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Formation of Hydroxyproline in Collagen

Proline is incorporated into peptides before it is hydroxylated.

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Collagen, a most important component of animal connective tissue, comprises about one-third of body protein. It is not only the most abundant, but one of the most unusual animal proteins, since it is devoid of cysteine and tryptophan and contains more than 30 percent glycine and such unusual amino acids as hydroxyproline and hydroxylysine. In animal tissues hydroxyproline and hydroxylysine are essentially found only in collagen. The formation of collagen is therefore of interest not only as a problem in protein biosynthesis, but also as one in the biosynthesis of these unusual amino acids.

In 1949 Stetten (*1*) made an observation that has long puzzled researchers in collagen biochemistry. She reported that when N^{15}-labeled hydroxyproline was fed to rats it was not incorporated into collagen, whereas in prior studies with Schoenheimer (*2*) she had shown that labeled proline could serve as a precursor of collagen hydroxyproline (Fig. 1). This finding did not appear to be in accord with the concept that free amino acids were the precursors of proteins. However, the observation was verified in experiments not only with intact animals but also with whole-cell preparations of a variety of tissues (*3, 4*). Subsequent studies by Sinex and Van Slyke (*5*) showed also that the hydroxylysine of collagen was chemically derived from free lysine and that labeled free hydroxylysine was not incorporated into the protein. Although these observations were generally accepted, investigators of collagen metabolism have continued to utilize the appearance of protein-bound hydroxyproline as an index of collagen biosynthesis. It is obvious, however, that two processes, protein synthesis and hydroxylation, are involved in the conversion of proline to protein-bound hydroxyproline. Stetten originally postulated that hydroxylation of proline took place in peptide linkage, but early attempts to demonstrate a hydroxyproline-deficient protein intermediate were unsuccessful. However, since Stetten's original observations, much has been learned about the mechanisms of protein synthesis, and it has become apparent that proline is hydroxylated at one of several intermediate stages during its activation and incorporation into the collagen molecule. Proline hydroxylation could occur at the stages of free proline, prolyl adenylate, prolyl-sRNA, ribosomal proline peptide, or protein-bound proline. Although it is now generally agreed that proline is hydroxylated after it has been activated, there is disagreement as to the exact nature of the substrate. Our group has published a number of reports indicating that hydroxylation occurs after the proline is incorporated into peptide linkage. These reports present evidence that a proline-rich, hydroxyproline-deficient protein accumulates when hydroxylation becomes the rate-limiting reaction and show that such a protein can serve as a substrate for hydroxylation. On the other hand, several laboratories have reported that in various systems in vitro hydroxyprolyl-sRNA (soluble RNA) appears after incubation with labeled proline (*6*). It is the purpose of this article to present and evaluate the experimental findings relating to the site and mechanism of proline hydroxylation.

Important information concerning the mechanism of proline hydroxylation has come from studies with several tissues. Minced guinea pig granuloma was first used for these studies by Robertson and Schwartz (*7*). Subsequently, Mitoma *et al.* (*4*) reported the use of chick embryos. These two tissues have been used most widely by investigators in this field.

Although ascorbic acid has long been implicated in the overall maintenance of connective tissue, the studies of Robertson and Hiwett (*8*) with minced granuloma from scorbutic guinea pigs were the first to implicate it in the biosynthesis of collagen and to suggest its role in the hydroxylation of proline. Subsequent studies by Stone and Meister (*9*) showed that granuloma minces directly require ascorbic acid for hydroxyproline formation. Concurrent studies by Lowther *et al.* (*10*) and Eastoe (*11*) with minced granuloma and by Prockop *et al.* (*12*) with intact chick embryos showed that collagen hydroxyproline-C^{14} appeared first in the microsomes when the tissues were incubated with proline-C^{14}. These three studies demonstrated that the synthesis of collagen, like that of other proteins, is a microsomal process. Furthermore, they could be interpreted as indicating that the hydroxylation of proline was also catalyzed by an enzyme in the microsomes.

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Mechanism of Hydroxylation

A great deal of information concerning the overall mechanism of hydroxylation has been obtained with intact chick embryos. Thus it was shown that the hydroxyl moiety of collagen hydroxyproline is derived from molecular oxygen and not from water (*13*). Data from one of the studies with O_2^{18} and H_2O^{18} are shown in Table 1. These studies provided the first evidence that proline is converted to hydroxyproline by an oxygenase enzyme. Further studies were carried out with proline-3,-4-H^3, from which a direct-displacement type of hydroxylation should release only one tritium atom from carbon No. 4. Reports from different laboratories were not in agreement, and some questioned the uniformity of labeling of the different batches of commercially prepared proline-3,4-H^3 (*14*). This problem was resolved, however, when Fujita *et al.* (*15*) synthesized *cis*- and *trans*-4-monotritio-proline and showed that only the tritium atom *trans* to the carboxyl group was lost during hydroxylation. This verified the hypothesis that hydroxyproline formation is catalyzed by an oxygenase and showed that the entering hydroxyl group directly displaces one hydrogen atom. It was later shown that *trans*-4-fluoroproline could be incorporated into collagen and converted to hydroxyproline; this finding indicates that the hydroxyl group can also displace a fluorine atom (*16*). It has already been shown that aromatic hydroxylases can displace fluorine atoms when they are substituted for those hydrogen atoms which are displaced by the hydroxyl group at the site of hydroxylation (*17*).

Nature of the Substrate

Although the above studies gave valuable information as to the mechanism of oxidation, they did not indicate the nature of the substrate for hydroxylation. Further advances in this laboratory were made possible by two important developments. The first was the introduction of a specific and reproducible method by which hydroxyproline could be measured and which became the basis for a procedure for the simultaneous assay of the radioactivity in the proline and hydroxyproline in tissue hydrolysates (*18*). The second and more important advance was made by Peterkofsky (*19*), who attained, for the first time, a cell-free

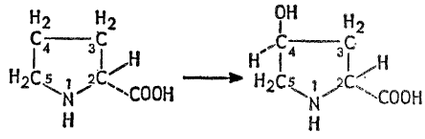

Fig. 1. Conversion of proline to hydroxyproline.

system which was capable of converting proline-C^{14} to collagen hydroxyproline-C^{14}. This was done with supernatant fractions derived from homogenates of whole 8- to 9-day-old chick embryos by centrifugation at 15,000*g*. When these two assay procedures were applied to the cell-free system so as to measure formation of protein-bound hydroxyproline from free proline-C^{14}, it was shown that the factors usually required for protein systhesis were required by the system. A requirement for oxygen was also demonstrated. However, it was observed that the appearance of protein-bound hydroxyproline-C^{14} did not begin for about 30 minutes, whereas incorporation of proline-C^{14} was essentially complete at this time. The lag between the appearance of proline and that of hydroxyproline in the chick-embryo protein made possible a number of interesting experiments (Fig. 2). First, there was no requirement for oxygen until proline-C^{14} had already been maximally incorporated into protein. Second, the effects of ribonuclease had already become negligible before hydroxyproline began to appear in the collagen. Consistent with this was the finding that whereas puromycin inhibited incorporation of proline-C^{14} into protein-bound proline and hydroxyproline when added at the start of the incubation, it had no effect when added at the end of the 30-minute lag period. This was before collagen hydroxyproline-C^{14} had begun to appear. These findings, which are summarized in Fig. 2, indicate that hydroxylation occurs after the prolyl-sRNA (soluble RNA) step of protein synthesis. If hydroxyprolyl-sRNA were a necessary intermediate, then ribonuclease should have degraded it and inhibited the appearance of collagen hydroxyproline. Addition of puromycin at the end of the lag period should also have prevented the incorporation of a hydroxyprolyl-sRNA into protein if such a moiety were accumulating during the lag period. This did not occur. Furthermore, the fact that oxygen was not needed during the lag period indicated that hydroxylation of a nonprotein proline intermediate was not the reason for the lag. The conclusion consistent with these findings was that a proline-rich, hydroxyproline-deficient protein, free or ribosome-bound, accumulated as a substrate for hydroxylation.

Table 1. Incorporation of O_2^{18} and H_2O^{18} into collagen hydroxyproline. Chick embryos were exposed to O_2^{18} or H_2O^{18} of known composition for 24 hours. The collagen was extracted, the hydroxyproline isolated, and its O^{18} content determined. The estimated values are approximations based on the O^{16} content of precursor and the extent of dilution.

Precursor	Excess O^{18} in hydroxyproline (atom percent)	
	Estimated	Found
H_2O^{18}	0.40	0.000
H_2O^{18}	.60	.007
O_2^{18}	1.00	.57
O_2^{18}	1.00	.36

Modification of Procedure

The above evidence would have been conclusive in itself had it been possible to isolate the small amounts of newly formed collagen or hydroxyproline-deficient intermediate from other proteins. However, the isolation procedure used in the above studies actually yielded newly synthesized protein which was only about 10 percent collagen, as shown by the ratio of proline-C^{14} to hydroxyproline-C^{14} (*20*). It was obvious that if the studies were based on pure collagen isolated from each incubation mixture definitive results could be obtained. This was not possible, however, since the amounts of newly formed collagen or proline-deficient protein in these studies were so small. The availability of a highly specific collagenase preparation offered an alternative approach to this problem. Collagenase obtained from *Clostridium histolyticum* has been shown to attack only collagen and gelatin. Keller and Mandl (*21*) tested many other proteins and found none which were substrates of the bacterial collagenase. We obtained this enzyme from commercial sources and purified it so that the preparation released peptides only from collagen and gelatin. We found that the proportions of proline and hydroxyproline in the peptides released by collagenase were the same as in the original protein, there being no fractionation of imino acids during proteolysis. The original assay procedure was therefore modified in the following manner.

After the fortified chick-embryo system had been incubated with proline-C^{14}, the proteins were precipitated with cold trichloroacetic acid, washed several times with cold trichloroacetic acid, extracted with hot trichloroacetic acid, and then dialyzed against water. Collagenase was then added and incubated with the isolated mixture of proteins to hydrolyze the collagen to peptides. To the resulting mixture of proteins and peptides was added an equal volume of 20-percent tannic acid, and the precipitated proteins were removed by centrifugation. The supernatant solution, which contained peptides derived solely from collagen, was hydrolyzed in 6*N* HCl, and the proline and hydroxyproline were isolated and assayed for radioactivity. About 50 percent of the proline, hydroxyproline, and glycine in collagen or gelatin appeared as peptides soluble in 10-percent tannic acid after such treatment.

When this procedure was followed in experiments in which chick embryo microsomes were incubated aerobically with proline-C^{14} for 2 hours (as in Fig. 2), the ratio of proline-C^{14} to hydroxyproline-C^{14} in the isolated imino acids approached unity, indicating that the imino acids released by the collagenase–tannic acid procedure were derived exclusively from collagen (*20*). Of even greater interest was the finding that, when the microsomes were incubated for only 30 minutes (through the lag period) in a nitrogen atmosphere, peptides of proline-C^{14} were released by the collagenase–tannic acid procedure but there were practically no hydroxyproline-C^{14}-containing peptides. Collagenase can degrade simple peptides having an internal -glycine-proline- sequence (*22*), and peptidyl hydroxyproline is not required for its action. These findings gave further proof, therefore, that a hydroxyproline-deficient "collagen-like" protein accumulated during incubation of the chick-embryo system with proline. The validity of this additional proof depended, of course, on the specificity of the bacterial collagenase. Although no other protein substrates have yet been found for this collagenase, the specificity of the overall method was established even further by utilizing as a control an amino acid which is not found in collagen. When tryptophan-C^{14} was incubated with the chick-embryo system, it was incorporated into protein, some of which was extracted into hot trichloroacetic acid. However, none of the protein-bound tryptophan-C^{14} was released as tryptophan-C^{14}-containing peptides by collagenase. Furthermore, none of the proline-C^{14} incorporated into *Escherichia coli* ribosomal protein was released as soluble peptides by the collagenase–tannic acid procedure. It may therefore be concluded that the hydroxyproline-C^{14}-deficient, proline-C^{14}-labeled collagenase substrate which accumulates in chick embryo microsomes on incubation with proline-C^{14} during the 30-minute lag period represents a protein or polypeptide related to collagen.

This conclusion was strengthened even further in subsequent studies by Peterkofsky and me (*23*). Chick embryo microsomes, prepared so that they contained only protein-bound proline-C^{14}, were isolated, washed to remove soluble materials, stored for some time, and subsequently incubated in oxygen, so that part of the protein-bound proline-C^{14} was converted to protein-bound hydroxyproline-C^{14}. Shortly thereafter, Prockop and Juva (*24*) reported similar experiments in which substantial quantities of bound proline-C^{14}, in a

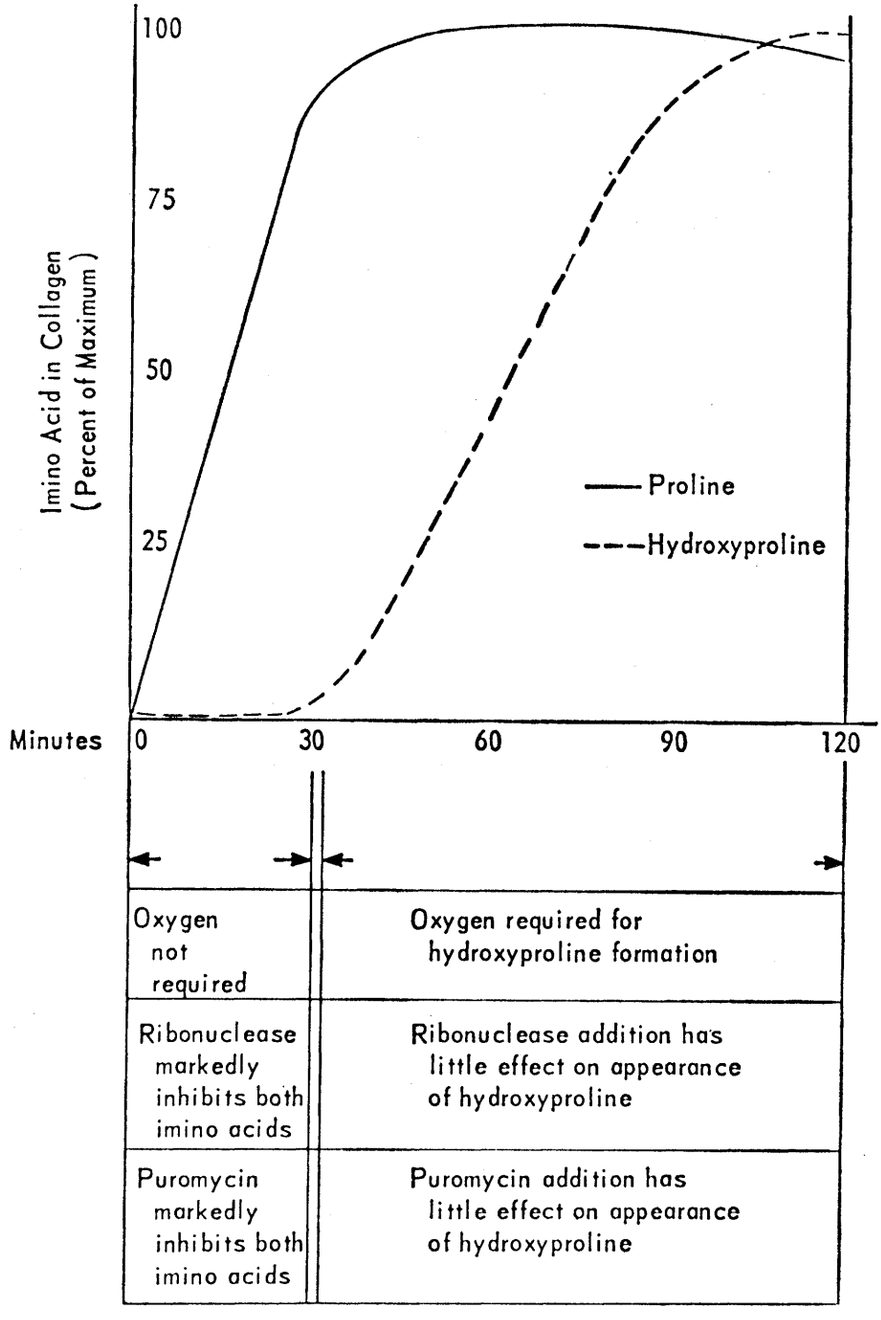

Fig. 2. Appearance of proline-C^{14} and hydroxyproline-C^{14} in microsomal protein of the cell-free chick-embryo system incubated with proline-C^{14}; effects of anaerobiosis, ribonuclease, and puromycin.

protein formed on incubation of chick embryo tibias with proline-C^{14}, were converted to protein-bound hydroxyproline-C^{14} when incubated with homogenates of whole chick embryos. More recently Juva and Prockop (*25*) incubated synthetic repeating polymers (pro-C^{14}-gly-pro)$_n$ (*26*) of molecular weight 4,000 and 15,000 with chick embryo homogenates and demonstrated that several percent of the proline-C^{14} was hydroxylated.

Incorporation of Labeled Proline

All these findings show conclusively that chick-embryo homogenates can accumulate a hydroxyproline-deficient, collagen-like protein on incubation with proline-C^{14} and that they contain a proline hydroxylase capable of hydroxylating proline residues within a protein or polypeptide chain.

If the proposed mechanism is correct, the hydroxyproline-deficient protein should accumulate in other collagen-forming systems as well as in chick-embryo homogenates. This is actually the case. We have recently examined a large number of collagen-forming systems, including guinea pig granuloma, hen oviduct, fetal rabbit skin, and 15-day-old chick embryos (*27*). All these tissues, minced or homogenized, incorporated proline-C^{14} into a protein which contained little or no hydroxyproline-C^{14} but which was degradable by collagenase. Such a protein accumulated in the tissues when hydroxylation was impaired for any reason such as lack of oxygen, deficiency of ascorbic acid, or destruction of the labile oxygenase (*27*).

A number of heretofore unexplained observations can now be explained. Thus Stone and Meister (*9*) reported that minced scorbutic granuloma produced a collagen which, upon incubation with proline-C^{14}, was labeled only in the proline residues. These observations are best explained by the formation, during incubation, of a hydroxyproline-C^{14}-deficient "collagen" which coprecipitates with "old" hydroxyproline-C^{12}-containing collagen already present in the tissue before incubation. Urivetzky *et al.* (*28*) reported similar findings with homogenates of fetal rabbit skin. They also degraded the collagen with collagenase and isolated the tripeptide gly-pro-hyp, which was labeled only in the proline residue. Their observations can be explained in the same way as those of Stone and Meister with one addition. The "old" collagen which Urivetzky *et al.* isolated after incubation most likely contained traces of newly formed proline-C^{14}-labeled, hydroxyproline-deficient protein. The gly-pro-hyp isolated from collagenase digests in which only the proline residue appeared to be labeled was most likely a mixture of unlabeled "old" gly-pro-hyp and traces of gly-pro-C^{14}-pro-C^{14} which was derived from the newly formed hydroxyproline-deficient intermediate. In our laboratory we have shown that gly-pro-hyp and gly-pro-pro are not readily separated by paper or column chromatography.

A few of the characteristics of the hydroxyproline-deficient protein are known. In all the systems investigated in this laboratory the protein remains associated with the microsomes, from which it can be extracted in a number of ways. The gelatinized hydroxyproline-deficient "collagen" is nondialyzable, is precipitable with tannic acid, and can be attacked by collagenase. Prockop and Juva (*24*) used various buffers to extract the hydroxyproline-deficient protein formed by chick embryo tibias in tissue culture. They found that it behaved as a large molecule (molecular weight approximately 100,000 to 200,000). Comparable studies were recently reported by Lukens (*29*). In studies with other tissues, dialyzable hydroxyproline-containing peptides have been detected during experimental studies of collagen biosynthesis (*30*).

Appreciable information is also available concerning the proline hydroxylase of chick embryo. Peterkofsky and I (*23*) showed that the enzyme was present in the microsomes. When microsomes were washed, activity was lost and could be partially restored by addition of ascorbic acid or reduced tetrahydropteridines plus a heat-stable factor from the supernatant; Fe^{++} was also implicated. Prockop and Juva (*24*) dissociated the enzyme activity from microsomes and observed stimulation by Fe^{++}. These and earlier findings suggest that proline hydroxylase is an oxygenase similar to the enzymes that hydroxylate phenylalanine, tyrosine, and tryptophan. In this instance, however, the substrate is not the free amino acid but part of a polypeptide.

Although the studies described above clearly indicate that proline is hydroxylated in peptide linkage during collagen biosynthesis, there have been a number of reports which are apparently inconsistent with such a mechanism. Mainly these have concerned the isolation of labeled hydroxprolyl-sRNA from a variety of systems which were incubated with proline-C^{14} (*6*). In this laboratory we looked carefully for such an intermediate since we, too, thought it was the most probable site for hydroxylation. However, as was pointed out above, we have been unable to isolate or detect hydroxyprolyl-sRNA in chick-embryo homogenates or extracts which were capable of converting proline to collagen hydroxyproline. There have been no reports that a hydroxyprolyl-sRNA can be incorporated into collagen, and recently Lukens (*31*) has reported many unsuccessful attempts to detect its formation.

More recently, Manning and Meister (*32*) presented evidence that, in minced guinea pig granuloma that has been incubated with proline-C^{14}, some hydroxyproline-C^{14} appears at the amino acyl end of a peptide chain that is still attached to the ribosome. They also reported that, in the presence of sufficient puromycin to inhibit proline incorporation, appreciable hydroxylation occurred when the proline was present in smaller ribosomal-bound peptides. They concluded that proline is hydroxylated while bound in peptide linkage and still attached to the ribosome.

Peptidyl Proline Hydroxylation

Our findings and those from the laboratories of Prockop, Lukens, and Meister can be explained by a mechanism which is shown diagrammatically in Fig. 3. Proline is activated and converted to prolyl-sRNA, which then attaches itself to the ribosome and is incorporated into a ribosomal-bound peptide. When the ribosomal-bound peptide reaches a definite size and contains appropriate sequences which can be recognized by the hydroxylase, certain proline residues are hydroxylated to hydroxyproline residues. However, in the absence of oxygen, ascorbic acid, or the hydroxylating enzyme (*33*) the ribosomes form a proline-rich, hydroxyproline-deficient protein. In the chick-embryo system this protein can be hydroxylated by subsequent introduction of appropriate conditions for hydroxylation.

Apparently the hydroxyproline-de-

ficient protein can be hydroxylated even after separation from the ribosome and after the addition of inhibitors of protein synthesis. Furthermore, some of the prolines in synthetic polymers such as $(\text{pro-gly-pro})_n$ can also be hydroxylated. It would appear, therefore, that the substrate specificity of proline hydroxylase is to a large extent determined by a peptide sequence. Conceivably the attachment to the ribosome may have some additional effect on the rate or site of hydroxylation.

Conclusion

Since lysine is no doubt hydroxylated by a comparable mechanism, a generalized scheme for hydroxylation may be considered. The ribosome accepts amino acyl sRNA molecules destined for collagen. As polymerization proceeds, two conditions begin to be met which allow proline and lysine residues in peptide linkage to become substrates for the hydroxylase: (i) the peptide grows to a minimum size, and (ii) the two amino acids become incorporated into definite sequences which can be recognized by the specific hydroxylases. In the presence of sufficient hydroxylating enzymes and cofactors, hydroxylation keeps pace with peptide synthesis so that when the protein chain is completed it is fully hydroxylated. Under such conditions hydroxylation occurs during the process of translation. When hydroxylation is limited, ribosomal peptide-synthesizing mechanisms are, for a time at least, unaffected and con-

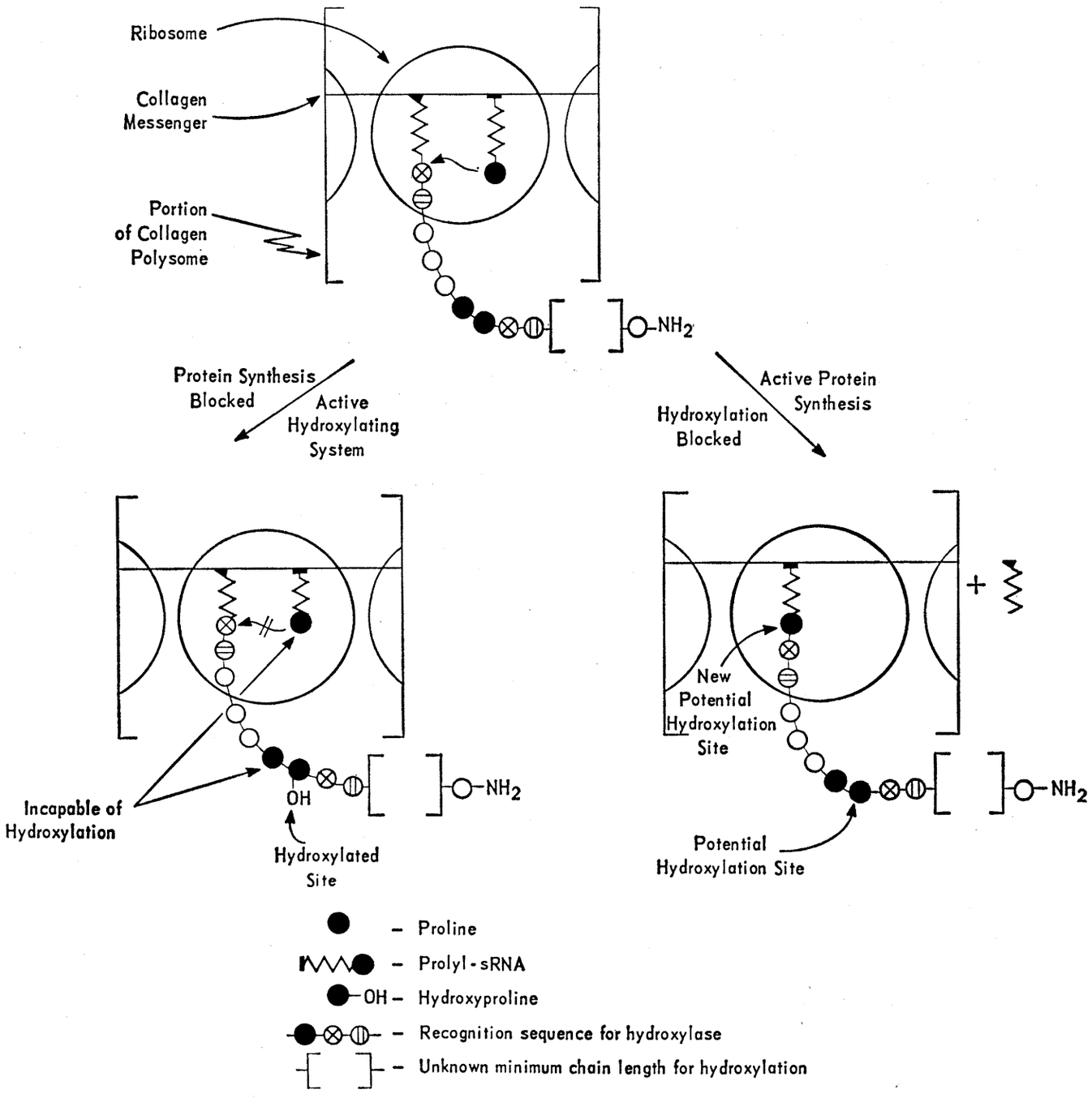

Fig. 3. Scheme for the hydroxylation of proline during collagen biosynthesis.

tinue to elaborate the usual chain, which is, however, deficient in or devoid of hydroxylated residues. No large quantities of unhydroxylated "collagen" have been accumulated experimentally. We have even suggested that the rate of hydroxylation may be a controlling factor in collagen synthesis (*23*). However, certain worms normally make a collagen in which almost all the imino acid residues are proline (*34*). It should be noted that collagen is composed of two different peptide chains and that each contains hydroxylated amino acids. The same enzyme may be responsible for hydroxylation of both chains.

The hydroxylation of an sRNA amino acid is a process at least as complex as the hydroxylation of an amino acid in peptide linkage. Hydroxylation of a partial or completed protein chain represents only one of many modifications of proteins that are known to occur. These include disulfide-bond formation, *N*-acetylation, *O*-phosphorylation, glycosylation, and *O*-sulfation. These modifications, which undoubtedly occur after the peptide chain of a protein has been completed, are important in developing the final specific structure of the protein.

Information about specific protein-modifying enzymes may prove to be of great practical value. In the field of cancer chemotherapy scientists have attempted to block protein synthesis at early stages by inhibiting the formation or the action of nucleic acids. The general idea is that tumor cells grow faster than normal cells and will therefore be more susceptible to inhibitors of protein synthesis. However, nucleic acid synthesis at this early stage is hardly a specific approach. In adult animals the turnover of most of the collagen is very slow (*35*), but collagen proliferates rapidly at sites of wounds, irritation, or tumor growth. It appears that the hydroxylases for "collagen" proline and lysine would be excellent targets for achieving specific inhibition of synthesis of this protein or at least for modifying its properties. In adult animals, inhibitors of these enzymes should have little effect except in tumors or the scar tissue of healing wounds. Should such inhibitors become available they will be of great value as tools and conceivably as therapeutic agents.

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36. I thank Dr. Fred Bergmann for his suggestions on the diagrammatic presentation shown in Fig. 3.