Cell-free Collagen Synthesis on Membrane-bound Polysomes of Chick Embryo Connective Tissue and the Localization of Prolyl Hydroxylase on the Polysome-Membrane Complex*

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SUMMARY

A fraction characterized as membrane-bound polysomes has been isolated from homogenates of 14-day-old chick embryo tibiae and other connective tissues by centrifugation between 700 to 15,000 × g. These polysomes, as well as free polysomes isolated by further centrifugation at 105,000 × g, synthesize collagen and noncollagen protein in a cell-free system, but the membrane-bound polysomes contain 70 to 90% of the collagen-synthesizing activity. Electron micrographs of the 15,000 × g pellet revealed vesicles of the endoplasmic reticulum with ribosomes attached to the surface. Homogenization in the presence of detergent resulted in disruption of these vesicles with formation of free polysomes. This conclusion was based on electron microscopy of the fractions and on the observation that collagen-synthesizing activity was lost from the 15,000 × g pellet derived from homogenates prepared with detergent but was recovered by centrifugation at 105,000 × g. Approximately 80% of the protein synthesized by membrane-bound polysomes in the cell-free system remained attached to the ribosomes; the remainder was released either into the soluble fraction or into the cisternae of the endoplasmic reticulum.

When membrane-bound polysomes were labeled with [14C]proline and reisolated free of soluble fraction, hydroxylation of proline in collagen-nascent chains could be obtained merely by incubating with ascorbate, Fe2+, and α-ketoglutarate, indicating that prolyl hydroxylase was present in the membrane-polysome complex. When free polysomes released by detergent were prelabeled with [14C]proline, reisolated, and incubated with hydroxylase cofactors, no hydroxylation of proline was obtained. Our results suggest that collagen is synthesized almost entirely on polysomes bound to the membranes of the endoplasmic reticulum and that prolyl hydroxylase may also be attached to these membranes. The location of the enzyme may serve to regulate the entry of collagen into the endoplasmic reticulum.

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Studies in several laboratories have supported the hypothesis that proteins destined for secretion are synthesized on polysomes attached to the membranes of the endoplasmic reticulum, whereas proteins that remain intracellular are synthesized on free polysomes in the cytoplasm (1). Sickevita and Palade (3) demonstrated that the synthesis of chymotrypsinogen by exocrine cells of the guinea pig pancreas occurs on membrane-bound polysomes, and work by Peters (3) illustrated that newly synthesized albumin was associated with the rough endoplasmic reticulum. Recently, Redman (4) compared the synthesis of serum proteins and ferritin by membrane-attached and free polysomes of the rat liver and found that 80% of the total serum protein was synthesized on the attached polysomes, whereas free polysomes were more active in the synthesis of ferritin, a protein which is not secreted. The cell-free synthesis of immunoglobulin by membrane-bound polysomes from lymph nodes has also been demonstrated (5).

There is some evidence that collagen, a secreted protein, also is synthesized on the membranes of the rough endoplasmic reticulum. Numerous studies have shown that cells actively synthesizing collagen contain an extensive and well-organized network of endoplasmic reticulum with attached ribosomes (6). Autoradiographic studies by Ross (7) of healing wounds indicated that newly synthesized radioactive collagen was associated with the endoplasmic reticulum and that most polysomes were attached to these structures in cells rapidly synthesizing collagen. Under scurvy conditions, where it is known that collagen synthesis is greatly diminished, polysomes were detached from the endoplasmic reticulum, and free ribosomes appeared in the cytoplasm. Goldberg and Green (8) obtained more direct evidence that collagen synthesis occurs on membrane-bound polysomes. They labeled 3T3 cells in culture with radioactive proline and found that most of the labeled hydroxyproline was in the nascent chains attached to polysomes derived from a fraction of the homogenate which consisted mainly of endoplasmic reticulum.

The present report describes the isolation of intact membrane-bound polysomes from bone and other connective tissues and the properties of a cell-free system in which collagen is synthesized in the presence of this fraction.
EXPERIMENTAL PROCEDURES

Materials—Fertile eggs were purchased from Truslow Farms and incubated at 37°C for either 14 or 18 days as needed. Uniformly labeled L-[3H]proline (200 µCi per µmole) was obtained from New England Nuclear Corporation. Pancreatin dihydrochloride, the sodium salts of ATP and GTP, creatine phosphate, creatine phosphokinase, and ribonuclease (protease-free) were purchased from Sigma Chemical Company.

Fractionation Procedures—All procedures were performed at 0-4°C unless otherwise noted. Tissue or femurs were removed from 14-day embryos (20 to 30 embryos), dissected free of skin and adhering tissues, and washed twice with a solution containing 0.25 M sucrose, 50 mM Tris-HCl, 1.0 mM MgCl₂, and 5.0 mM KCl (sucrose Buffer I). Excess solution was removed, the bones minced, resuspended in 2.5 volumes of sucrose Buffer I, and homogenized in a motor-driven Teflon pestle-glass vessel homogenizer (six strokes). The liquid extract was removed leaving bone particles in the vessel. The remaining particles were then resuspended and rehomogenized as above and the second extract added to the first. This procedure of homogenization released approximately 80% of the ribosomal RNA. The homogenates were centrifuged at 70,000 x g for 10 min to remove intact cells, nuclei, and mitochondria (9). The supernatant was decanted and again centrifuged at 70,000 x g. The supernatant from this step was centrifuged at 150,000 x g for 1 hour to obtain the free polysome fraction (P-105). In some experiments, P-15 and P-105 fractions also were obtained from tissue and femurs combined, or from skin and liver, by the same procedure. In two studies cultured cells derived from chick embryo frontal bone (3 x 10⁶ cells) and HeLa cells (2.3 x 10⁶ cells) were used. Cells were grown in modified Eagle’s minimal essential medium as described previously (10). When the chick frontal bone cells obtained a density of 7.5 x 10⁶ cells per plate (100 x 20 mm), sodium ascorbate was added to the growth medium to give a final concentration of 0.25 mM. After a 30-min incubation period the medium was removed, and the cells were scrapped from the plates in 5 ml of Tris-buffered 0.15 N NaOH, neutralized, and digested with protease-free bacterial collagenase under optimal conditions as described previously (10, 11). In this procedure, radioactive peptides derived from digestion of collagen remain soluble after precipitation of noncollagen protein with 5% trichloroacetic acid containing 1 mM t-proline to remove unincorporated [3H]proline. The precipitate was dissolved in 0.2 ml of 0.2 N NaOH, neutralized, and digested with purified protease-free bacterial collagenase under optimal conditions as described previously (10, 11). In this procedure, radioactive peptides derived from digestion of collagen remained soluble after precipitation of noncollagen protein with 5% trichloroacetic acid containing 1 mM t-proline to remove unincorporated [3H]proline. Each point was run in duplicate or triplicate with one of the sets serving as a minus collagenase blank, and the blank value was subtracted from the total radioactivity released into the supernatant to obtain collagenase-dependent release.

Analytical Methods—Radioactive proline and hydroxyproline were determined by a modification (11) of the method of Peterkosky and Procek (12). RNA in polysome-containing fractions was determined by extracting portions of the fractions with 5% trichloroacetic acid at 90°C for 15 min and then assaying an aliquot of the extract by reaction with orcinol (13). Protein was measured by the method of Lowry et al. (14).

Electron Microscopy—P-15 and P-105 fractions derived from bone homogenates were prepared for electron microscopic examination by fixation in 2.5% glutaraldehyde in 0.05 M phosphate buffer for 1 hour. The cells were then resuspended in 0.25 M sucrose containing 2 mM l-proline and incubated at 37°C for either 14 or 18 days as needed. Uniformly labeled L-[3H]proline (200 µCi per µmole) was obtained from New England Nuclear Corporation. The homogenates were centrifuged at 70,000 x g for 1 hour to obtain the P-105 fraction. Free amino acids were removed from the S-105 by gel filtration on a Bio-Gel P-10 column (1 x 22 cm) equilibrated with Buffer I. After the protein fraction first appeared in the eluate, 1.5 times the original volume was collected. The protein concentration of this fraction was approximately 15 mg per ml.

Cell-free Synthesis of Collagen and Noncollagen Protein—The resuspended polysome fractions (P-15 or P-105) were incubated for 15 min at 37°C in a total volume of 0.25 ml in the presence of the following components: 40 mM Tris-HCl (pH 7.6), 80 mM KCl, 4 mM MgCl₂, 0.25 mM ATP, 8 mM creatine phosphate, 25 µg of creatine phosphokinase, 0.1 mM GTP, 1.0 µM each of a mixture of amino acids minus proline, 0.1 mM dithiothreitol, 1 µCi of L-[3H]proline, and 1.4 mg of Bio-Gel P-10-treated liver S-105. The reaction was terminated by the addition of 5 µl of ribonuclease and incubated for an additional 10 min. An equal volume of cold 10% trichloroacetic acid containing 2 mM l-proline was added, and the precipitate was washed three times with cold 5% trichloroacetic acid containing 1 mM t-proline to remove unincorporated [3H]proline. The precipitate was dissolved in 0.2 ml of 0.2 N NaOH, neutralized, and digested with purified protease-free bacterial collagenase under optimal conditions as described previously (10, 11). In this procedure, radioactive peptides derived from digestion of collagen remained soluble after precipitation of noncollagen protein with 5% trichloroacetic acid containing 1 mM t-proline to remove unincorporated [3H]proline. Each point was run in duplicate or triplicate with one of the sets serving as a minus collagenase blank, and the blank value was subtracted from the total radioactivity released into the supernatant to obtain collagenase-dependent release.

RESULTS

Cell-free Collagen Synthesis by Membrane-bound and Free Polysomes—According to previous observations (9), it seemed likely that membrane-bound polysomes would sediment at less than 105,000 x g. Therefore 14-day chick embryo tissue homogenates were fractionated by removing nuclei, mitochondria, and other cell debris by centrifugation at 700 x g and then isolating fractions from the supernatant solution which sedimented at 15,000 x g (P-15) and 105,000 x g (P-105).
These fractions were resuspended and added to a cell-free protein-synthesizing system similar to one used previously (16) except that liver S-105 was used as the source of soluble factors. Both fractions incorporated [3H]proline into collagen and noncollagen protein. Incorporation was linear for 30 min and was proportional to microsomal RNA concentrations up to 400 \( \mu \)g per ml of incubation mixture. Nonlinearity above 400 \( \mu \)g RNA per ml was not due to limitation of liver supernatant since liver polysomes incorporated [3H]proline linearly up to approximately 1 mg of RNA per ml. It is most likely that the membrane-bound fraction contains inhibitors similar to those observed in lymph node microsomes (5). In the studies described in this report, the RNA concentration was kept at less than 400 \( \mu \)g per ml, and the incubation was carried out for 15 min.

Protein synthesis in this cell-free system required magnesium, RNA, and liver S-105 in addition to polysomes (Table I). Preincubation of the P-15 or P-105 fractions with ribonuclease (25 \( \mu \)g per ml, final concentration) or boiling these fractions almost completely abolished [3H]proline incorporation into both collagen and noncollagen protein. Addition of puromycin (0.2 to 2.0 mM) to the reaction mixture markedly inhibited protein synthesis (Table II), although the concentration required to inhibit [3H]proline incorporation completely in this system is somewhat higher than in other systems described.

After it was found that a low speed sedimented fraction contained protein synthetic activity, a more detailed fractionation study was carried out. Following the initial 700 \( \times \)g centrifugation, pellets were collected at 2,000 \( \times \)g (P-2), 15,000 \( \times \)g (P-15), 35,000 \( \times \)g (P-35), and 105,000 \( \times \)g (P-105), and each pellet resuspended in Buffer I and tested for protein synthetic activity in the cell-free system (Table III). Approximately 90% of the total collagen-synthesizing activity was found to sediment between 700 to 35,000 \( \times \)g, and there was an enrichment of collagen-synthesizing polysomes in these fractions. The total collagen

### Table I

**Requirements for protein synthesis in P-15 and P-105 fractions**

Homogenates were prepared from tibiae of 14-day-old chick embryos and P-15 and P-105 fractions isolated as described under "Experimental Procedures." The pellets were suspended in Buffer I, and 0.10 ml was incubated in the cell-free system for 15 min. Boiled fractions were boiled 5 min before the addition of the other components. For RNase treatment, 0.10 ml of the suspensions was incubated at 37° for 5 min with 2.5 \( \mu \)g of RNase in Buffer I before the addition of the other components, and the control was incubated with an equivalent amount of Buffer I.

| Treatment       | Collagen | Inhibition | Noncollagen protein | Inhibition |
|-----------------|----------|------------|---------------------|------------|
|                 | cpm      | %          | cpm                 | %          |
| Fraction P-15   |          |            |                     |            |
| Experiment 1    |          |            |                     |            |
| Complete        | 1308     | 0          | 291                 | 0          |
| Minus MgCl\(_2\) | 255      | 80.6       | 90                  | 69.2       |
| Experiment 2    |          |            |                     |            |
| Complete        | 954      | 0          | 234                 | 0          |
| Minus S-105    | 148      | 84.5       | 52                  | 86.2       |
| Boiled P-15    | 34       | 96.5       | 52                  | 77.9       |
| Experiment 3    |          |            |                     |            |
| Complete        | 667      | 0          | 273                 | 0          |
| Plus RNase      | 50       | 92.4       | 71                  | 73.8       |
| Fraction P-105  |          |            |                     |            |
| Experiment 1    |          |            |                     |            |
| Complete        | 344      | 0          | 213                 | 0          |
| Minus MgCl\(_2\) | 50       | 83.7       | 63                  | 70.4       |
| Experiment 2    |          |            |                     |            |
| Complete        | 186      | 0          | 153                 | 0          |
| Minus S-105    | 10       | 94.6       | 0                   | 100        |
| Boiled P-105   | 8        | 95.7       | 60                  | 60.8       |
| Experiment 3    |          |            |                     |            |
| Complete        | 223      | 0          | 149                 | 0          |
| Plus RNase      | 0        | 100        | 16                  | 89.3       |

### Table II

**Effect of puromycin on cell-free protein synthesis in P-15 and P-105 fractions**

Homogenates were prepared from 1.5 g of tibiae and femurs of 14-day-old embryos and fractions prepared as described under "Experimental Procedures." The pellets were suspended in 0.5 ml of Buffer I and 0.10-ml portions incubated in the cell-free system for 15 min at 37° with or without the addition of puromycin as indicated.

| Puromycin | Collagen | Inhibition | Noncollagen protein | Inhibition |
|-----------|----------|------------|---------------------|------------|
| m\(\mu\) | cpm      | %          | cpm                 | %          |
| Fraction P-15 |          |            |                     |            |
| 0        | 790      | 0          | 405                 | 0          |
| 0.2      | 500      | 36.7       | 182                 | 54.7       |
| 2.0      | 251      | 68.3       | 95                  | 76.5       |
| Fraction P-105 |        |            |                     |            |
| 0        | 512      | 0          | 368                 | 0          |
| 0.2      | 212      | 58.6       | 179                 | 51.4       |
| 2.0      | 0        | 100        | 90                  | 75.6       |

### Table III

**Distribution of collagen and noncollagen protein synthetic activity in fractions from differential centrifugation**

A homogenate was prepared from 2.2 g of tibiae and femurs and centrifuged at 200 \( \times \)g as described under "Experimental Procedures." The supernatant solution was centrifuged at 2000 \( \times \)g, and the pellet obtained is designated as the P-2 fraction. Similarly, further centrifugation of the supernatant solution sequentially at 15,000 \( \times \)g, 35,000 \( \times \)g, and 105,000 \( \times \)g yielded the P-15, P-35, and P-105 fractions. Each pellet was resuspended in 0.8 ml of Buffer I, and 0.10 ml of each suspension was incubated in the cell-free system for 15 min and assayed for radioactivity in collagen and noncollagen protein as described under "Experimental Procedures." Since the P-2 and P-15 pellets were considerably larger than the other two pellets, the final volume was greater than 0.8 ml, and the data in the table have been corrected to account for this in order to have a valid comparison of all fractions. The percentage of collagen synthesized was calculated by the formula

\[
\text{Collagen cpm} \times \frac{100}{\text{NCP cpm} \times 5.4} + \text{collagen cpm}
\]

in which the enriched content of imino acids in collagen (22%) as compared to noncollagen protein (NCP) (4.1%) is corrected for.

| Fraction | Collagen | Noncollagen protein |
|----------|----------|---------------------|
|          | Radioactivity | Distribution | Radioactivity | Distribution |
|          | cpm      | %          | cpm | %          |
| P-2      | 672      | 21.3       | 258 | 15.3       | 32.5       |
| P-15     | 1800     | 57.2       | 646 | 38.4       | 31.0       |
| P-35     | 317      | 10.1       | 122 | 7.3        | 32.5       |
| P-105    | 358      | 11.4       | 656 | 39.0       | 29.2       |
| Total    | 3147     | 100        | 1682| 100        | 25.7       |
synthesized by all of the fractions compared to synthesis of noncollagen protein was 25.7%, which is comparable to the relative rate of collagen synthesis in the tibia in vivo (17). It should be noted that the radioactivity incorporated into collagen is actually 90% of the total incorporation, but we have taken into account the greatly different content of amino acids in collagen and noncollagen protein when calculating the relative rate of collagen synthesis.

**Evidence that P-15 Fraction is Composed of Membrane-bound Polysomes**—Combined tibiae and femurs were homogenized in the presence or absence of 0.5% Triton X-100 and 0.2% sodium deoxycholate to determine if membrane-bound polysomes could be released. The results in Table IV show that when the homogenate was prepared in the presence of detergent, essentially all of the collagen and noncollagen protein-synthesizing activity formerly associated with the P-15 fraction no longer sedimented at 15,000 x g but appeared in the P-105 fraction. Homogenization in the presence of detergents did not affect total protein synthesis nor alter the rate of collagen synthesis. Direct treatment of the P-15 fraction with the same concentration of detergent resulted in recovery of about 66% of the collagen-synthesizing activity in the P-105 fraction.

Sections for electron microscopy were prepared from the P-15 and P-105 fractions of bone tissue homogenized in the presence or absence of detergent as described above. Micrographs revealed large vesicles of endoplasmic reticulum with ribosomes attached in the control P-15 fraction (Fig. 1A). This fraction contained very few mitochondria and very few, if any, free polysomes. Examination of the control P-105 fraction (Fig. 1B) revealed that it consisted mostly of free polysomes and small smooth vesicles. When the bone tissue was homogenized in sucrose Buffer I plus detergent prior to fractionation, electron microscopy showed a loss of membrane-bound polysomes from the P-15 fraction (Fig. 1C) which appeared in the P-105 fraction as free polysomes (Fig. 1D). Only some fibrillar type of material remained in the P-15 fraction (Fig. 1C).

**Table IV**

| Fraction | Collagen | Noncollagen protein | Collagen synthesized |
|----------|----------|---------------------|---------------------|
|          | Radio-activity incorporated | Activity in P-15 | Radio-activity incorporated | Activity in P-105 |
| P-15     | 1558     | 87.3                | 511                 | 66                   | 40.2 |
| P-105    | 272      |                      | 255                 | 16.5                 |     |
| Total    | 2130     |                      | 760                 | 34.0                 |     |
| P-15-D   | 68       | 4.5                 | 7                   | 0.9                  | 31.2 |
| P-105-D  | 1879     |                      | 768                 |                      | 31.2 |
| Total    | 1967     |                      | 775                 | 32.0                 |     |

*Radioactivity in noncollagen protein was insignificant and was not used for this calculation.

**Membrane-bound Polysomes in Various Tissues**—Various tissues were examined to determine whether collagen synthesis on membrane-bound polysomes is characteristic of connective tissue. The results in Table V indicate that most of the collagen-synthesizing activity (70 to 90%) in several connective tissues is associated with the membrane-bound polysomes. This is true even in tissues where the relative rate of collagen synthesis is very low (1.5% in skin and 2.4% in cultured frontal bone cells). In HeLa cells, where there is no detectable collagen synthesized, 74% of the total protein is synthesized by polysomes in the P-105 fraction.

Although the polysomes from skin and cultured frontal bone fibroblasts synthesize a relatively low amount of collagen, the specific activity of collagen synthesis by membrane-bound polysomes is similar to that of the tibia and femur. It appears that the difference between these tissues is that the specific activity for noncollagen protein synthesis is much higher in the P-105 polysomes of skin and cultured fibroblasts.

**Localization of Newly Synthesized Collagen**—In another study, the effect of detergent on the release of newly synthesized protein from the membrane-bound polysome complex was examined. P-15 polysomes were incubated in the cell-free system, allowed to incorporate [14C]proline into protein under the usual conditions, and then chilled to 0°C. The incubation mixture was divided into two portions and detergents were added to one portion. Both portions were centrifuged to give three fractions (P-15, P-105, and S-105) which were analyzed for collagen and noncollagen protein.

The results in Table VI show that newly synthesized collagen and noncollagen protein remained almost completely associated with the membrane complex in the control whereas newly synthesized protein was dissociated from the detergent-treated fraction. Sixty to 70% of the detergent-disassociated radioactivity was recovered in the free polysome fraction, indicating that most of the nascent chains had not been released from the polysomes. Approximately 7% (131 cpm) of the collagen radioactivity appeared in the S-105 fraction of the untreated sample, indicating that either some chains had been released into the soluble fraction or dissociated from the membranes during recentrifugation. After detergent treatment, radioactivity in both collagen and noncollagen protein increased in the S-105 fraction, indicating that approximately 9% of the collagen and 14% of the noncollagen protein had been transferred into the cisternae of the endoplasmic reticulum since intracisternal chains would only become soluble after dissolution of the membranes.

**Localization of Prolyl Hydroxylase in Membrane-bound Polysome FRACTION**—It has previously been observed that a ribosome-containing fraction from chick embryo homogenates contained prolyl hydroxylase (18, 19) and that proline in nascent chains attached to polysomes or removed by puromycin contained hydroxyproline (8, 20, 21). These observations, together with our findings that collagen synthesis occurs mainly on membrane-bound polysomes, suggested that prolyl hydroxylase might be associated with the polysomes or membranes of the endoplasmic reticulum. In order to study this possibility, we carried out an experiment in which both membrane-bound (P-15) polysomes and detergent-dissociated polysomes were used to synthesize [14C]proline-labeled collagen in the cell-free system described above. The polysome fractions were then resolated by centrifugation at 105,000 x g and incubated with or without the prolyl hydroxylase cofactors: ascorbate, Fe3+, α-ketoglutarate, and serum albumin (22). The fractions were digested with...
FIG. 1. Homogenates of tibiae from 14-day-old embryos were prepared with and without detergents and fractionated as described in Table IV. The pellets were sliced and fixed as described under “Experimental Procedures.” A, P-15 fraction from control homogenate; B, P-105 fraction from control homogenate; C, P-15 fraction from tissue homogenized with detergent; D, P-105 fraction from tissue homogenized with detergent. \( \times 40,000 \)

collagenase and the digests analyzed for radioactivity in proline and hydroxyproline. The results which are presented in Table VII indicate that in the absence of hydroxylase cofactors there is no hydroxylation in either the P-15 fraction or in the polysomes released from this fraction by detergent. When cofactors were added, hydroxylation occurred only in the untreated P-15 fraction but not in the free polysome fraction, suggesting that prolyl hydroxylase was associated with the polysome-membrane complex.

**DISCUSSION**

The results presented in this report clearly demonstrate that collagen-synthesizing polysomes are associated with the membranes of the rough endoplasmic reticulum. Approximately 90% of the total cell-free collagen-synthesizing activity of connective tissue is found in fractions that can be isolated by sedimentation between 700 and 35,000 \( \times g \) (Table III). Based on the observations of other investigators (5, 8), fractions isolated at this centrifugal force should contain membranes of the endoplasmic reticulum. All of the studies reported here were carried out with the fractions that sedimented between 700 and 15,000 \( \times g \) (P-15, membrane-bound polysomes) and between 15,000 and 105,000 \( \times g \) (P-105, free polysomes).

Examination of the P-15 fraction by electron microscopy confirmed that this fraction consisted mainly of polyribosomes attached to membranes of the endoplasmic reticulum (Fig. 1A)
In the cell-free system, and assayed for radioactivity in collagen procedures. Data from P-15 fractions were corrected for differences in volume compared to the P-105 fractions as described in the legend to Table I.

| Tissue          | Fraction | Percent activity in P-15 | Specific activity (cpm/μg RNA) | Per cent collagen synthesized |
|-----------------|----------|--------------------------|-------------------------------|-------------------------------|
| Tibia           | P-15     | 67.3                     | 33.6                          | 29.6                          |
|                  | P-105    | 9.6                      |                               |                               |
| Femur           | P-15     | 73.4                     | 54.3                          | 31.8                          |
|                  | P-105    | 12.0                     |                               |                               |
| Skin            | P-15     | 69.4                     | 26.1                          | 1.5                           |
|                  | P-105    | 9.7                      |                               |                               |
| Cultured frontal bone cells | P-15     | 88.2                     | 25.2                          | 2.4                           |
|                  | P-105    | 1.3                      |                               |                               |

While the P-105 consisted of free polysomes and small vesicles with no apparent ribosomes attached.

Both the P-15 and P-105 fractions were active in a cell-free protein-synthesizing system, and they both showed several characteristic requirements for protein synthesis (Table I). Preincubation of either fraction with ribonuclease or addition of puromycin markedly inhibited protein synthesis by both the membrane-bound and free polysomes. Further evidence that labeled proline was incorporated into peptide linkages was demonstrated by the fact that a portion of the [14C]proline was in trichloroacetic acid-precipitable protein that could be specifically digested by purified collagenase. When the necessary cofactors for hydroxylation were added, [%]hydroxyproline was also released from the product after collagenase digestion (Table VII).

Additional evidence that the polysomes in the P-15 fraction are membrane bound was obtained by either homogenizing the tissue in the presence of detergents (Table IV) or by treating the isolated P-15 fraction directly with detergents. In both instances the collagen-synthesizing activity was shifted from the P-15 to the P-105 fraction, indicating that polysomes were released from membranes. Protein-synthesizing activity was not lost when the tissues were homogenized in the presence of detergents, but if isolated P-15 fraction was treated with detergents approximately 30% of the total activity was lost. Examination by electron microscopy of the P-15 and P-105 fractions obtained after homogenizing in the presence of detergent confirmed the conclusion that the polysomes were released from membranes by this treatment (Fig. 1, C and D).

Examination of a variety of tissues and cells revealed that in

### Table V

| Tissue          | Fraction | Percent activity in P-15 | Specific activity (cpm/μg RNA) | Per cent collagen synthesized |
|-----------------|----------|--------------------------|-------------------------------|-------------------------------|
| Tibia           | P-15     | 61.0                     | 13.7                          |                               |
|                  | P-105    | 5.6                      |                               |                               |
| Femur           | P-15     | 72.7                     | 21.4                          |                               |
|                  | P-105    | 4.9                      |                               |                               |
| Skin            | P-15     | 15.3                     | 68.7                          | 329.0                         |
|                  | P-105    | 329.0                    |                               |                               |
| Cultured frontal bone cells | P-15     | 31.3                     | 68.7                          |                               |
|                  | P-105    | 58.4                     |                               |                               |
| HeLa cells      | P-15     | 26.4                     | 18.7                          |                               |
|                  | P-105    | 12.9                     |                               |                               |

### Table VI

| Reisolated fraction | Control | Triton-treated | Control | Triton-treated |
|---------------------|---------|----------------|---------|----------------|
| P-15                | 1676    | 166            | 469     | 130            |
| P-105               | 50      | 1207           | 65      | 360            |
| S-105               | 131     | 287            | 9       | 81             |
| Total               | 1857    | 1600           | 534     | 580            |

### Table VII

| Experimental conditions | Proline | Hydroxyproline | Ratio of proline to hydroxyproline |
|-------------------------|---------|----------------|-----------------------------------|
| P-15                    | 3009    | 893            | 3.4                               |
| Plus cofactors          |         |                |                                   |
| Minus cofactors         | 4220    | 71             | 60.4                              |
| P-105-D                 | 1540    | 0              | ∞                                 |
| Plus cofactors          |         |                |                                   |
| Minus cofactors         | 2200    | 0              | ∞                                 |

Homogenates from tibiae and femurs of 14-day-old embryos were prepared and fractionated to give a P-15 fraction which was incubated for 15 min in a 3-ml reaction mixture with the components of the cell-free system. After incubation, the reaction mixture was divided in half, and one half was digested with equal volume of Buffer I containing 0.5 μg buffer but also containing 0.5% Triton X-100-0.2% deoxycholate. The solutions were then centrifuged at 15,000 × g and 105,000 × g to yield P-15, P-105, and S-105 fractions from both control and detergent-treated samples. The pellets were resuspended in 0.5 ml of 0.05 M Tris-HCl (pH 7.6), and these fractions and the S-105 fractions were treated with 10 μg/ml of RNase for 10 min and assayed for collagen and noncollagen protein as described under "Experimental Procedures."
those tissues that synthesize collagen, 70 to 90% of the collagen-synthesizing activity is associated with membrane-bound polysomes. In skin and cultured fibroblasts, where the relative rate of collagen synthesis is low, most of the noncollagen-synthesizing polysomes are in the P-105 fraction while most of the collagen-synthesizing activity is in the P-15 fraction (Table V). It is also of interest that the specific activity of the membrane-bound polysomes that synthesize collagen in skin and cultured fibroblasts is comparable to the specific activity observed in the tibia and femur although the latter tissues synthesize about 30% of collagen. In addition, the rate of collagen synthesis by polysomes of skin and tibia in this cell-free system relative to the synthesis of noncollagen protein is in the same range as that observed when these tissues were labeled in ovo (17). For example, in the skin from 8- to 14-day-old embryos the relative collagen synthesis was between 3 to 7% and in the 14-day tibia the relative collagen synthesis was about 30% in ovo. The fact that the relative rate of collagen synthesis in this cell-free system reflects that observed in ovo indicates that we are recovering a representative sample of the population of polysomes from these tissues. In HeLa cells, which do not synthesize collagen and have very little rough endoplasmic reticulum (23), most of the protein-synthesizing activity is in the free polysome fraction.

Prolyl hydroxylase is usually purified from the soluble fraction of tissues (22, 24), and it has been suggested (25) on the basis of this finding and other observations that the first step in collagen biosynthesis is the synthesis of a polypeptide chain in which prolyl and lysyl residues are not hydroxylated (procollagen). Procollagen would then be released and subsequently hydroxylated by the soluble hydroxylases. It has been shown, however, that a substantial amount of the enzyme is present in the particulate fraction of tissues (18, 19, 26), and this data together with conclusive evidence that hydroxyproline occurs in nascent collagen chains (8, 20, 21) suggested that prolyl hydroxylase is normally associated with the endoplasmic reticulum but may be dissociated during homogenization procedures and thus found in the soluble fraction. Our findings strongly support this conclusion.

In the cell-free system which we have used, collagen polypeptides synthesized on membrane-bound polysomes are not released from ribosomes, yet the proline in these chains can be hydroxylated in the absence of soluble fraction when the necessary cofactors are added. When polysomes were released from membranes by detergent treatment, they still synthesized collagen, but the proline in these chains was not hydroxylated even in the presence of cofactors. Lazarides and Lukens (27) also observed that purified polysomes isolated after homogenization of 8-day chick embryo wings and legs with detergent contained no hydroxylase. Our observations suggest several possibilities for the localization of prolyl hydroxylase within the membrane-polysome complex. (a) Hydroxylase is attached to either nascent chains or ribosomes but is inactivated or dissociated from its original site by detergent treatment. (b) Hydroxylase is associated with the membranes of the endoplasmic reticulum and is solubilized by the detergent. A third possibility that the enzyme may be within the cisternae but not attached to the membranes seems unlikely since we found that in our cell-free system most of the newly synthesized hydroxylated collagen chains remained attached to the polysomes (Table VI). Since the polysomes are attached to the outer surface of the endoplasmic reticulum vesicles (Fig. 1A) it seems reasonable to assume that a large portion of these chains are outside the vesicles and could not be acted on by an internal enzyme.

In our studies, detergent was no longer present during the incubation with hydroxylase cofactors; however, we cannot discount the possibility that the prior treatment with detergent inactivated the hydroxylase. This possibility would have to be determined by treating a prolyl hydroxylase preparation with detergent and then resolubilizing the enzyme. However, since all the factors necessary for peptide synthesis remained active after detergent treatment (Tables IV and VII), it seems unlikely that the hydroxylase would be inactivated specifically by this treatment.

Although our data are consistent with the first two possibilities, the known specificity of detergent for membrane disruption leads us to favor the second (b). A postulated sequence of events for collagen biosynthesis would then be as follows. As procollagen is synthesized on the membrane-bound polysome complex, the nascent chain is bound to prolyl hydroxylase which would act as a gateway on the surface of the membrane. As hydroxylation proceeds, the chain would move past the enzyme and enter the cisternae of the endoplasmic reticulum. The enzyme lysyl hydroxylase may also be associated with the membrane in a similar manner. The transport of newly synthesized protein into the cisternae of the endoplasmic reticulum has been postulated as the first step in protein secretion (2), and evidence has been obtained to support this hypothesis by the use of a cell-free system from pigeon pancreas in which the enzyme amylase is transported into the cisternae after synthesis on the ribosomes (28). The procollagen chains would accumulate in the cisternae of the endoplasmic reticulum and would then be packaged in secretory vesicles and, in a process involving microtubules (29-31), would be transported to the cell surface and secreted. There is insufficient evidence at present to determine when glycosylation of hydroxylysine occurs. In the absence of hydroxylation cofactors, nascent unhydroxylated procollagen chains would bind to the membrane-associated hydroxylase, and because of the high affinity of the enzyme for the substrate (25) a stable complex would be formed, thus impeding further movement of the polypeptide chain through the membrane. It has been observed that unhydroxylated collagen chains synthesized in the presence of the iron chelator aedipyridyl were released from polysomes to almost the same extent as normally hydroxylated collagen chains (32) although secretion of collagen is almost completely inhibited by the chelator in several cell types (33, 34). Therefore, it seems most likely that the initial unhydroxylated collagen chains synthesized would saturate the hydroxylase substrate-binding sites and any further chains synthesized, being blocked from entering the membrane, would be released into the cytoplasm. The proposed sequence of events could explain the decreased rate of secretion in cells producing underhydroxylated collagen chains either because of treatment with aedipyridyl (33, 34) or because of ascorbate deprivation (35).

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Cell-free Collagen Synthesis on Membrane-bound Polysomes of Chick Embryo Connective Tissue and the Localization of Prolyl Hydroxylase on the Polysome-Membrane Complex

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