isolated. Kivirikko and Prockop,[113] on the other hand, found the enzyme to be a mixture of several

aggregate forms and isolated components with molecular weights of 550,000 and 200,000 daltons. Their preparation had a pH optimum of 7.4 and rapidly lost enzymic activity in dithiothreitol above 50 μM concentrations. Both preparations, however, were specific for hydroxylating lysine residues in protocollagen[113,114] and a wide range of synthetic polypeptides[113,115] and neither contained any peptidyl proline hydroxylase activity.[113,114,116]

Using a synthetic polypeptide as substrate, Kivirikko and Prockop[113] determined the K_m values for the cofactors ferrous ion, α-ketoglutarate, and ascorbate. They were all slightly different from those found for the peptidyl proline hydroxylase (Table 2).

Free lysine is not a substrate for the lysyl hydroxylase.[115] The tripeptide ILE-LYS-GLY is a very weak substrate. The hexapeptide (ILE-LYS-GLY)$_2$ is a better substrate, and maximal activity was obtained with a longer peptide ALA-ARG-GLY-ILE-LYS-GLY-ILE-ARG-GLY-PHE-SER-GLY. This dodecapeptide was synthesized specifically to match a naturally occurring collagenous sequence in which glycosylated hydroxylysine appears. Ascaris cuticle collagen and the cyanogen bromide peptides derived from it did not serve as a substrate. This was surprising in view of the fact that the same peptides do serve as substrates for peptidyl proline hydroxylase. In addition, the peptides prepared from Ascaris cuticle collagen did not inhibit hydroxylation of the dodecapeptide, indicating that neither the Ascaris collagen nor its cyanogen bromide peptides bind to the enzyme.

All of the sequences, synthetic or cyanogen bromide peptides, which were tried and which served as substrates for the enzyme contain a glycine residue immediately following the lysine to be hydroxylated; that is, the hydroxylated lysine is in the same number three position of the collagen triplet as is the proline which is hydroxylated. In this case, however, neighboring residues have a marked effect on facilitating hydroxylation. In the synthetic peptides studies by Kivirikko et al.,[115] the residue just preceding the lysine to be hydroxylated always contained a bulky hydrophobic group from phenylalanine, isoleucine, or methionine. On the other hand, a peptide derived from the α1-chain of lathrytic rat skin (α1-CB8-HA1) was not as good a substrate for hydroxylation as any of the intact soluble denatured collagens. Although this peptide contains 99 amino acid

TABLE 2

A Comparison of K_M Values for Fe^{+2}, α-Ketoglutarate, and Ascorbate in Hydroxylation of Peptidyl Proline Hydroxylase and Peptidyl Lysine Hydroxylase under Comparable Conditions

	K_M Value (M)	
Cofactor	Peptidyl proline hydroxylase[98]	Peptidyl lysine hydroxylase[113]
Fe^{+2}	5×10^{-6}	1×10^{-6}
α-Ketoglutarate	1×10^{-5}	5×10^{-5}
Ascorbate	2×10^{-4}	5×10^{-5}

Adapted from Kivirikko and Prockop.[98,113]

residues, lysine only occurs twice in position three and both times with the same sequence GLY-ALA-LYS-GLY. Apparently, the alanine side chain is not sufficiently nonpolar to assist in the binding of the enzyme to the peptide. An insufficient length of the peptide sequence can be ruled out in this case very clearly since the dodecapeptide with only 12 amino acids was fully hydroxylated under the same circumstances. Thus, in the case of peptidyl lysine hydroxylase, the sequence specificity appears to be more critical than chain length, as demonstrated not only with this peptide but also by the lack of reactivity of the enzyme with Ascaris cuticle collagen and its cyanogen bromide peptides.

As in the case of PPH, the peptidyl lysine hydroxylase does not act on collagen in the triple-helical state with the same ease with which it hydroxylates denatured collagen peptide chains.[77,88,116] The same type of sequence studies of cyanogen bromide peptides containing potentially hydroxylatable lysine residues[105] have been carried out as in the case of studies on the hydroxylation of proline residues in such peptides. Again, not all lysine residues in position three and which are susceptible to hydroxylation with exogenous lysine hydroxylase are found to be hydroxylated in collagens from the same preparation. This lysine-hydroxylysine microheterogeneity may be the result of variations in extent or timing of triple-helix formation during the assembly of the nascent peptide chains. Nothing is known as of this time about the intracellular localization of the enzyme. However, the finding that the peptidyl lysine hydroxylase will not further hydroxylate native collagen suggests very strongly that the hydroxylation must occur prior to helix formation

and, therefore, the lysine hydroxylase must be acting roughly in the same position both location-wise and time-wise as the peptidyl proline hydroxylase, and this places it as in the membrane-bound state on the cisterna side of the endoplasmic reticulum.

It has been difficult to separate the effects of hydroxylation of proline and lysine and their role in secretion of the completed collagen. When prolyl hydroxylase is inhibited experimentally, lysyl hydroxylase is also inhibited because of the similarity in their cofactor requirements. One possibility for selectively inhibiting prolyl hydroxylase without disturbing the lysyl hydroxylase system is in the observation that ascorbic acid deficiency inhibits proline hydroxylation to a much greater extent than lysyl hydroxylation.[61] However, since ascorbic acid deficiency does not completely shut down hydroxylation to the extent necessary to inhibit secretion, this method of inhibition may not add much to studies on the role of hydroxylysine in the secretion of collagen.

DL-*trans*-4,5-Dehydrolysine substitutes for lysine in the activation of transfer-RNA^{lys} and is thereby incorporated into the collagen molecule.[89,117] This lysine analog has no significant effect on the activation of other amino acids with respect to their t-RNAs. The analog was incorporated directly into collagen peptides. It was found that whatever lysine and proline were incorporated in the presence of the DL-*trans*-4,5-dehydrolysine were hydroxylated to a normal extent, and, as with the proline analogs tested, the dehydrolysine-containing molecules were not extruded at the normal rate.[117] Since the proline-incorporating system is separate from that for incorporation of lysine, one must assume that these molecules contain a normal amount of proline and hydroxyproline. Christner and Rosenbloom[117] estimated that there was a 1:1 replacement of lysine by DL-*trans*-4,5-dehydrolysine. Thus, it could be argued from these data that hydroxylysine and its glycosylated derivatives are also a necessary condition for normal extrusion of the collagen molecule. In this case there is no evidence that hydroxylysine or its subsequent glycosylation has any effect on triple-helix formation so that the two effects of hydroxylysine formation and triple-helix formation via proline hydroxylation represent separate requirements for secretion.

Levene et al.[61] found that ascorbic acid does not seem to activate peptidyl lysine hydroxylase as it does peptidyl proline hydroxylase. This does not rule out the possibility that some other molecule serves such a function, but no information is available that peptidyl lysine hydroxylase requires activation like its counterpart, peptidyl proline hydroxylase.

J. Glycosylation of Lysine Residues

The final synthetic stage in completing the chemistry of the collagen molecule is in the glycosylation of selected hydroxylysine residues. Obviously, glycosylation occurs at some time subsequent to the initial hydroxylation of these same lysine residues. Glucose and galactose are the only sugars which have been found linked to hydroxylysine in mammalian collagen, although other sugars have been found in lower organisms.[118-122] In mammalian collagen the sugars are present either as galactose or glucosyl galactose, and the linkage between the collagen and the galactose is the unusual $\alpha 1 \rightarrow$ 2-O-glycosidic bond,[119] a bond resistant to most glycosidases. The enzymes uridine diphosphate galactosyl transferase and uridine diphosphate glucosyl transferase have both been isolated from connective tissues, and these enzymes are specific for hydroxylysine.[119,123,124] Both enzymes require Mn^{+2} ion. Only galactose is attached directly to hydroxylysine; the glucosyl transferase requires the presence of a galactosyl residue for attachment.

In contrast to the rather narrow range of degree of proline hydroxylation and, as a matter of fact, in the total content of hydroxylysine in most interstitial collagens, there is a wide variation in the extent of glycosylation of collagens from different sources. The invertebrate collagens generally have relatively large amounts of carbohydrate.[122] Mammalian interstitial collagens, on the other hand, have very low levels of glycosylation with just a few modified hydroxylysine residues per α-chain. The basement membrane collagens[119-119b] are very highly glycosylated, but these collagens also contain a very high content of hydroxylysine compared to the interstitial collagen. In some cases the disaccharide, glucosyl galactose is found. In other collagens, or other positions on the same chain, only a galactosyl residue is attached to a hydroxylysine. There is little information at this point to indicate the basis for mono- or disaccharide formation, although tissue specificity may be involved. The sequences

of the peptides which contain the disaccharide linked to hydroxylysine have been determined in three cases.[125] These sequences, GLY-MET-HYLYS(GAL-GLU)-GLY-HIS-ARG, GLY-PHE-HYLYS(GAL-GLU)-GLY-ILE-ARG, and GLY-ILE-HYLYS(GAL-GLU)-GLY-HIS-ARG, are all similar in that as a requirement perhaps for hydroxylation they have a bulky nonpolar group preceding the hydroxylysine and a basic region following in the third position of the next triplet. Much work remains to be done to characterize many more peptides containing monosaccharide units and other disaccharide-containing peptide regions before definitive substrate specificities for the galactosyl transferase can be delineated. It is clear, however, that the variability in glycosylation provides another source for the microheterogeneity of collagen systems.

We have already noted from the work of Christner and Rosenbloom[117] that replacement of lysine by DL-*trans*-4,5-dehydrolysine would block hydroxylation and prevent the formation in that place of a modified or glycosylated group. In such cases, in the absence of an inhibition of proline hydroxylation, the collagen formed was still secreted more slowly than the control, and, as suggested earlier, it appeared that this had no relationship to triple-helix formation. The conclusion was that glycosylation was required for normal collagen extrusion. On the other hand, the secretion time of a particular collagen does seem to be related to the amount of glycosylation which takes place. Interstitial collagens in which the degree of glycosylation is relatively low are secreted from the cells about 20 min after completion of the nascent chains. In basement membrane collagens, with their much higher degree of glycosylation, secretion is a much slower process, approaching an hour or more.[126] Grant et al.[127] have shown that completely glycosylated molecules remain with chick lens cells for 30 min after glycosylation has been completed. There is no information on whether the glycosylation occurs on separated, disordered chains or on the triple-helical fully assembled collagen molecule. It would appear, however, that glycosylation occurs quite rapidly after the formation of hydroxylysine, and this might mean that glycosylation would occur on nascent, disordered chains. Bosmann and Eylar[123,124] did use their isolated galactosyl transferase to glycosylate disordered or denatured collagen chains, and there is no doubt that the disordered chains are good substrates for the enzymes.

PROCOLLAGEN AND DISULFIDE BOND FORMATION

A. Characterization of Procollagen

We turn now to reconsider studies referred to briefly in introducing the topic of intracellular events. Unfortunately, it is not possible to present a definitive description of procollagen. One major reason for this uncertainty lies in the difficulty of isolating a truly homogeneous preparation of collagen. The chemical microheterogeneity of a particular collagen has been noted in the previous discussions. There are still two forms of heterogeneity which have not been considered. It appears that the conversion of procollagen to collagen proceeds in a stepwise fashion with at least one enzymic degradation and possibly two such steps. Enzymic degradation can also occur artifactually during the isolation of the procollagen unless very special precautions are taken at all stages to minimize such degradation.[11] Most of the studies are clouded by either the naturally occurring or artifactual degradation processes, and the various degradation products are difficult to separate cleanly. Another element adding to the complexity of the system is the fact that it now appears that most tissues manufacture more than one form of collagen, that is, collagens which differ in chain composition. Each of these collagens, in the case of a mixed population, must go through the procollagen to collagen conversion, multiplying the number of potential intermediates that one might find. In spite of all these complexities and large number of possible components, considerable insight has been gained into the nature of the procollagen molecules and their composition and construction. However, the discussion which follows must be viewed with some tentativeness since the nagging question of homogeneity and purity of the components examined still is unresolved.

The experimental evidence for the existence of a precursor of collagen, procollagen, came from radiolabeling studies aimed at following the biosynthesis of collagen in cultured fibroblasts,[3,4,66] isolated fibroblasts,[5,6] and embryonic bone.[7,8] In these studies the cells or tissues were labeled with a radioactive amino acid precursor, usually proline, lysine, or glycine, and then the proteins secreted into the culture medium, in the case of cells, or

extracted from the collagenous tissue in dilute acid, in the case of the bone system, were examined. The collagenous proteins, as gauged generally by the content of hydroxyproline, were examined by CM-cellulose chromatography,[3,7,8] molecular sieve chromatography,[3,5-7,66] and a variety of analytical SDS-acrylamide gel electrophoretic procedures.[4,8] All such studies require the denaturation of the procollagen, and, in general, components with molecular weights larger than α-chains were isolated along with components clearly representing aggregates of the α-chains. The estimated size of the procollagen molecule ranges from a low value of around 360,000 daltons[8] to a value between 500,000 and 600,000 daltons.[4] The procollagens generally were recognized by the collection of counts of radioactivity, and not enough material was available for extensive biochemical characterization or reliable determination of amino acid content. Concurrent with these studies, a genetic disease of the connective tissue of cattle had been described in which the major physical anomaly was the extreme fragility of the skin of these animals. This syndrome was termed dermatosparaxis. The dermatosparactic collagen differed from normal collagen quite considerably in properties such as increased solubility, a decrease in its ability to form stable fibrils, in net amino acid composition, and in the chromatographic patterns yielded upon CM-cellulose chromatography or acrylamide gel electrophoresis. From such studies it became evident that the dermatosparactic collagen was more closely related to procollagen than to the normal finished collagen.[21,128,129] Although the relationship between the procollagenlike material and procollagen was not clear, the availability of large amounts of dermatosparactic procollagen made this tissue an attractive material for investigation. Lenaers et al.[21] designated the intermediate chains which they isolated from dermatosparactic collagen as p-α1 and p-α2. They determined the molecular weights of these chains as 105,000 and 101, 000 daltons, respectively, as determined by sedimentation equilibrium. In contrast, the pro-α1 and pro-α2 chains of procollagen extracted from chick embryo tendon cells[130,131] or human fibroblasts in culture[20,22] seem to have molecular weights in the range of 120,000 to 125,000 daltons. From this evidence alone it appeared that the dermatosparactic collagen was an intermediate between the primary procollagen and the final collagen form.

An enzyme, procollagen peptidase, exists in normal tissues. This enzyme cleaves the extrahelical portion from the procollagen,[132,133] leaving intact helical collagen molecule with only a small NH_2-terminal nonhelical region. The procollagen peptidase is absent in dermatosparactic tissues, or reduced to a low level of activity. Lapiere and his colleagues[21,32,128,129,129,140] have therefore treated dermatosparaxis as an enzyme deficiency disease in which the properties of the collagen are modified by the failure of the enzyme to truncate or modify the procollagen sufficiently. The implication is that the procollagen as initially produced is entirely normal and unaffected. As indicated above, however, the normal procollagen used as the substrate for procollagen peptidase has a higher molecular weight than the intermediate p-α-chains, but the substrate used in these experiments was also artifactually shortened by enzymic degradation during its preparation.[11] Thus, it is uncertain at this time whether the same enzyme makes the initial cleavage in procollagen or if another enzyme removes a part of procollagen before action of the procollagen peptidase. It is also a possibility, which has not been considered in any detail, that the noncollagenous propeptide extension on collagen in dermatosparactic animals may not be identical to the normal procollagen and hence the deficiency might be in both the enzyme and the collagen molecule itself. At any rate, there is strong circumstantial evidence that dermatosparactic collagen represents an intermediate form or stage in the conversion of procollagen to collagen.

The remarks above apply to the procollagen isolated from that group of collagens known as interstitial collagen. Other procollagens have been found in specialized tissues in which the native collagen molecules differ in both size and amino acid composition from the interstitial type. Basement membrane procollagen synthesized by embryonic chick lens in vitro[26,126,127] has been extensively studied. The α-chains of basement membrane collagens are higher in molecular weight than the α-chains of interstitial collagens and contain considerably more 3-hydroxyproline and a very much larger content of hydroxylysine and glycosylated hydroxylysine residues. The procollagen of basement membranes is likewise considerably larger than the interstitial procollagen with the molecular weight estimated at 140,000,[127] compared to the 125,000 value for the interstitial

procollagen. The time required for synthesis and secretion of the basement membrane procollagen is also longer, 1 hr, although there is no simple explanation for this lag period. Initially it was thought that the postsynthetic glycosylation step might be at the root of the delay in secretion. However, it has been shown that glycosylation occurred very shortly after synthesis and at least a half hour before the final secretion.[126] In other respects, the snythesis and processing of basement membrane procollagen appear to proceed in the same fashion as that for the interstitial procollagens.

Fibroblasts from the skin of a dermatosparactic calf were isolated and established as a clonal line in culture, and these cells secrete procollagen into the culture medium. Since the culture medium does not contain an active procollagen peptidase in this case, the procollagen molecules are retained in their supposedly primary state. Two genetically distinct procollagen molecules appear to be present. One procollagen has a typical composition, yielding a 2:1 mixture of a pro-α1- and pro-α2-chains on denaturation. These chains have molecular weights of about 120,000 daltons. The second form of procollagen has pro-α-chains with molecular weights of 150,000 daltons, and it converts only into a single type of α-chain upon denaturation, its molecular formula thus being $[\text{pro-}\alpha]_3$. Church et al.[32] first suggested that the higher molecular weight precursor molecule, that is, the procollagen with pro-α-chains on the order of 150,000 daltons, would be an earlier precursor of the pro-α-chains and have a noncollagenous or propeptide mass about two times greater than that of the propeptide of the more usually observed pro-α-chains. However, they were able to show quite clearly that the two pro-α-chains isolated differed in primary structure.

In a similar study, Church et al.[31] examined the collagen produced by a clonal line of Schwann cells. Schwann cells are of neuroectodermal rather than mesenchymal origin. These cells, at an early stage in culture, produce procollagen polypeptides with molecular weights of 105,000, 120,000, and 155,000, as well as finished collagen molecules with the usual-sized α1-and α2-chains. As the cultures ages, the Schwann cells ceased making the lower molecular weight collagen and exclusively began to produce the 155,000 dalton form. The procollagen containing this very high molecular weight form was rapidly digested by pepsin or chymotrypsin under conditions where collagen in the helical conformation would not be digested, indicating that the very high molecular weight procollagen is not in the helical conformation. On the other hand, the Schwann cells do produce an active form of procollagen peptidase and at an early stage in cloning normal α1-and α2-chains are found in the culture medium. The authors suggested that the collagenlike material observed in peripheral neurinomas may be produced by the Schwann cells and that this tissue is composed of collagen related to this high molecular weight procollagen form. The neurinoma collagen fibers have an abnormal type of structure more closely related to the fibrous long spacing form (FLS) than the native form of 670 Å banded native fibers, further suggesting that this unusual form of collagen has impaired or different fibril formation properties. Since Schwann cells normally produce a form of basement membrane, the abnormal procollagen may be related to a basement membrane type. However, the 155,000 dalton polypeptide containing collagen may represent a type unique to Schwann cells. Church et al.[31] speculate, however, that the Schwann cells are unable to convert this high molecular weight procollagen to collagen, much as in the dermatosparactic situation. Furthermore, from the generalized protease susceptibility of the collagen related polypeptide, they proposed that a defect exists in the processing of the collagen into its triple-helical molecular form. Another possibility is that the Schwann cell procollagen may be associated very closely with a non-collagenous macromolecule, as found in the case of cartilage procollagen.[134]

Still another possibility lies in the fact that there may be a carboxyl terminal extension on the procollagen molecule as well as the amino terminal extension. Tanzer et al.[13] have demonstrated that the high molecular weight procollagen isolated from the culture medium of the clonal line of calf dermatosparactic cells contains a carboxyterminal noncollagenous peptide extension or appendage equivalent in size to that of the amino terminal extension. This carboxyterminal extension may modify the aggregation properties of the procollagen, although it does not prevent that procollagen from forming segment long spacing precipitates normal in appearance except for the extended carboxyl terminal region.

B. Disulfide Bond Formation

One of Speakman's[2] fundamental postulates was that the extrahelical propeptide at the amino

terminal region of the α-chains served to register the newly synthesized peptides into their proper alignment. How this is accomplished is yet to be determined conclusively. One may consider, of course, ionic interactions, hydrogen bonding, hydrophobic bonding, and disulfide bond formation as potential bases for chain ordering. Early evidence for the existence of cysteine in procollagen led to speculation that the latter, through the formation of disulfide bonds, could be the primary registration mechanism since this amino acid does not occur in the triple-helical portion of the collagen molecule.

The amount of cysteine in pro-α1- and pro-α2-chains is still an unsettled question. Lenaers et al.[21] found six to eight half-cystine residues in their p-α1 and only one half-cystine residue in p-α2 in the dermatosparactic procollagen. Dehm et al.[135] demonstrated that ^{14}C-cysteine was incorporated into procollagen but made no estimate of the number of residues involved. They did find partial aggregation and that prior treatment with mercaptoethanol eliminated this, suggesting the presence of disulfide linkages between some pro-α-chains. However, because some pro-α-chains could be isolated even in the absence of mercaptoethanol or an equivalent reducing agent, there was doubt that all three pro-α-chains were linked to an equivalent extent, at least, by disulfide bonds. Bornstein et al.[23] and Ehrlich and Bornstein[136] supported this observation. Chick embryo calveria were labeled with ^{35}S-cysteine. The procollagen was extracted in 0.5*N* acetic acid and examined on CM-cellulose in the presence of 4 *M* urea. The labeled cysteine was incorporated only into pro-α 1-chains and not into pro-α2. This evidence, along with the fact that complete dissociation of the pro-α-chains was accomplished with 4 *M* urea alone, argued against disulfide bond formation as being the stabilizing mechanism necessary for registration of the procollagen α-chains. However, since ^{35}S-cysteine was incorporated into pro-α1-chains, intrachain disulfide bonds were not entirely ruled out.

The conclusions drawn from these studies must be reevaluated. Monson and Bornstein[11] have recently shown that the acid extraction procedure leads to artifactual partial proteolytic degradation of the amino terminal propeptide (and perhaps also the carboxyl terminus) of the procollagen molecule, giving a shortened molecule which nevertheless still contains some of the extrahelical amino acid residues equivalent to the p-α-chains of Lenaers et al.[21] When the extraction conditions were sufficiently altered so that enzymic degradation was minimized, a procollagen in which all three chains are disulfide linked was obtained.[11] Fessler et al.[25] also isolated a three-chain disulfide-bonded precursor form of collagen from chick calveria. Veis et al.[137] also demonstrated that in rat skin extracted under conditions where enzymic degradation of procollagen was inhibited, two disulfide-linked forms of procollagen can be isolated. These recent findings reconcile the data on chick embryo calveria procollagen with that found previously for human fibroblasts in tissue culture,[9,10,20,22] chick embryo fibroblasts in culture,[34] and isolated chick embryo tendon cells.[24]

The conclusion that in all these procollagens disulfide bonds linked the three α-chains initially is thus based on the following evidence:

1. Chromatography in various systems of SDS-gel electrophoresis shows the presence of very high molecular weight aggregates of proteins related to collagen. Treatment with disulfide bond-breaking reagents eliminates this high molecular weight material and pro-α-chains are observed.[9-11,20,22,26,131,135,138]

2. Labeled cysteine is incorporated into procollagen.[22,24,25,34,134]

3. Amino acid analyses of isolated procollagen α-chains show the presence of cysteine.[10,21,130]

Intact procollagen contains about 15 residues per 1,000 amino acid residues,[130] and the isolated propeptide fragment contains on the order of 23 residues cysteine per mole.[10] There is little doubt that the proteins examined are all related to collagen. Three methods have generally been used to demonstrate this critical point: (1) the presence of nondialyzable hydroxyproline,[11,20,22,24-26,34,135,136] (2) digestability of the material with highly purified bacterial collagenase,[9,23,135] and (3) the reaction of the isolated material with antibodies prepared against procollagen or the propeptide.[10]

Thus, although there were initial suggestions that not all three pro-α-chains were linked by disulfide bonds, the weight of evidence indicates that in the initial undegraded procollagen molecule this is the case. The model proposed by Goldberg

and Sherr[9] based on procollagen isolated from both cultured cells (intracellular) and culture medium (extracellular) seems to best fit the available data. The cells synthesize procollagen α-chains in which the cysteine residues are not all at the same distance from the amino terminus in both the pro-α1- and pro-α2-chains. The cysteine in the pro-α2 is more proximal to the amino terminus. During the intracellular assembly, disulfide bonds form linking all three chains together. In addition, helix formation occurs. Goldberg and Sherr do not speculate about either the subcellular localization of this event or the order of occurrence of disulfide bonding and helix formation. The molecule is next thought to be secreted in the disulfide-bonded triple-helical form. Two or more extracellular proteases then sequentially process the molecule. The first peptidase cleaves a segment proximal to the amino terminus which contains the disulfide bond linking pro-α2 to pro-α1. However, the two pro-α1 chains are still linked together by another disulfide bond. Another peptidase or the same enzyme acting sequentially then cleaves the remaining propeptide from the modified procollagen, leaving only the helical segment and its near neighbor telopeptides. Thus, the disulfide bond linking the pro-α1-chains together is lost. Goldberg and Sherr found on SDS-gel electrophoresis components which correspond in molecular weight and disulfide bonds to account for all the intermediates postulated in the above model.[9,22] Further evidence to support this hypothesis that all three chains are linked by disulfide bonds came from a study[10] in which the precursor molecule was digested with collagenase and the remaining propeptide portion isolated. The molecular weight of the propeptide portion was 75,000 to 80,000 daltons. Reduction and alkylation gave a molecular weight of about 25,000 daltons for the component chain.

Other studies focusing on the propeptide portion of procollagen have shown that antibodies can be made specifically to the propeptide portion and that these will not recognize the triple-helical portion of collagen or the small noncollagenous telopeptide extensions which remain after the action of procollagen peptidase.[139] Digestion intermediates of procollagen are also not recognized. Thus, the immunogenic portions are very close to the amino terminus of procollagen. Amino acid analyses have been carried out on the propeptide fragment remaining after collagenase digestion[10] on the pro-α1 segment remaining after collagenase digestion[140] and on the cyanogen bromide (CNBr) isolated propeptide α1-CL1 fragment.[141] Although there are differences in the various values obtained (Table 3), the data all agree on the presence of half-cystine and a high content of serine, aspartic acid, and glutamic acid. The molecular weights reported for the propeptide range from 12,000[142] to 25,000 daltons, although the lower molecular weight must be viewed with considerable caution as the extraction of the procollagen was accomplished in 0.5*N* acetic acid without protease inhibitors,[142] conditions under which, as we have seen, proteolytic attack on the propeptides occurs.

C. Localization of Disulfide Bond Formation and Triple-helix Assembly

Schofield et al.[138] have attempted to probe the relationship between disulfide bond formation and triple-helix assembly. Using a chick embryo tendon cell system, they found that some of the secreted procollagen was disulfide bonded but that approximately 20 to 30% existed in a triple helix without disulfide bonds. On the other hand, intracellular procollagen was disulfide bonded, and it appeared that the intracellular processes of disulfide bonding and triple-helix assembly closely paralleled one another. There was evidence that these two processes did not occur until the pro-α-chains were released from the ribosomes. In a clever experiment, utilizing α,α′-dipyridyl inhibition which leads to the synthesis of non-triple-helical protocollagen at 37°C, they found that disulfide bond formation still occurred. Thus, while disulfide bonding may be necessary for helix formation, helix formation is not a prerequisite for disulfide bond formation. Schofield et al.[138] argued that disulfide bond formation must be a relatively large event in the assembly of the procollagen molecule as it was only after chase periods of 9 to 12 min following a 4-min pulse of labeled cysteine that most of the intracellular pro-α-chains were found to be disulfide bonded. Harwood et al.[131] approached the same problem by labeling cells with radioactive proline and then doing a classical subcellular fractionation. Corroborating Diegelman et al.,[17] they demonstrated that procollagen was synthesized on membrane-bound polysomes. They then isolated the rough endoplasmic reticulum, with its associated polysomes, and smooth endoplasmic reticulum. Since

TABLE 3

Compositions of Propeptide Fragments from Various Collagens
Residues/peptide

Amino Acid	Intact disulfide linked propeptide. Human fibroblast[a] (Ref. 10)	Additional peptide of pα1 chain, dermatosparactic[a] (Ref. 140)	Additional peptide of proα1 chain, chick cranial bone[b] (Ref. 141)
Lysine	44	7.0	6
Histidine	11	1.9	4.5
Arginine	25	5.2	5
Aspartic acid	85	27	23
Threonine	43	11	9
Serine	54	3.3	32
Glutamic acid	79	34	29
Proline	37	20	13
Glycine	63	16	33
Alanine	56	2.8	10
Half-cystine	23	16	6.4
Valine	42	18	9
Methionine	11	–	–
Isoleucine	28	4.7	7.3
Leucine	48	5.8	8
Tyrosine	24	1.8	3.6
Phenylalanine	25	0.7	3
Tryptophan	Not determined	2.9	Not determined
4-Hydroxyproline	–	–	2
Hydroxylysine	–	–	1.1
Total residues/peptide	698	178.1	204.9

[a]Direct determination on fragment.
[b]Calculated difference between pro-α1-chain and α1.

the nascent and intracisternal proteins were radiolabeled, they could follow the state of aggregation of these by monitoring radioactivity either with or without mercaptoethanol treatment. After a 4-min incubation with the radioactive proline, the rough endoplasmic reticulum was isolated and treated with SDS and iodoacetimide to prevent nonspecific aggregation. Gel chromatography of this material showed no radioactive label in peptides greater than 125,000 daltons, the size of the postulated primary pro-α-chain. When this experiment was repeated following a 2-hr incubation with the labeled proline, approximately half of the label appeared in components with molecular weight greater than 300,000 daltons. Reduction with mercaptoethanol prior to chromatography again yielded a majority of the radioactivity in the elution position of pro-α-chains. Isolation of the smooth endoplasmic reticulum from these same cells and examination of the protein related to it without prior reduction indicated that over 90% of the radioactivity was in large molecular weight aggregates. Because no aggregation of pro-α-chains was seen after the 4-min labeling period, Harwood et al.[131] concluded that disulfide bond formation does not occur with the nascent polysome bound polypeptides. They proposed that the pro-α-chains are released into the cisternae of the rough endoplasmic reticulum where disulfide bond formation begins. By the time the procollagen molecule has reached the next stage, having been transported to the smooth endoplasmic reticulum, the process of disulfide bonding has been completed, as evidenced by the fact that the protein isolated from the smooth endoplasmic reticulum bounded subcellular vesicles is almost totally disulfide bonded. Taken together, then, these two sets of experiments from different laboratories indicate strongly that both disulfide bond formation and triple-helix assembly occur after the release of the pro-α-chains from the ribosome and probably within the cisternae of the endoplasmic reticulum.

Nothing is known yet about the time of

glycosylation in relation to the events of the synthesis of peptide-bound hydroxylysine, to the release of pro-α-chains from the ribosomes, to the entry of these pro-α-chains into the cisternae of the endoplasmic reticulum, to disulfide bond formation, triple-helix assembly, or to secretion from the cell. Glycosylation could occur at any time after the synthesis of hydroxylysine up to or during the movement of the helical procollagen molecule from the rough to the smooth endoplasmic reticulum. If the glucosyl and galactosyl transferases which utilize peptide-bound hydroxylysine as substrates are at all analogous to the enzymes which glycosylate other glycoproteins, they probably exist within the confines of the endoplasmic reticulum. Moreover, since there is some evidence that the prolyl hydroxylase acts only on the unfolded pro-α-chains and not on triple-helical procollagen,[77] it is likely that glycosylation may occur prior to helix formation. This is, however, only speculation, as no experimental evidence is available to either localize the enzymes intracellularly or to kinetically determine the time of glycosylation other than the experiments relating to basement membranes, in which it was found that glycosylation occurs at an early stage long before the molecule is secreted from the cells.

PROCOLLAGEN SECRETION

A. Intracellular Packaging

In all respects, except perhaps for the glycosylation of the procollagen, we can consider that the procollagen molecule is fully assembled and in its native conformation by the time that it leaves the cisternae of the endoplasmic reticulum and begins its transit from the cell. Several intracellular routes of secretion have been postulated, all based mainly upon electron microscope autoradiography. The first proposal was made by Revel and Hay.[143] They suggested that the collagen was transported from the endoplasmic reticulum to the Golgi region, where secretory vacuoles were formed, moving the membrane-bound collagen to the plasma membrane surface where exocytosis occurred by fusion of the vesicles with the plasma membrane. This form of merocrine secretion via the Golgi is similar to that of the secretory pathway for other glycoproteins. While recognizing the possibility of this pathway, Ross and Benditt[142] suggested that the procollagen could pass directly from the cisternae of the rough endoplasmic reticulum to the cell surface by an intermittent fusion of the cisternal membrane with the plasma membrane or by small vesicles arising from the cisternae, circumventing the Golgi system entirely. The validity of this study, however, is in question since it has been shown recently that the hamster fibroblasts used in Ross and Benditt's study do not have a well-developed Golgi apparatus and the secretory pathway in this system may thus be unique rather than general. Salpeter[144] also attempted to follow the collagen secretory pathway in cartilage cells from regenerating limbs of adult newts via electron autoradiography of samples taken at various times following the injection of ^{3}H-proline. Salpeter's data definitely implicated the rough endoplasmic reticulum as the site of synthesis of some ^{3}H-proline-labeled product. There was, however, a considerable diffusion of label in the ground cytoplasm even at very early time periods, and label was also seen in the Golgi. Upon a detailed analysis, no definite flow of radioactivity from one cellular organelle to another was observed, and Salpeter concluded that some secretory components could leave the cell directly from the ground cytoplasm. While the data were not incompatible with the hypothesis that some of the radioactive products were secreted via the Golgi system, the data appeared to be incompatible with the hypothesis that all secretion went through the Golgi apparatus.

The basic problem in all these studies, as well as that of Cooper and Prockop,[145] is that the studies were done in tissues which synthesize and secrete more than one macromolecular product, such as the glycosaminoglycans and proteoglycans of the cartilage system.[144,145] The synthesis of these extracellular products was thus superimposed upon the synthesis of the procollagen, and these other proteins undoubtedly also contained proline. Because there was no way by electron microscopic examination to distinguish between the labels in any one of the macromolecular cell products, it was necessary to search for tissue types or cell types which synthesize or export only procollagen. The synthesis of intracellular proteins in these systems may still be an interfering factor, but the fact that they are not secreted, as well as differences in turnover times, minimize the interferences. Three laboratories have therefore turned to highly specialized tissues in an attempt to resolve the question of the intracellular pathway of procollagen secretion.

Hay and Dodson[146] have examined the secretion of collagen by corneal epithelium. These cells are very active in producing large sheets of collagen and basal lamina which constitute the corneal stroma. The corneal epithelial cells are synthetically active and are rich in secretory granules, secretory organelles, vacuoles, and intracellular clefts. Weinstock and Leblond[147] have used the odontoblasts of rat incisors. These cells are highly polarized and have a well-developed separation between nucleus, supernuclear rough endoplasmic reticulum, Golgi, and an apical rough endoplasmic reticulum. In these cells it is relatively easy to follow the migration of radioactive material from a region close to the nucleus to the distal portion of the cell where secretion of the extracellular matrix occurs. Weinstock and Leblond have also followed collagen secretion in the osteoblasts of alveolar bone.[148] In these studies ^{3}H-proline was used as a tracer followed by electron microscope autoradiography. Bornstein[149] has begun using ferritin-labeled antibodies to procollagen in conjunction with electron microscopy to follow collagen secretion in both osteoblasts and fibroblasts. Since the antibodies to procollagen are specific for this macromolecule,[139] this method does not suffer from the drawback of multiple proline-labeled cell products.

In all of these cases, the Golgi apparatus and its associated vesicles were implicated directly in the secretion of procollagen. The Golgi vesicles were reported to contain filamentous threads while still associated with the Golgi, and later as the vesicle traversed the cytoplasm these threads appeared to aggregate into rodlike bundles of dimensions appropriate to aggregates of collagen molecules. According to Weinstock and Leblond,[147] the newly synthesized procollagen is transported to the Golgi either by way of a transitional element or by fuzzy-coated intermediate vesicles budding off from the rough endoplasmic reticulum. These are then sequestered into highly distended Golgi sacules. The procollagen appears in the form of entangled threads within these distended sacules. Subsequently, the contents of the sacule become organized, changing from a roughly spherical to a cylindrical shape, and the threads become aligned in parallel fashion. Finally, as depicted in Figure 2, these sacules flatten and separate from the Golgi and lie free in the cytoplasm. These free prosecretory granules undergo further reorganization and finally as separate secretory granules migrate to the odontoblastic process where they release their contents into the predentin. The work of Hay and Dodson[146] and Bronstein[149] also supports the strong involvement of the Golgi apparatus in the secretion of collagen.

Additional, but somewhat more indirect, evidence supporting the idea that the Golgi apparatus is involved in procollagen secretion comes from the studies of Trelstad.[150,151] Using the corneal epithelium of chick embryo, he demonstrated that the epithelial cells underwent some striking changes at different stages during embryonic development. The cells are engaged in active collagen synthesis at two stages: first, at stage 28, day 5 and second at stage 41, day 15. The first period of collagen synthesis corresponds to the laying down of the primary corneal stroma, while during the second stage Bowman's membrane is elaborated. At the onset of both of these periods of rapid collagen synthesis, the Golgi of the corneal epithelial cells assumed a basal position within the cell. At all other times the Golgi was located predominantly apical to the nucleus. The inference was that the Golgi apparatus migrated within the cell from an apical to the basal position in order to engage in the secretion of the collagenous elements of both the primary stroma and Bowman's membrane, which are located in a position basal to the cells.

Trelstad also noted two different types of vacuoles in these corneal cells during the time of elaboration of the primary stroma. The first type of vacuole contained cross-striated aggregates which appeared to be collagen-like. These vacuoles were found near the cell membrane within the cell. The collagen-like aggregates were at the center of the vacuole and had diameters of 200 to 600 Å and lengths of 3,000 to 6,000 Å. Amorphous material was found at the periphery of the vacuole. The second type of vacuole was found to be cylindrical in shape, measuring an average of 1,000 Å by 6,000 Å. The fibrillar material contained in these vacuoles was densely packed, but no cross-striations could be discerned. Since the principal product of the corneal epithelial cells was collagen, Trelstad concluded that the collagen was being concentrated and excreted from the cell via these vacuoles. It could not be determined from the electron micrographs whether the vacuoles were derived from the Golgi apparatus. However, the Golgi apparatus was a permanent organelle within the same region of the cells as the

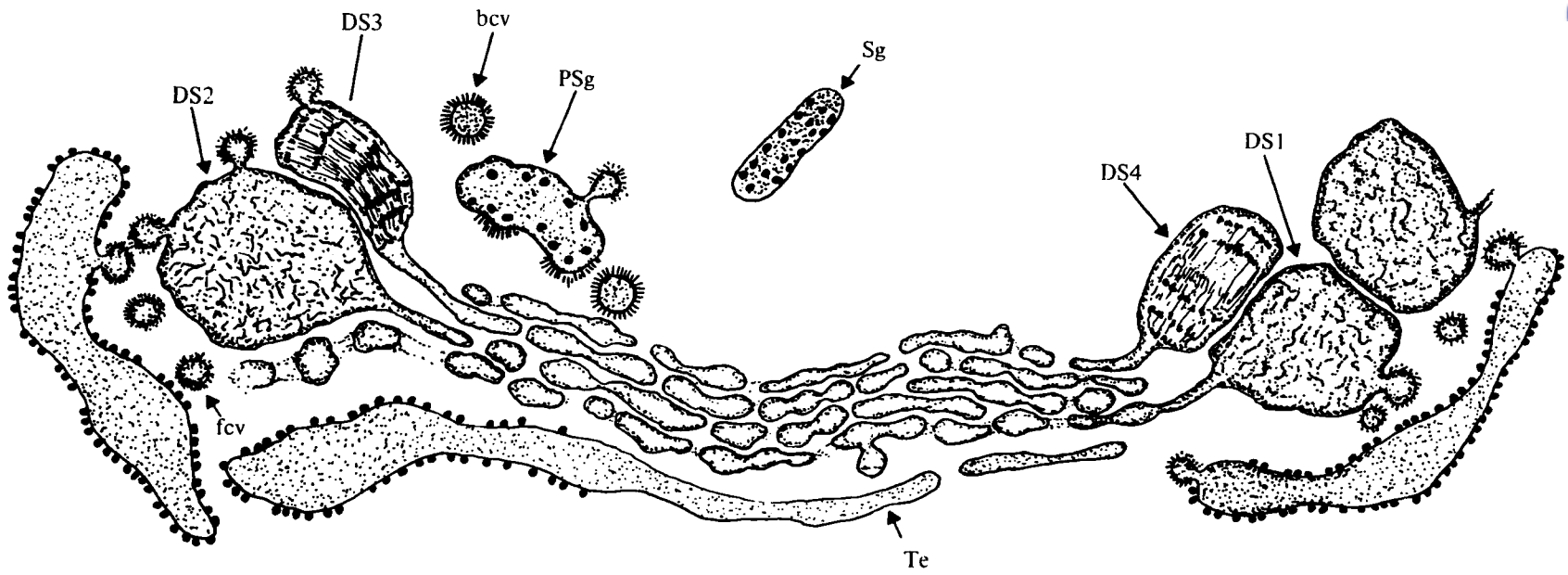


FIGURE 2. Schematic drawing of a portion of the Golgi apparatus in the odontoblast depicting the presumed sequence of events in the transport and packaging of procollagen. In this scheme, transitional elements (Te) or fuzz-coated vesicles (fcv) from the endoplasmic reticulum move into the Golgi, where they form distended vacuoles (DSl, DS2). The structures within the vesicles are in the form of entangled threads. The saccule contents gradually organize (DS3, DS4). The more organized structures containing dense aggregates of parallel collagen molecules are referred to as prosecretory granules (PSg) and, finally, as secretory granules (Sg). Bristle-coated vesicles (bcv) also appear to bud off from the prosecretory granules. (From Weinstock, M. and Leblond, C. P., *J. Cell. Biol.*, 60, 92, 1974. With permission.)

vacuoles. These data, along with the regular change in position of the Golgi apparatus during active collagen secretion, led Trelstad to support the hypothesis that collagen was secreted via the Golgi apparatus. Vacuoles containing recognizable collagen structures and related to the locus of the Golgi apparatus have been observed in less specialized cells of mesenchymal origin,[152-154] and one may readily extrapolate these observations to suggest that in all collagen-producing cells, procollagen is secreted in vacuoles originating from the Golgi apparatus.

B. Transport of the Secretory Granules. Involvement of Microtubules

Microtubules are the intracellular structural proteins, and the microtubular structures seem to be involved in the secretion of material contained in many types of vacuoles and granules.[155,156] The evidence indicated above that the procollagen secretory pathway involves membrane-bound secretory sacules related to the Golgi suggests that microtubules may also be involved in their secretion. Indeed, when cells[157,158] or whole tissues such as cranial bone[158,159] are incubated with microtubule disruptive drugs, the secretion of procollagen is impaired. When either 3T3 fibroblasts in culture[158] or isolated chick embryo tendon cells[157] were used, there was no inhibition of incorporation of radiolabeled amino acids into collagenous or noncollagenous protein. However, the collagenous proteins which were not secreted in the presence of colchicine or vinblastine were found retained within the cells[157,158] as procollagen and were not immediately degraded. In addition, neither colchicine nor vinblastine had any effect on the extent of hydroxylation of proline[157-159] of this retained procollagen. Presumably there was also no effect on lysine hydroxylation. When cranial bones were incubated with the microtubule disruptive agents, the situation was more complicated than in the cell culture experiment since there was a decreased incorporation of labeled amino acids into both collagenous[158,159] and noncollagenous proteins.[158]

The observation that microtubules seem to be involved in procollagen secretion argues for a merocrine model of secretion. Unless microtubule disruption affected the secretion of all types of cell products by drastically altering cell function, passage of the procollagen molecules directly from the rough endoplasmic reticulum or via the soluble cytoplasm through the plasma membrane should not have been affected by the presence of colchicine or vinblastine. Presumably the role of microtubules in secretion is that of a transport conduit along which the secretory vesicles move from their place of biogenesis within the Golgi apparatus to their eventual fusion with the plasma membrane and dumping of their contents from the cell. Secretory vesicles cannot traverse the cell, and secretion is inhibited when the microtubular transport system is disrupted.

REGULATION OF COLLAGEN BIOSYNTHESIS

Very little is known about the mechanisms by which collagen synthesis and transport are controlled. The relevant questions which may be asked are the following: Is collagen biosynthesis under any type of hormonal control? What directs the fibroblasts or any of the collagen synthesizing cells to begin synthesis of the m-RNA molecules which code for procollagen? What factors determine the rate at which procollagen is made? Is the controlling mechanism at the level of transcription or translation? Is there a feedback mechanism that stops or at least decreases the rate of collagen biosynthesis? Is such control at the level of fibril formation? Does the propeptide portion of procollagen upon cleavage from the molecule after exocytosis participate in synthetic regulation? Answers to these questions must be left for the future as almost no information has been obtained concerning most of them.

Hormonal control is one area of regulation of collagen biosynthesis in which some data are available, although these data are scanty and somewhat in conflict.

Since a considerable amount of connective tissue, of which collagen is the major protein, is elaborated during the growth phase of a young animal, one might expect those hormones which promote growth to enhance collagen synthesis. When the effects of growth hormones were investigated in vitro, however, either no effect was observed[160] or a decrease in the specific activity of radioactive hydroxyproline extractable in neutral salt and acetic acid[161] was seen. Since the specific activity of peptide-bound proline in these same fractions was increased, the data were interpreted as reflecting an inhibition of the peptidyl proline hydroxylase. Ascorbic acid had been added

to the granuloma minces used in this study, so there was no possibility of a vitamin C deficiency. Skin minces showed a decrease in specific activity of both proline and hydroxyproline in the presence of growth hormone, while bone fragments showed an increase in proline but a decrease in hydroxyproline-specific activity. Since growth hormone probably plays some role, direct or indirect, in the synthesis of collagen in vivo, some factor necessary for its action in vitro must have been lacking in these studies.

Serotonin[162] and estradiol[163] also have an effect on collagen metabolism. Serotonin increases collagen synthesis but in a nonspecific manner due to an increase in 30 S ribosomal RNA and t-RNA synthesis. The estradiol effects are tissue-specific. In tendon, faschia, and aorta, estradiol caused a decrease in specific activity of radiolabeled hydroxyproline isolated after administration of radiolabeled proline. In tendon this occurred within 24 hr and remained significant up to 144 hr after estradiol administration. In faschia and aorta, however, no effect was seen until 144 hr after the administration of the radioactive proline. Thus, the decrease in hydroxyproline-specific activity 6 days after treatment must have reflected an increased degradation of already formed collagen.

The effects of insulin on bone collagen metabolism have been examined recently using organ culture of embryonic chick tibia[164] or neonatal rat tibiae.[160,165] Perlish et al.[164] found that in the presence of 3.2 units/ml of insulin there was a short-term, that is, 2- to 4-hr, increase in collagen synthesis but no long-term effect, that is, over a 24-hr period. On the other hand, Schwartz et al.[165] found no short-term, 3- to 6-hr, effect of insulin in concentrations ranging from 0.01 to 10.0 units/ml of culture medium. When a continuous flow system was used for constant renewal of the culture medium, however, an increase in collagen synthesis was found at longer time periods.[160,165] For these long incubations, physiological doses of insulin (0.01 units/ml) were found to be effective. Schwartz et al. speculated that a primary effect on protein synthesis might have been the mode of action. Other possibilities include a decreased degradation of collagen due to residual insulin after removal of the tissue from the animal.

Many other studies dealing in a more general way with hormonal treatment influences on wound healing or involution of the uterus all lead to the conclusion that various hormones do have an effect on collagen biosynthesis and remodeling, but it is not clear whether this is a control specifically directed at the collagen system. Obviously this field is a fertile one for further investigation.

EXTRACELLULAR CONVERSION OF PROCOLLAGEN TO COLLAGEN AND THE FORMATION OF THE FIBER SYSTEM

When the collagen-containing secretory granules merge with the cell membrane and spill their contents out into the extracellular space, the collagen is still in the form of the undegraded precursor, procollagen. Electron micrographs[147,151] show that at this stage the procollagen is organized into bundles 3,000 or 6,000 Å in length and arranged in an antiparallel fashion. It appears that in the 6,000 Å bundle the propeptide segments are aligned along the center of the bundle. Hence, the packing arrangement in the larger bundles is such that the amino terminal regions are all oriented toward the center of the 6,000 Å spindle and the carboxyl terminal regions point in opposite directions on either side of the central band. In the odontoblast,[147,148] the first step in the formation of the extracellular fiber and fibril systems is thus the dissolution of these antiparallel collagen molecule bundles and their conversion and reorganization into a parallel alignment. This, perhaps, is the stage at which the enzyme procollagen peptidase makes its primary cleavage and serves in releasing individual molecules from the aggregated state in the secretory granules.

In the cell culture system, the procollagen is not cleaved to give polypeptides with molecular weights on the order of α-chains. Instead, it remains in the culture medium as the procollagen molecule. Since culture conditions attempt to approach physiological conditions, collagen molecules of the usual length would be expected to spontaneously precipitate in the form of native fibrils. Indeed, some collagen is usually present in fibril form around the cells in the cell layer. This fibrillar collagen is closer to the partially degraded collagen of the dermatosparactic type than it is to complete procollagen, indicating that partial degra-

dation takes place in the cell culture system. The accumulation of a partially degraded form of collagen in the cell layer suggests that the first stage procollagen peptidase is localized at the cell membrane. The difference between the culture and in vivo situations may well be that in culture the procollagen escapes from the vicinity of the cell membrane before the enzyme has occasion to act completely. In this case, the appearance of the partially degraded collagen or p-collagen is rather clear evidence that the conversion of procollagen to collagen takes place normally in more than one step and that the deficiency in dermatosparaxis is in the enzyme which carried out the second rather than the first conversion process.

Although the early studies of the conversion of procollagen to collagen[8,11] indicated that the procollagen was rapidly converted to collagen in most instances, more recent evidence suggests that the methods used for extraction of the collagen in those experiments led to the artifactual appearance of collagen as a result of a procedural degradation of the amino terminal propeptides.[11] More recent work in our own laboratory[137,166-168] has demonstrated that the conversion of procollagen to collagen in vivo, at least in rat skin, must be a multistep process with at least two conversion steps. Intact procollagen and an intermediate form, approximately the same size but not identical in properties to the dermatosparactic type of p-collagen, can be isolated from rat and bovine skin during the period of rapid growth of the animal. The intermediate form in bovine skin was present in sufficiently large amount that the collagen could be isolated and prepared in SLS form. The electron micrographs of this material (Figure 3) show quite conclusively that there is an amino terminal extension of approximately 120 nm in addition to the length of the normal-sized collagen molecules.[166] Pulse labeling studies of actively growing rats[137,168] showed that the labeled proline moved from procollegen to the intermediate collagen. The peptide chains from isolated intermediate were larger than those of the final α-chain size. The intermediate chains were designated as heavy α-chains, or h-α-chains, and the intact intermediate as H-collagen. These observations suggested a precursor-product relationship in going from procollagen to H-collagen to collagen. The collagen containing the heavy chains, that is, the H-collagen, appeared to renature much more readily than the final form of normal length α-chains, and it was suggested that the intermediate had a rather special role in fibril formation. Native collagen fibers could be produced easily from the H-collagen. These data are in conflict with the work on dermatosparaxic collagen where it appears that the p-collagen is less effective than collagen in forming collagen fibrils. This position is rather difficult to accept since the intermediate p-collagen isolated from dermatosparactic skin has about the same solubility as normal collagen and is present in the skin in the form of native fibers, although it is claimed that p-collagen aggregates more slowly than normal collagen.[21] In contrast, the complete procollagen, as obtained from the cloned dermatosparactic cells by Tanzer et al.,[13] does not form fibers readily. The intact propeptide does therefore appear to inhibit fibril formation.

After the conversion of procollagen to H-collagen, fibrils form. If, as Veis et al.[168] suggest, the h-propeptide segment facilitates fibril formation, then it is logical to suppose that the final step in enzymic conversion to collagen occurs after the microfibril forms. This, in turn, implies that the enzyme-susceptible end-regions must be readily accessible on the microfibril surface or, alternatively, that the enzyme is bound directly to the fibrils. A discussion of the construction of the collagen fiber is beyond the scope of this review; however, two facts are of special interest. The end-regions of the collagen molecule, as in H-collagen or collagen, are very important in establishing intermolecular contacts leading to fibril formation.[169,170] Although collagen molecules trimmed as far as possible by enzymes to leave only the triple-helical section still form fibrils with the native periodicity, the conditions for fibril formation in vitro must be greatly altered.[171] Thus, while ionic and hydrophobic interactions are sufficient to order collagen-helical segments in the quarter-stagger array, the end-region supplies some specific interaction potential for registration of end-overlapped molecules necessary for microfibril formation. After formation and stabilization of the fibrous form, the final procollagen peptidase can act to remove the final noncollagen end-region remnant. From this point of view, the nonhelical segments of collagen should be considered as having an extracellular as well as several intracellular roles – an extracellular fibril registration function as well as intracellular chain registration and transport functions.

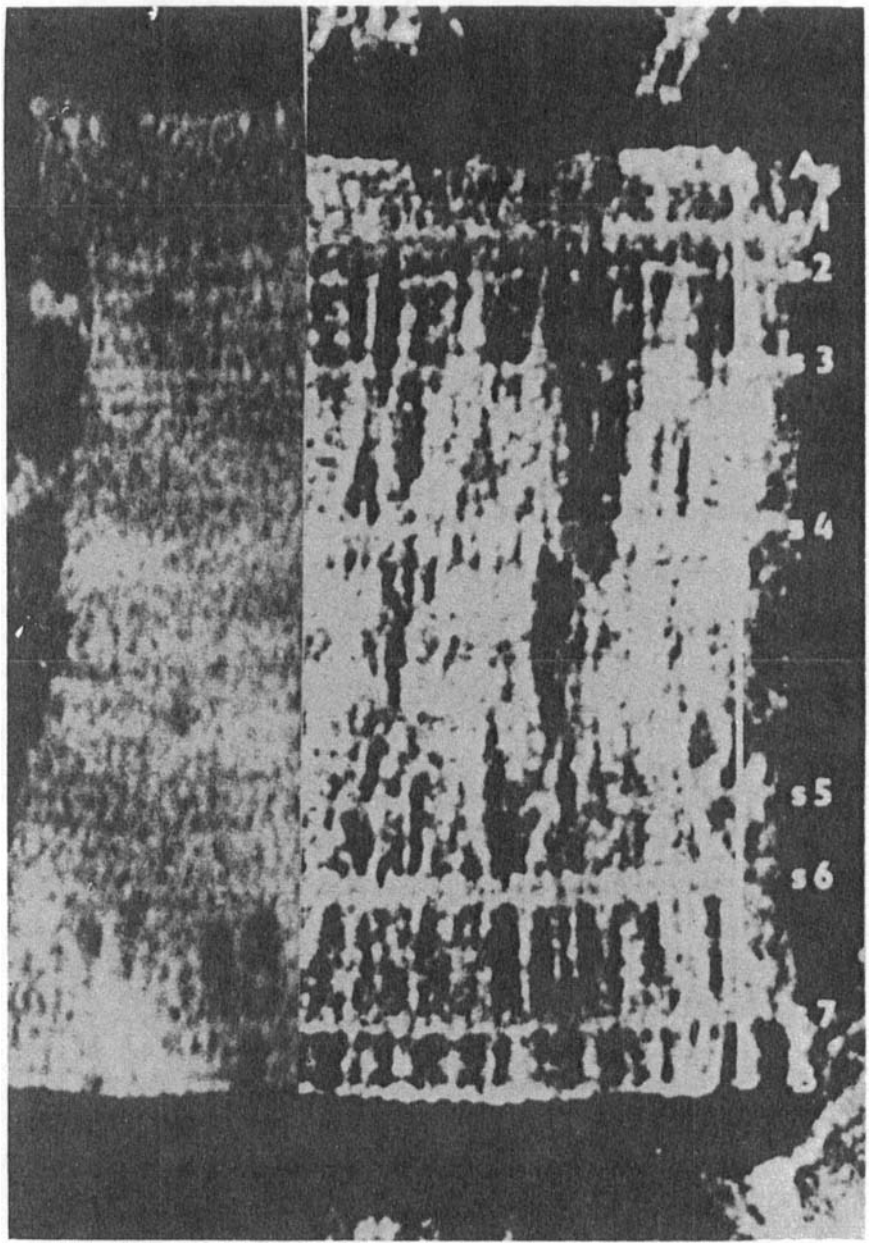
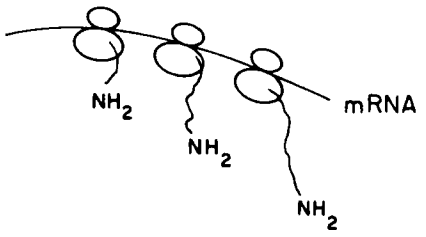


FIGURE 3. A comparison of segment-long-spacing forms of collagen (right side) and H-collagen (left side). The -COOH terminal region of the H-collagen is obviously extended by ~120 mm. (From Veis, A., Anesey, J., Yuan, L., and Levy, S. J., *Proc. Natl. Acad. Sci. U.S.A.*, 70, 1464, 1973. With permission.)

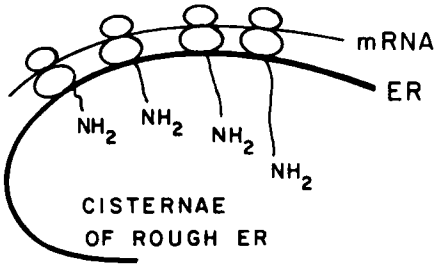
SUMMARY

Figure 4 outlines the processes which have been discussed here. As indicated, there are at least ten steps or stages in the synthesis and assembly of the interstitial collagens. Specialized collagen synthesis, such as in basement membrane and possibly cartilage, probably requires further modifications. Obviously, knowledge of the biosynthetic process has increased markedly in the past few years. However, it should be pointed out that many gaps in our understanding of the system still exist. For example, at the very first step, little is known of the factors which control initiation of chain synthesis. Is there a feedback loop which normally signals overproduction of collagen fiber? In step 2, what factors are involved in binding the ribosomes to the ER membrane? Is there any ordering of

1. Synthesis initiates on ribosomes, nonhelical NH_2-terminal propeptide begins to form.



2. Ribosomes attach to surface of rough ER; chains penetrate to cisternae and continue to elongate.



3. Hydroxylation of nascent chains; activation of PPH within lumen of ER.

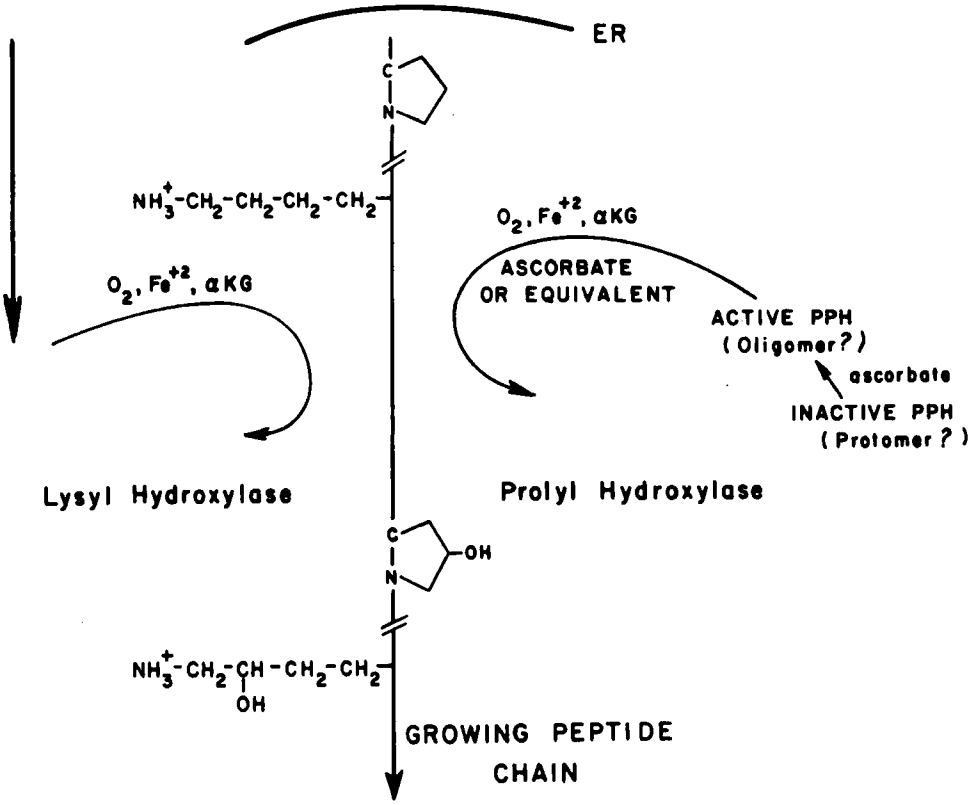


FIGURE 4. A schematic conceptualization of the flow of events in the biosynthesis of collagen.

4. Release of chains from ER attachment, disulfide bond formation, chain registration, continuation of hydroxylation. Glycosylation?

5. Triple-helix formation Hydroxylation reactions cease. Glycosylation?

6. Formation of secretory granule with loose aggregates of procollagen molecules in thin filaments.

7. Condensation of contents of secretory granules into ordered antiparallel arrays of procollagen molecules; translocation to cell surface.

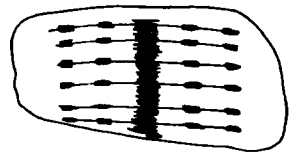
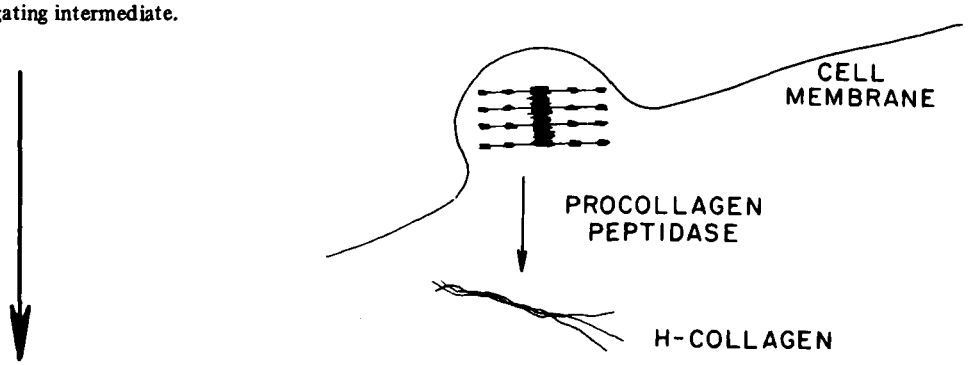
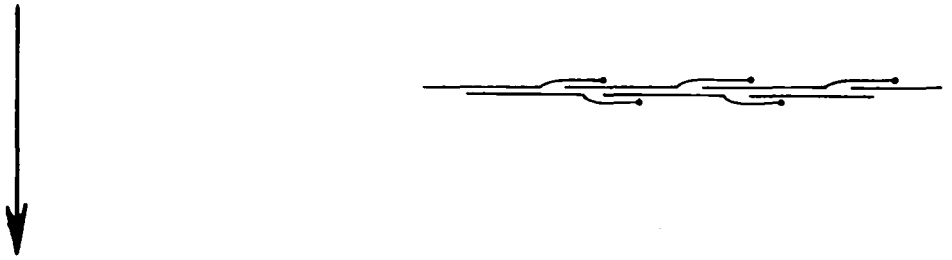


FIGURE 4. (continued)

8. Release of collagen into extracellular space. Procollagen peptidase 1 acts at this stage producing aggregating intermediate.



9. Aggregation of H-collagen to form microfibrils. Final removal of propeptide fragment.



10. Stabilization of fibril system by cross-linking. Appearance of the functional form, cross-linked collagen fiber.

FIGURE 4. (continued)

polysomes producing α1(I) and α2-chains on the ER surface? At step 4, how do the chains find each other and interact to form a 3-peptide strand complex before triple-helix formation? Where does glycosylation begin and terminate? What is the function of glycosylation? At steps 6 and 7, what factors are involved in the formation of the secretory granules and the organization of ordered procollagen aggregates within them? Finally, where does procollagen peptidase act and how do the aggregates reorganize from antiparallel to parallel fibrillar arrays? What are the roles of other ground-substance components in fibrillar organization?

These open questions indicate that many exciting fundamental studies remain to be carried out as we attempt to gain further understanding of the collagen system.

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