*TRANSFER OF PUROMYCIN-CONTAINING POLYPEPTIDES THROUGH THE PLASMA MEMBRANE OF CARTILAGE CELLS SYNTHESIZING COLLAGEN**

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The hydroxyproline and hydroxylysine in collagen are synthesized by an unusual pathway[1, 2] in which proline[3–9] and lysine[10] are hydroxylated after they are incorporated into a large polypeptide precursor of collagen called protocollagen.[7, 11, 12] The protocollagen recovered from embryonic tissues in which the hydroxylating reactions are inhibited is comparable in size to the complete α chains of collagen,[5, 12, 13] and it serves as a substrate for the synthesis of both hydroxyproline and hydroxylysine by a purified enzyme from chick embryos.[14, 15] The enzyme hydroxylates both proline and lysine residues in protocollagen,[10, 14, 15] and both hydroxylations[14, 15] require atmospheric oxygen, ferrous iron, α-ketoglutarate, and ascorbate.[5, 7, 10, 16–21] The name "protocollagen hydroxylase" (protocollagen, ascorbate:oxygen oxidoreductase (proline and lysine hydroxylating) EC 1.14.2) has been suggested for this enzyme.[15]

When the hydroxylation of protocollagen in isolated tissues is prevented with anaerobic conditions[4] or a chelator for ferrous iron,[18] incorporation of proline continues at 70–90 per cent of the control rate for several hours, but the protocollagen accumulates intracellularly.[22] When the hydroxylase activity of the tissue is restored, the appropriate proline and lysine residues in the accumulated protocollagen are hydroxylated, and the molecules are extruded through the plasma membrane into the extracellular matrix.[22] The results suggest that the polypeptides cannot be extruded until the appropriate proline and lysine residues are hydroxylated.

High concentrations of puromycin have been shown to inhibit the synthesis of hydroxyproline by embryonic tissues to a greater extent than the incorporation of proline into polypeptides.[11, 23] Nondialyzable polypeptides recovered from cartilage incubated with puromycin were lacking in hydroxyproline, but susceptible to collagenase.[11] The present paper reports observations which indicate that the puromycin-substituted polypeptides do not accumulate within cells synthesizing collagen, but they are extruded into the matrix more rapidly than collagen even though they contain essentially no hydroxyproline and probably no hydroxylysine.

Materials and Methods.—Materials: Puromycin dihydrochloride-(*O*-methyl-H^3), 1100 μc per μmole; and DL-lysine-4,5,-H^3, 3 μc per μmole, were purchased from New England Nuclear Corp. Other materials were as described previously.[4, 5, 11]

Incubation of embryonic tibiae: Tibiae which consisted almost entirely of unossified cartilage were removed from 10-day-old embryos, and they were incubated in 2.5 ml of a synthetic medium as described previously.[4, 5] After incubation under the conditions indicated below, the tissues were either taken for autoradiographs, enzymatic hydroxylation, or radioactive assays.

Radioactive assay: The tissues were homogenized and the homogenates were dialyzed extensively against running tap water. The samples were hydrolyzed and then assayed for total C^{14} or H^3 content, as described previously.[24] Assays for radioactive hydroxyproline were performed by oxidation to pyrrole in a specific chemical procedure.[25]

Preparation of autoradiographs: At the end of the incubation, tissues were fixed immediately in a chilled mixture of ethanol:acetic acid (3:1) or ethanol:acetic acid:chloroform (6:3:1) for 3 hr, and then they were transferred to a 2% glutaraldehyde solution in 0.01 *M* sodium phosphate, pH 7.4, for storage. The glutaraldehyde-fixed tissues were dehydrated and embedded in paraffin blocks, and 5-μ-thick sections were prepared. The sections were mounted on glass slides and coated with Ilford Nuclear Research emulsion, Type KII. After exposure for 7–21 days, the autoradiographs were developed for 5 min with D-19 (Kodak) developer. The sections were then stained with Alcian blue for microscopic examination.

Hydroxylation with purified protocollagen hydroxylase: After incubation, the tibiae were homogenized in 6 ml cold distilled water, and the homogenates were centrifuged at 100,000 $\times$ *g* for 1 hr at 4°C. The supernatant fractions were dialyzed against several changes of 4 liters of 1 *M* KCl and 0.05 *M* Tris-HCl buffer, pH 7.6. The 100,000 $\times$ *g* sediments were extracted with 5 ml of 7 *M* urea at 100°C for 20 min.[12] The urea extracts were dialyzed against several changes of 1 liter of 7 *M* urea, and then against several changes of 1 *M* KCl and 0.05 *M* Tris-HCl buffer, pH 7.6. Aliquots of the dialyzed 100,000 $\times$ *g* supernatants and the dialyzed urea extracts of 100,000 $\times$ *g* sediments were then incubated with purified protocollagen hydroxylase, as described previously.[14, 15] The protocollagen hydroxylase was purified through the calcium phosphate gel step.[15] After incubation the samples were hydrolyzed and assayed for total C^{14} and hydroxyproline-C^{14} as described above.

Studies on E. coli and reticulocytes: E. coli adapted to a low Mg^{++} medium[26] were allowed to grow logarithmically to a concentration of 3 $\times$ 10^8 cells/μml. Ten-ml aliquots of the culture were incubated in bubbler flasks at 37° for 60 min with or without 50 μg/ml puromycin and with 5 μg/ml carrier L-proline and 1.0 μc L-proline-C^{14}. The samples were then centrifuged at 3000 $\times$ *g* for 10 min in the cold. The supernatant fractions and the sedimented cells were dialyzed separately against cold water, and the dialyzed samples were then hydrolyzed and assayed for total C^{14} content.[24]

One ml of packed rabbit reticulocytes[27, 28] were incubated with 5 μc lysine-H^3 in 3 ml of medium with or without 50 μg/ml puromycin. After incubation for 2 hr at 37°, the samples were centrifuged at 2000 $\times$ *g* for 15 min at 4°, and the supernatant and sediment fractions were precipitated with 5% trichloroacetic acid (TCA). The TCA precipitates were washed 5 or 6 times with 5 ml 5% TCA containing 1 mg/ml carrier lysine. The precipitates were then hydrolyzed, decolorized,[29] and assayed for total H^3 content.[24]

Results.—Effect of puromycin on the incorporation of proline-C^{14} and the synthesis of hydroxyproline-C^{14} by embryonic cartilage: When cartilagenous tibiae from ten-day-old chick embryos are incubated with proline-C^{14}, about two thirds of the incorporated C^{14} is recovered in collagen, and essentially all the C^{14} is recovered as proline-C^{14} and hydroxyproline-C^{14}.[11] The rate of incorporation increases with time during the first 20–30 minutes of incubation, and then it proceeds almost linearly for over four hours.[11, 30] Puromycin, 50 μg per milliliter, inhibited the incorporation of proline-C^{14} into nondialyzable fractions by over 90 per cent.[11] The synthesis of hydroxyproline-C^{14}, however, was depressed to a greater extent than the incorporation of proline-C^{14}, and after incubation for 120 minutes hydroxyproline-C^{14} was only about 1 per cent of the total nondialyzable C^{14} (Figs. 1*A* and *B*).

Hydroxylation of puromycin-containing polypeptides with purified protocollagen hydroxylase: Tibiae were incubated with puromycin and proline-C^{14}, and then nondialyzable fractions of the tibiae were incubated with purified protocollagen hydroxylase. Before incubation with protocollagen hydroxylase, hydroxyproline-C^{14} accounted for less than 1 per cent of the nondialyzable C^{14} in the 100,000 $\times$ *g* supernatant fraction. After incubation with the enzyme, 21 per cent of the total C^{14} was recovered as hydroxyproline-C^{14}. The extent to which proline-C^{14} in the sample was converted to hydroxyproline-C^{14} was similar to that observed with

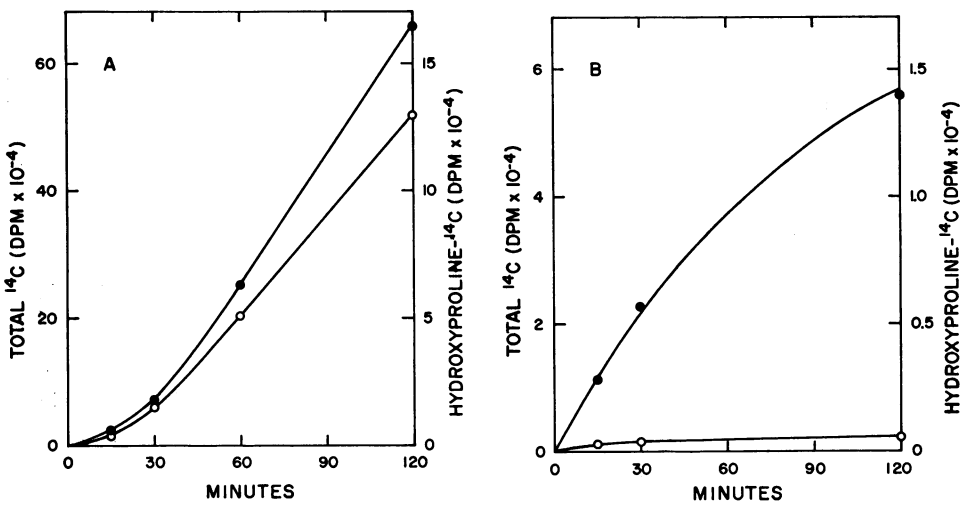

FIG. 1.—Effect of puromycin on the incorporation of proline-C^{14} and the synthesis of hydroxyproline-C^{14} by embryonic cartilage. Isolated tibiae were preincubated in the medium at 37° for 1 hr and then proline-C^{14} was added. In order to examine the effect of puromycin, the antibiotic was added 20 min before addition of the proline-C^{14}. Other conditions as described in text. (*A*) Tibiae incubated with 2 μc proline-C^{14} under control conditions. (*B*) Tibiae incubated with 10 μc proline-C^{14} and 50 μg/ml puromycin. Symbols: total nondialyzable C^{14} (●—●); nondialyzable hydroxyproline-C^{14} (○—○).

proline-labeled protocollagen-C^{14} in the 100,000 × *g* supernatant fraction from embryonic cartilage. Also, the ratio of hydroxyproline-C^{14} to total C^{14} was similar to the control value observed in normal cartilage which was incubated with proline-C^{14} under conditions where the hydroxylation was not inhibited (Table 1).

Labeled polypeptides which served as substrates for the synthesis of hydroxyproline-C^{14} were also present in urea extracts of the 100,000 × *g* sediments of tissues incubated with puromycin. Before incubation with the enzyme, hydroxyproline-C^{14} was 0.5 per cent of the total C^{14}, and after incubation with the enzyme, it was 17.1 per cent of the total C^{14}.

Rapid extrusion of puromycin-containing polypeptides: In embryonic cartilage incubated with proline-H^3, most of the label was over the cells in 15 minutes and significant amounts of radioactivity did not appear over the extracellular matrix until after 30–60 minutes (Figs. 2*A* and *C*). In the presence of puromycin, however, the tritium was rapidly transferred to the matrix, and most of the tritium was

TABLE 1

HYDROXYLATION OF PUROMYCIN-CONTAINING POLYPEPTIDES AND PROTOCOLLAGEN WITH PURIFIED PROTOCOLLAGEN HYDROXYLASE

Sample	Total C^{14} for hydroxylation (dpm)	Hypro-^{15}C (dpm)	$\frac{\text{Hypro-}C^{14}}{\text{Total }C^{14}} \times 100$ (%)
Puromycin-treated, before hydroxylation	15,000	105	0.7
Puromycin-treated, after hydroxylation	15,000	3,210	21.4
Protocollagen, before hydroxylation	50,000	250	0.5
Protocollagen, after hydroxylation	50,000	10,500	21.0
Control tissue*	150,000	34,500	23.0

Conditions for assay as described in text. Control tissue sample was the nondialyzable 100,000 × *g* supernatant fraction from tibiae incubated with proline-C^{14} without any inhibition of protein synthesis or of the hydroxylation of proline.

* Sample was hydrolyzed and assayed directly without incubation with protocollagen hydroxylase.

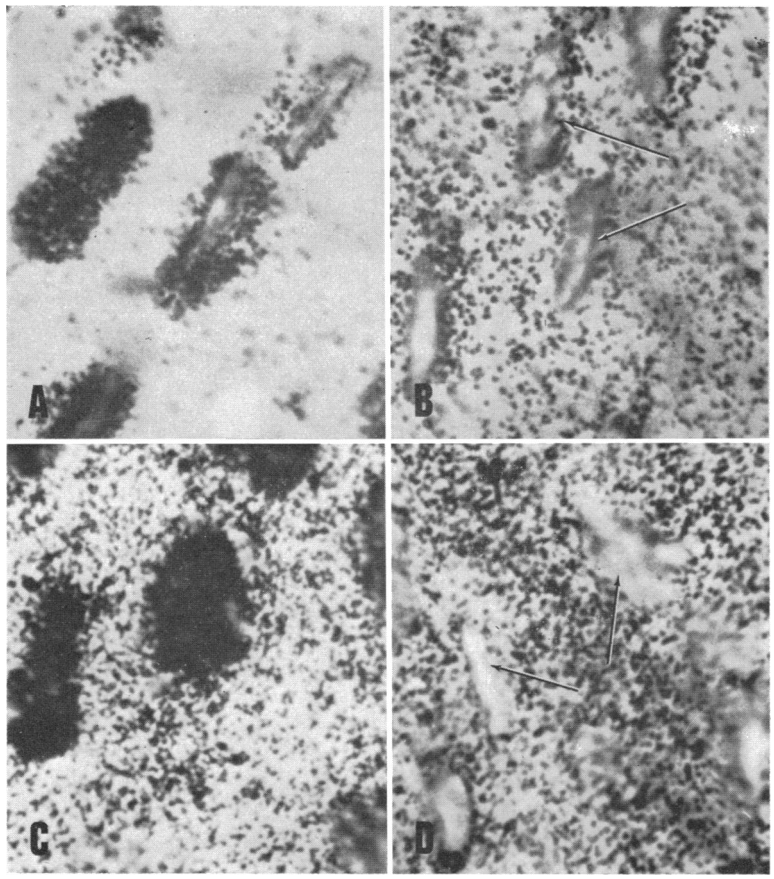

FIG. 2.—Effect of puromycin on the intracellular and extracellular distribution of polypeptides. Tibiae were incubated with radioactive proline and puromycin as described in Figure 1 except proline-H^3 was used instead of proline-C^{14}. Autoradiographs were prepared as described in text. (*A*) Incubation under control conditions with 20 μc proline-H^3 for 15 min. (*B*) Incubation with 50 μg/ml puromycin and 200 μc proline-H^3 for 30 min. Arrows indicate location of cells. (*C*) Incubation under control conditions with 20 μc proline-H^3 for 60 min. (*D*) Incubation with 50 μg/ml puromycin and 200 μc proline-H^3 for 60 min. Arrows indicate location of cells.

extracellular at 15, 30 (Fig. 2*B*), and 60 minutes (Fig. 2*D*). The four different cell types in the tissue[22] all showed a decrease in the amount of intracellular label. In control experiments free proline-H^3 did not appear in the autoradiographs.

In order to establish that the extracellular proline-containing polypeptides also contained puromycin, tibiae were incubated under similar conditions with puromycin-H^3 as the source of radioactive label. As shown in Figures 3*A* and *B*, most of the radioactive puromycin was over the matrix after incubation for 30–60 minutes. Because only one molecule of puromycin-H^3 is incorporated per polypeptide,[26, 31] and because of the lower specific activity of the puromycin-H^3, the amount of radioactivity incorporated into the tissue was less than that incorporated when proline-H^3 was used, but the distribution of label was essentially the same.

Failure of puromycin to enhance the extrusion of completed protocollagen polypep-

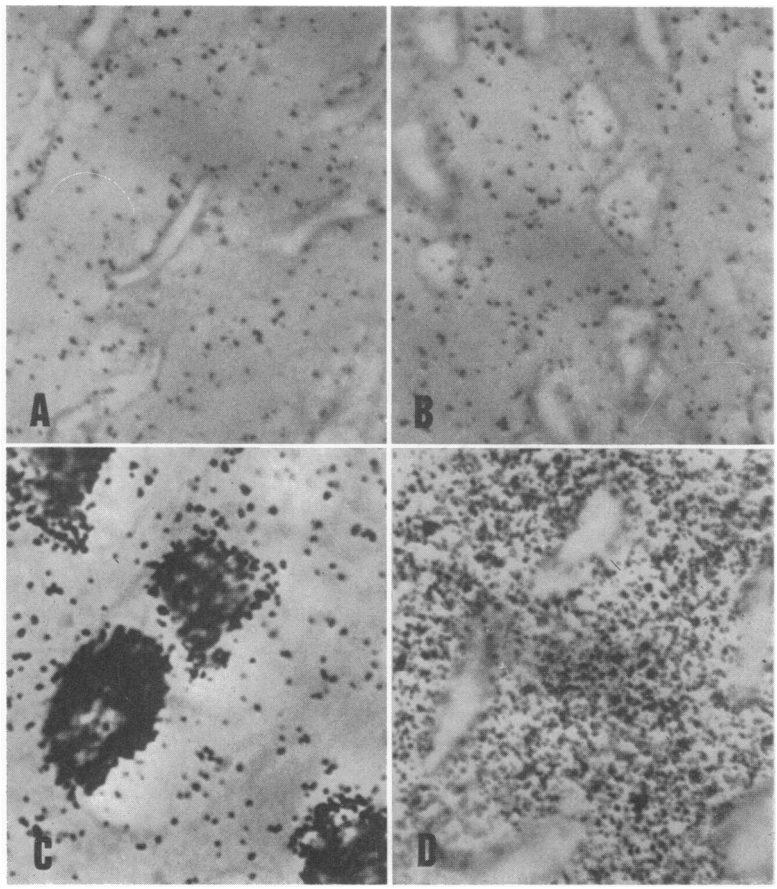

FIG. 3—Distribution of polypeptides containing puromycin-H^3 in embryonic cartilage. Isolated tibiae were preincubated in the medium at 37° for 2 hr and then 200 μc puromycin-H^3 (50 μg/ml) were added. (*A*) Incubation with puromycin-H^3 for 30 min. (*B*) Incubation with puromycin-H^3 for 60 min. (*C*) Failure of puromycin to enhance the extrusion of completed protocollagen polypeptides. Tibiae were preincubated with 10^{-3} *M* α,α'-dipyridyl for 30 min, and then 20 μc proline-H^3 was added for a labeling period of 2 hr. The medium was then replaced with fresh medium containing 120 μg/ml carrier L-proline, 10^{-3} *M* α,α'-dipyridyl, and 50 μg/ml puromycin, and the incubation was continued for a third hour in order to determine whether the puromycin had any effect on the distribution of labeled protocollagen which had accumulated in the cells. The autoradiographs were indistinguishable from those obtained with samples incubated with proline-H^3 and α,α'-dipyridyl but no puromycin. (*D*) Failure of α,α'-dipyridyl to affect the transfer of puromycin-containing polypeptides to the extracellular matrix. Tibiae were preincubated with 50 μg/ml puromycin and 10^{-3} *M* $\alpha.\alpha'$-dipyridyl for 30 min and then 300 μc proline-H^3 was added to the medium for a labeling period of 2 hr. The medium was then replaced with fresh medium containing 50 μg/ml puromycin, 10^{-3} *M* α,α'-dipyridyl, and 120 μg/ml carrier L-proline, and the samples were incubated for a third hour.

tides: Completed protocollagen polypeptides accumulate intracellularly when the hydroxylation of proline and lysine in protocollagen is prevented.[22] In order to determine whether puromycin had any significant effect on the plasma membrane, tissues were labeled with tritiated proline in the presence of α,α'-dipyridyl, and then 50 μg per milliliter puromycin was added to the incubation medium. If puromycin markedly increased the permeability of the membranes to large polypeptides, the accumulated protocollagen-H^3 should have passed into the extracellular matrix. The labeled protocollagen remained in the cells (Fig. 3*C*). Control experiments indicated that treating the tissues with α,α'-dipyridyl does not in itself inhibit the extrusion of polypeptides (Fig. 3*D*).

Effect of puromycin in E. coli and rabbit reticulocytes: When *E. coli* or rabbit reticulocytes were incubated with 50 μg per milliliter puromycin, the incorporation of labeled amino acids into protein was inhibited over 90 per cent. Small differences in the intracellular and extracellular distribution of labeled polypeptides were observed, but over 90 per cent of the TCA-precipitable or nondialyzable radioactivity was intracellular. The results suggested, therefore, that the effect of puromycin on the extrusion of polypeptides is not observed in cells which do not synthesize extracellular proteins.

Discussion.—Two related aspects of the results presented here appear to be of interest: (*a*) the reasons why the polypeptide precursors of collagen synthesized in the presence of puromycin do not contain hydroxyproline, and (*b*) the rapid transfer of the polypeptides synthesized in the presence of puromycin into the extracellular matrix of the tissue.

Puromycin inhibits protein synthesis by substituting for aminoacyl-RNA in the assembly of polypeptides on ribosomes.[26, 31] In *E. coli* the incorporation of puromycin into nascent polypeptides terminates the growth of polypeptide chains, and incomplete polypeptides containing puromycin are released from the ribosomal complexes.[26, 31] Puromycin apparently inhibits protein synthesis in embryonic cartilage by the same mechanism,[11] and in the presence of puromycin incomplete polypeptides accumulate in the tissue.[6, 11, 23]

It was originally suggested[11, 23] that the incomplete polypeptide precursors of collagen synthesized in the presence of puromycin did not contain hydroxyproline because they were of insufficient size to serve as substrates for protocollagen hydroxylase. The enzyme is not inhibited by puromycin,[5, 7] and it does not hydroxylate free proline,[4] the tripeptide gly-pro-pro, or poly-L-proline.[14] Preparations of the synthetic polytripeptide $(\text{pro-gly-pro})_n$ are hydroxylated,[7, 14, 32] but measurements of K_m values[33] indicated that preparations with a molecular weight of 1,800 are not hydroxylated as readily as preparations with molecular weights of 4,000–15,000. The results presented here suggest that the original interpretation of the effect of puromycin on the synthesis of collagen hydroxyproline[11, 23] should be modified, and that the smaller size of polypeptides synthesized in the presence of puromycin cannot in itself explain why they are not hydroxylated in the tissue. Although the K_m values for the puromycin-substituted polypeptides could not be measured, the labeled polypeptides isolated from cartilage incubated with puromycin were hydroxylated by purified protocollagen hydroxylase to about the same extent as protocollagen. Autoradiographs demonstrated that puromycin-containing polypeptides rapidly leave cells synthesizing collagen. It seems probable,

therefore, that the rapid egress of the puromycin-containing polypeptides at least in part explains why they are not hydroxylated by the intracellular enzyme. Recent studies have demonstrated that the hydroxylation of protocollagen occurs after the complete polypeptides are released from ribosomal complexes, and that a significant pool of protocollagen is present in connective tissue cells under normal conditions.[9] The polypeptides synthesized in the presence of puromycin apparently bypass the intracellular pool in which the hydroxylation normally occurs.

At the moment no simple explanation can be offered as to why puromycin produced the rapid extrusion of polypeptides into the extracellular matrix. In preparations of rat liver microsomes, puromycin was shown to produce a transfer of peptides from ribosomes into the interior of microsomal vesicles, and the results suggested that the only requirement for the transfer of nascent polypeptides across the microsomal membranes in liver is that they be released from ribosomes.[34, 35] The effect of puromycin on the release of polypeptides from ribosomes cannot in itself explain the rapid extrusion of polypeptides observed here, since, when the intracellular hydroxylation of protocollagen is inhibited, complete polypeptides which have been released from ribosomes continue to accumulate in the ground cytoplasm for two days or longer.[36]

Puromycin apparently has no significant effect on the cell membranes, because addition of puromycin to tissues in which protocollagen had accumulated did not release protocollagen into the matrix. The effect of puromycin on the extrusion of polypeptides might be explained by the assumption that the plasma membrane is permeable to small polypeptides and that the polypeptides released from ribosomes by puromycin are small enough to pass the barrier to extrusion. However, significant amounts of nondialyzable polypeptides from puromycin-treated cartilage had molecular weights greater than 10,000.[11] It appears more likely, therefore, that COOH-terminal substitution of the polypeptides with puromycin is a more critical factor than their size in the rapid extrusion of these polypeptides. Several investigators have suggested that carbohydrate substitution of proteins may be essential for the extrusion of extracellular proteins from cells.[37] The hydroxyl groups of hydroxyproline or hydroxylysine may either replace the need for a carbohydrate or they may be required for the binding of sugars[38] in the extrusion of collagen. It is possible that puromycin substitution of polypeptides specifically replaces the need for the hydroxyl groups in the extrusion of collagen.

Summary.—Inhibition of collagen synthesis by puromycin results in the synthesis of incomplete polypeptides which do not contain hydroxyproline but are rich in proline. Proline in the incomplete polypeptides was hydroxylated by purified protocollagen hydroxylase, the enzyme which normally hydroxylates complete polypeptide precursors of collagen. Accordingly, the smaller size of the puromycin-containing polypeptides does not in itself explain why they are not hydroxylated in the tissues.

Normally, the complete polypeptide precursors of collagen are not extruded from the cells until appropriate proline and lysine residues are hydroxylated to hydroxyproline and hydroxylysine. Autoradiographs indicated that the incomplete polypeptides synthesized in the presence of puromycin are extruded more rapidly than collagen, even though they do not contain hydroxyproline. It seems probable that the rapid egress of the puromycin-containing polypeptides at least in part explains

why they are not hydroxylated by the intracellular enzyme. Extrusion of the puromycin-containing polypeptides might be explained by their small size, but it seems more likely that the COOH-terminal substitution with puromycin circumvents the requirement for hydroxyl groups in their transfer through the plasma membrane.

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