Degradation of Newly Synthesized Collagen*

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Explants of rabbit lung parenchyma maintained in serum-free medium incorporate \(^{14}C\)proline and \(^{14}C\)lysine into collagen as \(^{14}C\)hydroxyproline and \(^{14}C\)hydroxylysine at a rate constant for at least 24 h. Evaluation of the size distribution of the \(^{14}C\)hydroxyproline and \(^{14}C\)hydroxylysine containing peptides within the explants demonstrated that 20 to 40% of the \(\alpha\) chains of newly synthesized collagen were destroyed within minutes of being synthesized. Since the percentage of newly synthesized collagen destroyed did not increase with time of incubation, the mechanisms which mediate this process operate on the collagen molecule during a short interval in the sequence of collagen synthesis, secretion, and maturation. The degradation of newly synthesized collagen was not due to an extracollagenase; none could be detected in the culture medium and intact collagen added to the cultures was not degraded. It occurred so rapidly (30% of the labeled collagen was destroyed 8 min after the addition of \(^{14}C\)proline to the cultures) that it is unlikely to be the result of phagocytosis and digestion within phagolysosomes. The relative amount of degraded collagen was also independent of the addition of serum to the cultures and the use of inhibitors of proteolysis during the postincubation analytic procedures. In addition, this degradative process was age-invariant; the relative amount of newly synthesized collagen destroyed by explants of lung from old rabbits was identical to that destroyed by explants from young rabbits. Degradation of newly synthesized collagen is probably a normal process, independent of collagenase or phagocytosis, by which tissues modulate the quantity or quality, or both, of collagen being secreted from the cell.

In most tissues, the structure and function of the extracellular matrix depends upon the amount, kind, and distribution of collagen. Although the general features of collagen synthesis are well understood, less is known about the mechanisms of collagen destruction (1-11). Collagen is in a triple helical configuration as it is secreted from the cell (1, 2). In the extracellular milieu, only one enzyme, collagenase, is capable of attacking this triple helix (4-7). Since the original description by Gross and Lapière (8), it has been shown that collagenase is the primary means by which extracellular, mature collagen fibrils are destroyed. Trypsin will attack type III collagen near the site attacked by collagenase (9), but since trypsin is not found in the extracellular matrix, this is not considered "physiologic." Besides collagenase, the only other mechanism suggested for collagen degradation is that of phagocytosis (6, 10-12), in which extracellular collagen is incorporated into a phagolysosome and destroyed by acid proteases such as cathepsin B1 (13-15).

The present study evaluates a third mechanism by which significant quantities of collagen can be destroyed. Unlike collagenase-mediated or phagocytic mechanisms, this process acts only on newly synthesized collagen. The timing, pattern of action, and resistance to extracellular inhibitors of proteolysis suggest that it represents the destruction of collagen either intracellularly or soon after its secretion from the cell.

EXPERIMENTAL PROCEDURES

Materials—New Zealand white rabbits were obtained from B & H Rabbity, Rockville, Md. All studies were done with 1-week-old rabbits except for the age and in vivo studies which also used 10-week-old and 6-month-old rabbits. Pentobarbital was from Medic-Tech, Inc., Elkwood, Kan. Dulbecco's modified Eagle's (DME) medium and phosphate-buffered saline (NaCl/\(P\), \(pH\) 7.4; 140 mM NaCl, 3 mM KCl, 8 mM Na\(_2\)HPO\(_4\), 1.5 mM KH\(_2\)PO\(_4\), 0.9 mM CaCl\(_2\), 0.4 mM MgCl\(_2\)) were prepared by the NIH Media Unit; in experiments in which \(^{14}C\)lysine was used to label collagen, lysine was omitted from the DME medium. Serum was obtained from 2-month-old rabbits and dialyzed against NaCl/P (100 volumes, twice). Penicillin and streptomycin were from Difco Laboratories, Detroit, Mich.; Fungizone was from North American Biologicals, Miami, Fla.; ascorbic acid and Norit A were from Fisher Scientific, Fair Lawn, N. J.; iodoacetic acid was from Calbiochem, La Jolla, Calif.; \(\beta\)-aminopropionitrile fumarate, phosphorylase A, ovalbumin, and chymotrypsin were from Sigma Chemical Co., St. Louis, Mo.; Aquasol was from New England Nuclear, Boston, Mass.; diethylaminoethyl (DEAE)-cellulose (DE52) was from Whatman, Clifton, N. J.; and PA-28 and PA-35 chromatography resins were from Beckman Instruments, Palo Alto, Calif. All radioisotopes were from Schwarz/Mann, Orangeburg, N. Y.: \(\[^{3}H\]proline (300 mCi/ mmol), \(5[^{3}H]proline (40 Ci/mmol), [5,5,\[^{3}H\]hydroxyproline (54 mCi/ mmol), \(\[^{14}C\]hydroxyproline (1 Ci/mmol), and \(\[^{14}C\]lysine (300 mCi/ mmol). Tissue homogenization was carried out with a Polytron (Brinkmann Instruments, Westbury, N. Y.) equipped with a microtip. Dialysis tubing (1 cm width, type 8) was obtained from Union Carbide, Chicago, Ill.

Incubation of Lung Explants—Rabbits were killed with pentobarbital, the lungs were dissected free, and the trachea, large airways, and blood vessels were removed. At 4°C, the lung parenchyma was rinsed twice in NaCl/P and minced (<1 mm pieces), and tissue fragments were rinsed three times in NaCl/P. The tissue was divided into approximately 3-g (wet weight) portions and incubated in culture medium (equal volumes of DME medium and NaCl/P with ascorbic acid (0.5 mM), penicillin (100 units/ml), streptomycin

1 The abbreviations used are: DME medium, Dulbecco's modified Eagle's medium; NaCl/P, phosphate-buffered saline.
(100 µg/ml), and Fungizone (5 µg/ml) at 37°C in a 95% O₂, 5% CO₂ atmosphere with gentle shaking in a Dubnoff incubator. After 45 min, the medium was discarded and the tissue was portioned as required by the type of experiment to be performed.

In continuous labeling experiments, approximately 300 mg (wet weight) of tissue was placed in a glass vial with 2 ml of culture medium containing [14C]proline and incubated at 37°C as described above. At appropriate times the vials were removed from the incubator, the explants were rinsed four times with 10-ml portions of NaCl/P₄ (4°C) and placed in 4 ml of 0.5 M acetic acid. The tissue was then homogenized and aliquoted as previously described (16, 17).

In pulse-chase studies utilizing labeled proline, approximately 3 g (wet weight) of explants were incubated at 37°C in 15 ml of culture medium supplemented with 10 mM proline, and incubated for varying times. At the end of the chase period 2 ml of NaCl/P₄ (37°C) to the explant container and rinsing with gentle suction. The tissue was then divided into 300-mg (approximate wet weight) portions, placed in glass vials with 2 ml of medium supplemented with 10 mM proline, and incubated for varying times. The end of the chase period 2 ml of 1 M acetic acid (4°C) was added to each vial, the tissue was homogenized as described above, and the homogenates were portioned into 1-ml aliquots.

Pulse-chase studies utilizing labeled lysine were carried out as described above except that the pulse lasted 90 min, the culture medium contained no lysine, and B-aminopropionitrile (50 µg/ml) was included to prevent lysine derived covalent cross-linking.

Quantitation of [14C]Hydroxyproline, [14C]Hydroxylysine, and [14C]Hydroxylysine - Samples were lyophilized to dryness, hydrolyzed in 6 N HCl (20 h, 115°C), decolorized with Norit A, filtered (Whatman No. 2 paper), and evaporated to dryness.

In most cases [14C]hydroxyproline was quantitated using a column (0.9 x 55 cm) of Beckman PA-28 resin eluted with 0.2 mM citrate buffer, pH 3.25, at 55°C with a flow rate of 60 ml/h. The samples were redissolved in 0.2 N citrate buffer, pH 2.2, and filtered (0.45-µm Swinnex filters, Millipore, Bedford, Mass.) prior to being applied to the column. [14C]Hydroxylysine was >98%. Further proof of the identity of [14C]hydroxyproline eluting from the column was determined by summing the fractions making up the radioactive peak eluting around 40 min. Recovery of [14C]hydroxyproline applied to the column was >98%. Further proof of the identity of the [14C]hydroxyproline came from three sources: 1) the [14C]hydroxyproline co-chromatographed with chemically pure hydroxyproline; 2) when the peak of [14C]hydroxyproline was collected and rerun on the same column, it eluted at an identical time and the recovery was >98%; and 3) when the peak of [14C]hydroxylysine was collected and rerun on the same column, it eluted at an identical time and the recovery was >98%.

In initial experiments it was found that [14C]proline alone yielded radioactivity in the hydroxyproline region of the PA-28 column. In addition, a significant quantity of radioactivity remained on the column for long periods and was difficult to remove. For these reasons, [14C]hydroxyproline was determined using a modification of the method of Juva and Prockop (18). When it was necessary to assay [14C]hydroxyproline in the presence of large quantities of [14C]proline, it was critical to determine whether [14C]proline was artificially detected as [14C]hydroxyproline. To account for this phenomenon, varying amounts of [14C]proline were assayed for [14C]hydroxyproline; typically less than 0.05% of [14C]proline was detected as [14C]hydroxyproline.

A standard curve of [14C]proline incorporated into protein as [14C]hydroxyproline per unit of time as adjusted for tissue DNA and specific activity of the free [14C]proline and free nonradioactive proline using methods that have been detailed elsewhere (16, 17). The ratio of free [14C]proline to free total proline (radioactive + nonradioactive) was used as an estimate of the specific activity of the intracellular pool of [14C]proline available for protein synthesis (16).

The rate of synthesis of collagen was quantitated as the amount of [14C]proline incorporated into protein as [14C]hydroxyproline per unit of time as adjusted for tissue DNA content and specific activity of the free [14C]proline. The rate of synthesis of noncollagen protein was quantitated as the amount of [14C]proline incorporated into protein per unit of time as adjusted for tissue DNA and specific activity of the free [14C]proline (16, 17).

Quantitation of Degradation of Newly Synthesized Collagen - Following incubation by the continuous or pulse-chase method, homogenates of the lung explants were divided into aliquots to determine the amount of labeled collagen that was degraded during the incubation period. Depending on the labeled amino acid precursor used, the total amount of newly synthesized collagen (intact and degraded) was represented by the total amount of labeled hydroxyproline or hydroxylysine found in the tissue homogenate. The amount of newly synthesized collagen that was not degraded during the incubation period was determined by placing aliquots of the homogenate into dialysis bags (1 x 25 mm): dialyzing against two changes of 100 volumes of 0.5 M acetic acid at 4°C for a total of 86 h, and measuring the amount of labeled hydroxyproline (or hydroxy-

Fig. 1. Quantitation of [14C]Hydroxyproline and [14C]Hydroxylysine by ion exchange chromatography: A. [14C]hyroxyproline was quantitated using a column (0.9 x 55 cm) of Beckman PA-28 resin eluted with 0.2 N citrate buffer (pH 3.25, 55°C, 66 ml/h flow rate). Two-milliliter fractions were collected. Standards were run every 20th sample; the peak of [14C]hydroxyproline varied +2 min from run to run. The [14C]hydroxyproline standard co-chromatographed with chemically pure hydroxyproline. B. [14C]hydroxylysine was quantitated using a column (0.9 x 15 cm) of Beckman PA-35 resin eluted with 0.2 N citrate buffer (pH 4.25, 55°C, 66 ml/h flow rate). Two-milliliter fractions were collected. Standards were run every 20th sample; the peak of [14C]hydroxylysine varied +2 min from run to run. The [14C]hydroxylysine co-chromatographed with chemically pure hydroxylysine.
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lysine) remaining in the dialysis bag. The percentage of newly synthesized collagen that was degraded during the incubation was calculated using the following formula: per cent = \((\text{amount of labeled hydroxyproline in 1 ml of homogenate before dialysis}) - (\text{amount of labeled hydroxyproline in the dialyzate after dialysis})) \times 100\%/(\text{amount of labeled hydroxyproline in 1 ml of homogenate before dialysis})

In some experiments, the percentage of newly synthesized collagen that was degraded was determined by quantitating the labeled hydroxyproline retained by the dialysis bag and also the labeled hydroxyproline that passed through the dialysis bag. In these studies, 1-ml aliquots of explant homogenate were dialyzed against 100 volumes of 0.5 mM acetic acid, 0.1 mM unlabeled hydroxyproline and 0.5% N-acetylsulfatide, 20 mM NaCl/P, for one change, 4°C. Using the material remaining in the dialysis bag, the percentage of degraded collagen was determined by: per cent = \((\text{amount of labeled hydroxyproline in 1 ml of homogenate before dialysis}) - (\text{amount of labeled hydroxyproline in the dialyzate after dialysis})) \times 100\%/(\text{amount of labeled hydroxyproline in 1 ml of homogenate before dialysis})

Size Distribution of Newly Synthesized Collagen Peptides - Minced lung tissue, 1 to 1.2 g wet weight, was incubated with \(^{13}C\) proline (0.5 wCi/ml), washed, minced, homogenized in 10 ml of a solution of 8 M urea, 1% \(\beta\)-mercaptoethanol, 50 mM Tris, pH 7.4. In order to extract the labeled hydroxyproline within the explant, the homogenate was incubated at 37°C for 2 h and then at 4°C for 40 h. The mixture was centrifuged at 20,000 \(\times g\) for 30 min at 4°C and the precipitate was discarded; 98% of the total labeled hydroxyproline was present in the supernatant. The size distribution of the peptides containing \(^{13}C\) hydroxyproline was determined by electrophoresing 200 \(\mu\)l of the extract on cylindrical gels (10% acrylamide, 0.26% bisacrylamide, 7 h, 6 mA/cm). The gels containing the labeled proteins were cut into 1-cm pieces, hydrolyzed in 6 N HCl for 20 h, and assayed for \(^{13}C\) hydroxyproline (16, 18).

To determine the size distribution of dialyzable peptides containing labeled hydroxyproline, portions of minced lung tissue, 500 to 800 mg wet weight, were incubated with \(^{13}C\) proline (0.5 wCi/ml), washed, minced, homogenized in 5 ml of 0.5 M acetic acid. The entire homogenate was dialyzed for 24 h against 60 ml of 0.5 M acetic acid containing 0.1 M hydroxyproline as carrier. The dialyze was evaporated to dryness, redissolved in 5 ml of 0.5 M acetic acid, and applied to a column of Bio-Gel P-2 (1.6 x 90 cm, 400 mesh; Bio-Rad Laboratories) eluted with 0.5 M acetic acid; stained with Coomassie blue and destained in 7.5% acetic acid, 5% methanol (19). The gels containing the labeled proteins were cut into 1-cm pieces, hydrolyzed in 6 N HCl for 20 h, and assayed for \(^{13}C\) hydroxyproline (16, 18).

Effect of Animal Age on Degradation of Newly Synthesized Collagen

To determine whether the degradation of newly synthesized collagen was an age-dependent process, explant cultures of lungs from 1-week-old and 6-month-old rabbits were pulse-labeled for 4 h with \(^{3}H\) proline and chased for up to 48 h. The percentage of newly synthesized collagen that was degraded was quantitated using pre- and postdialysis levels of \(^{3}H\) hydroxyproline as described above.

RESULTS

Explants of rabbit lung parenchyma maintained in serum-free medium incorporated proline into protein at a constant rate for at least 24 h. Approximately 8 to 10% of the labeled proline residues within the newly synthesized proteins were found as labeled hydroxyproline (Fig. 2).

Labeled hydroxyproline could be detected as early as 8 min after the labeled proline was added to the explant incubation (Fig. 3). However, only 60 to 70% of the newly synthesized hydroxyproline containing peptides were of sufficient size to be retained by a dialysis bag; the remaining 30 to 40% was found in the dialysate (Fig. 3). Since greater than 95% of the labeled hydroxyproline in these explants is found in newly synthesized collagen (16), this finding suggests that under these conditions, approximately one-third of all collagen synthesized by lung parenchyma is degraded rapidly.

Furthermore, the relative amount of newly synthesized collagen that was degraded was independent of the time of
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A. COLLAGEN SYNTHESIS

B. NON COLLAGEN PROTEIN SYNTHESIS

Fig. 2. Evaluation of the viability of the lung explant cultures over a prolonged period. Portions of minced lung from 1-week-old rabbits were divided into two groups. One group was incubated for 1 h, the medium was changed to include [14C]proline and the incubation continued up to an additional 3 h. The second group was incubated for 20 h, the medium was changed to include [14C]proline, and the incubation continued up to an additional 3 h. Following incubation, the tissue was analyzed for: A, the incorporation of [14C]proline into collagen (the rate of collagen synthesis/cell in the explants cultured for 1 h prior to the addition of label was 1.4 nmol of hydroxyproline/mg of DNA/h; the rate of collagen synthesis/cell in the explants cultured for 20 h prior to the addition of label was 1.3 nmol of hydroxyproline/mg of DNA/h); and B, the incorporation of [14C]proline into noncollagen protein (the rate of noncollagen protein synthesis/cell in the explants cultured for 1 h prior to the addition of label was 15.6 nmol of proline/mg of DNA/h, the rate of noncollagen protein synthesis/cell in the explants cultured for 20 h prior to the addition of label was 15.3 nmol of proline/mg of DNA/h).

incubation. To demonstrate this, the fate of collagen synthesized by the explants was followed by using a pulse-chase technique in which the explants were pulsed with [14C]proline for 20 min and chased with 10 mM unlabeled proline for up to 24 h. Under these conditions, the per cent [14C]hydroxyproline that was dialyzable remained stable at 25 to 40% throughout the entire chase period. Combining the data from three separate experiments involving 29 explants evaluated at eight different time points demonstrated that the mean per cent dialyzable [14C]hydroxyproline was 30 ± 3% (Fig. 4). Significantly, the per cent dialyzable [14C]hydroxyproline did not increase with time. Actually, the combined data suggested it fell slightly over the first 2 h following the chase with unlabeled proline; for chase times of 0 to 1 h, the per cent dialyzable hydroxyproline was approximately 35%; for chase times greater than 1 h, the per cent dialyzable hydroxyproline was approximately 27%.

Thirty minutes after labeled proline was added to lung explants, the molecular weight distribution of the peptides containing labeled hydroxyproline ranged from >90,000 to <200 (Fig. 5). However, most of the labeled hydroxyproline

Fig. 3. Synthesis of collagen in explant cultures of rabbit lungs. Portions of lung parenchyma from 1-week-old rabbits were incubated in culture medium containing [14C]proline (12.5 μCi/ml) for 8, 16, 24, or 32 min. Three separate incubations were done at each time point. Following incubation, the tissue was homogenized in 4 ml of 0.5 M acetic acid and aliquots of the homogenate were analyzed for [14C]hydroxyproline, DNA, and the tissue pool of free [14C]proline and total proline. Additional aliquots were dialyzed and the retentate and dialysate were analyzed for [14C]hydroxyproline. Newly synthesized collagen peptides, represented by [14C]hydroxyproline, were quantitated in three fractions: total, that which was retained by the dialysis bag, and that which passed through the dialysis bag. In each fraction, the amount of newly synthesized collagen was expressed in relation to tissue DNA content and specific activity of free [14C]proline in the tissue (16).

Fig. 4. Time course of destruction of newly synthesized collagen following a 20-min labeling period. Explant cultures of lung from 1-week-old rabbits were pulse-labeled with [14C]proline for 20 min, washed, and incubated with unlabeled proline for 0 to 24 h. Following incubation, the samples were homogenized and analyzed for [14C]hydroxyproline before and after dialysis. Per cent dialyzable hydroxyproline (OHPro) = [(total amount of [14C]hydroxyproline before dialysis) - (amount of [14C]hydroxyproline after dialysis)] × 100/[total amount of [14C]hydroxyproline before dialysis]. Data from three separate experiments (○, ●, ▲) are shown. Analyses for [14C]hydroxyproline were done using a Beckman PA-28 resin (○, ●) or by a modification of the method of Juva and Prockop (▲) (16, 18).

To validate that the "chase" with unlabeled proline prevented further synthesis of labeled protein, "chased" explants were evaluated for the amount of labeled protein appearing per unit time. In a typical experiment, the chase procedure reduced the amount of labeled protein appearing to less than 1.0% of that during the pulse period.

All statistical data will be given as mean ± standard error of the mean.
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In addition, the presence of rabbit serum in the explant cultures did not alter the percentage of newly synthesized collagen that was destroyed (Fig. 6), even when chase periods of up to 20 h were used. Since the medium with added serum completely inhibited purified rabbit alveolar macrophage collagenase (Table I), this also suggested that the destruction of newly synthesized collagen did not result from the presence of an active collagenase in the extracellular space of the explants.

When purified labeled collagen (type I) or procollagen (pro-type I, pro-type III) was incubated with lung explants for 0 to 24 h and the explants homogenized and dialyzed as in a typical experiment, 99 ± 2% of the labeled hydroxyproline recovered was in a nondialyzable form (Fig. 7). Thus, degradative

![Fig. 5. Size of peptides containing labeled hydroxyproline found in lung explants. Two methods were used to evaluate the molecular weight distribution of these peptides: electrophoresis on sodium dodecyl sulfate acrylamide gels (for Mr = 100,000 to 14,000) and gel filtration on a Bio-Gel P-2 column (for dialyzable peptides of Mr = 14,000 to <200). The data from these two methods were combined to display the relative amount of labeled hydroxyproline present in each of six Mr ranges (K = 10): >90K (intact a chains), 65K to 90K, 14K to 65K, 2K to 14K, 200 to 2K, and 20 to 200 (free hydroxyproline). Of the total labeled hydroxyproline, 42% had a Mr of <90,000. Of this, 76% was of Mr <200 and 24% was of Mr between 90,000 and 200.

was contained within peptides >90,000 (58% of the total) or <200 (32% of the total). Thus, most of the collagen peptides within the explant were either intact a chains or had been degraded to molecular weights of <200.

Even though the degradation of newly synthesized collagen was such that the collagen molecule was broken down to very small pieces, very little of the labeled 14C-hydroxyproline "leaked" out of the explant either during the incubation or during the NaCl/Pi wash of the tissue. When explants were incubated for 30 min with 14C-proline, less than 1% of the total 14C-hydroxyproline in the explant (intact + degraded) was removed from the tissue in the NaCl/Pi wash used at the end of the 14C-proline "pulse" period (data not shown).

**Evaluation of Destruction of Newly Synthesized Collagen**

Several studies were performed to characterize or modify the process of apparent degradation of newly synthesized collagen by rabbit lung explants.

To ensure that it was the collagen a chain that was being degraded, explants of lung from 1-week-old rabbits were incubated with 14C-lysine for 90 min and chased with 10 mM unlabeled lysine for 7 h. The tissue homogenate was then analyzed for 14C-hydroxylysine before and after dialysis.

Analysis of explant culture media after 30 min and 24 h of culture failed to reveal an active or latent collagenase capable of destroying type I collagen fibrils (Table I). Furthermore, active or latent collagenase could not be detected in the homogenate of explants incubated for 30 min. Thus, during the time in which the explants destroyed 20 to 40% of the newly synthesized collagen, there was no detectable collagenase in the tissue or the incubation medium.

**Collagenase activity in lung explant cultures**

<table>
<thead>
<tr>
<th>Source</th>
<th>Activity*</th>
<th>cpm released</th>
</tr>
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<tbody>
<tr>
<td>Rabbit alveolar macrophage culture mediuma</td>
<td>760</td>
<td>10</td>
</tr>
<tr>
<td>+Dialyzed rabbit serum (10%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung explant culture medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At 30 min of culture</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>+Activatione</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>At 24 h of culture</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>+Activatione</td>
<td>80</td>
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<tr>
<td>Homogenate of lung explantc</td>
<td>70</td>
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<tr>
<td>+Activationd</td>
<td>80</td>
<td></td>
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<tr>
<td>Trypsin control</td>
<td>190</td>
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</table>

* Activity is expressed in counts per min of 14C-collagen fibrils solubilized after 20 h; 760 cpm is equivalent to 6.2 µg of collagen digested/h (20).

* From medium of 10⁶ cells (20).

* From medium of explants containing approximately 10⁶ cells.

a To evaluate for a putative latent collagenase, the sample was incubated with trypsin as previously described; following activation, the trypsin was inhibited with soybean trypsin inhibitor (20).

* From a homogenate of approximately 10⁶ cells.

* Trypsin control = 5 µg of trypsin (20); counts per min of collagen fibrils released below this level are not significant.

**Fig. 6.** Effect of serum on the destruction of newly synthesized collagen in lung explant cultures. Explants of lung from 1-week-old rabbits were incubated with ³H-proline in culture medium either with ("+ serum") or without ("control") 10% rabbit serum. Prior to use, the rabbit serum was dialyzed against NaCl/Pi. Culture medium with 10% dialyzed rabbit serum completely inhibits rabbit alveolar macrophage collagenase (see Table I). Two experiments were carried out: 1) 20-min incubation with ³H-proline and 2-h chase with 10 mM unlabeled proline; 2) 4-h incubation with ³H-proline and 20-h chase with 10 mM unlabeled proline. Following incubation, the explants were homogenized and analyzed for ³H-hydroxyproline (ÖHPro) before and after dialysis.
enzymes which could alter added collagen or procollagen were not detectable during either the incubation or the subsequent analytic procedures.

Since all explants were homogenized in 0.5 m acetic acid immediately after incubation (yielding a typical pH of 3.0), only proteases active at acid pH could attack newly synthesized collagen during analysis. In the presence of iodoacetic acid, known to inactivate cathepsin B1 (14), the percent dialyzable labeled hydroxyproline was unchanged (control, 25 ± 6%; + iodoacetic acid, 24 ± 3%; p > 0.8).3

As additional confirmation that proteases possibly released during homogenization were not responsible for the degradation of labeled collagen in these studies, explants heated to 100°C for 20 min showed the same percent degradation as controls (control, 25 ± 6%; + heat, 25 ± 2%; p > 0.1).3 When explants were heated to 100°C in the presence of 2% sodium dodecyl sulfate and then homogenized, the results did not differ significantly from controls (control, 24 ± 4%; + heat + sodium dodecyl sulfate, 25 ± 8%; p > 0.9).3

Newly synthesized collagen was not destroyed in the lung explants because they were "dying" in vitro. There were no significant differences between the rates of synthesis of collagen (or of noncollagen proteins) incubated for 4 or 23 h (Fig. 2). Thus, the lung explants were "viable" during these studies, at least in terms of protein synthesis.

Evaluation of the 3H]hydroxyproline containing proteins found within lung after a 4-h in vivo labeling period in rabbits demonstrated that an average of 31% of the 3H]hydroxyproline was dialyzable (data not shown). Thus, a significant percentage of newly synthesized collagen in vivo was dialyzable, suggesting the destruction of newly synthesized collagen within the explants reflected in vivo events.

**Relationship of Degradation of Collagen to Animal Age**—The age of the rabbit had no effect on the percentage of newly synthesized collagen degraded in the lung explants (Fig. 8). As with explants of lung of younger animals, lung explants from 6-month-old rabbits destroyed approximately 30% of the total collagen synthesized. After a 4-h pulse, the percentage of dialyzable 3H]hydroxyproline was unchanged throughout a chase of 0 to 40 h. In vivo labeling studies corroborated that destruction of newly synthesized collagen was age invariant. While an average of 31% dialyzable 3H]hydroxyproline was found in lung 4 h after labeling of 1-week-old rabbits with 3H]proline, an average of 28% dialyzable 3H]hydroxyproline was found in lung 4 h after labeling 10-week-old rabbits with the same tracer (data not shown).

**DISCUSSION**

Twenty to 40% of the newly synthesized collagen in rabbit lung explants is destroyed within minutes of its synthesis. The striking feature of this process is that it operates on the collagen molecule only during a very short time interval in the sequence of collagen synthesis, secretion, and maturation; the percentage of newly synthesized collagen that is destroyed does not increase with time after synthesis. However, those collagen molecules that are attacked are degraded extensively, most to peptides of molecular weights less than 200. Most likely, this process operates upon the α chain portion of the newly synthesized collagen molecule and is independent of collagenase or collagen phagocytosis. The following arguments support these concepts.

1. The finding of small peptides containing labeled hydroxyproline represents degradation of the collagen α chain, not only the NH₂-terminal precursor piece of procollagen. Although the "Pn" precursor peptide of collagen does contain 7 hydroxyproline residues within its 132 amino acid length, "normal" degradation of this peptide could not account for all of the dialyzable hydroxyproline in lung explants for several reasons. (a) The α chain contains 90 to 114 residues of hydroxyproline (16, 24-26) compared to 7 within each Pn piece;
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if the Pn piece were the only source of dialyzable hydroxyproline, the maximum percent dialyzable hydroxyproline could never be more than 6 to 8%, yet it was always >20% (Fig. 4). (b) When explants were labeled with lysine, an average of approximately 34% of the labeled hydroxylysine found in the explant was dialyzable; since the Pn precursor piece of collagen does not contain hydroxylysine, the only collagen-derived source of both hydroxyproline and hydroxylysine is the α chain itself.

2. The dialyzable peptides containing labeled hydroxyproline are not incompletely synthesized collagen α chains that were still "nascent" on polyribosomes when the incubation was terminated. If they were, then the molecular weights of the labeled hydroxyproline containing peptides would be uniformly distributed (27). Instead, the vast majority were found in two populations: greater than 90,000 daltons (intact collagen α chains) and <200 daltons (Fig. 4).

3. Degradation of newly synthesized collagen by rabbit lung explants is not mediated by an extracellular collagenase. First, the addition of rabbit serum to the explants had no effect on collagen degradation (Fig. 6), even though the same amount of serum completely inhibited collagenase produced by a cell obtained from rabbit lung (Table I). Second, no collagenase (active or latent) was found in the explants or explant media during the period in which large quantities of collagen were degraded (Table I). Third, if an extracellular collagenase were responsible for collagen degradation in this system, we would expect it to destroy more collagen as the incubation continued. In fact, the percent degradation of newly synthesized collagen was invariant with time (Fig. 4).

4. Degradation of newly synthesized collagen in these rabbit lung explants was not mediated by a process of secretion followed by phagocytosis and degradation within phagolysosomes. As with an extracellular collagenase, time invariance of the phenomenon argues against phagocytosis and phagolysosomal degradation. In addition, the rapidity of the degradation of newly synthesized collagen (30% at 8 min after synthesis was detected, Fig. 3) argues strongly against a process in which synthesis, secretion, and phagocytosis precede destruction; the time of synthesis of an intact pro-α chain is 5 to 8 min with secretion taking at least 20 to 30 min longer (27-29). Finally, if the cells within the explant were actively phagocytizing extracellular collagen we might expect that labeled collagen added to the explant would also be phagocytized and destroyed; however, it was not (Fig. 7).

5. Degradation of newly synthesized collagen is not an "artifact" of the experimental system (e.g., due to enzymes released or activated during preparation of the explants, during incubation because the explants were dying, or after incubation due to homogenization of the tissue or subsequent analytic steps). The studies arguing against collagenase or phagocytosis also argue against "artifact"; these are: (a) the time invariance of collagen destruction; (b) intact collagen added to the culture was recovered quantitatively; and (c) serum had no effect. In addition, when isoaoic acid, heat, or heat plus sodium dodecyl sulfate were used to inactivate any proteolytic enzymes present during the analytic steps, there was no change in the percent dialyzable hydroxyproline.

6. The collagen α chain is the only known protein synthesized by lung that contains hydroxyproline and hydroxylysine. Elastin contains hydroxyproline, but this represents less than 4% of the total hydroxyproline present in lung (16). The only other protein in lung that could contribute to the dialyzable hydroxyproline is a newly described glycoprotein found in lung of patients with alveolar proteinosis (30). However, this glycoprotein is found in only small amounts in normal animals (31, 32) and it does not contain hydroxylysine (30). Thus, the dialyzable, labeled hydroxylysine found in the lung explants most likely comes from degraded collagen α chains.

Significance of Rapid Degradation of Newly Synthesized Collagen—On the basis of these studies, it is clear that a significant proportion of collagen synthesized by lung parenchymal cells is degraded within minutes of its synthesis. Evaluation of diverse in vitro and in vivo studies in the literature suggests this may be a generalized phenomenon characteristic of tissues that synthesize collagen.

1. Degradation of newly synthesized collagen is found in tissues other than rabbit lung explants. Studies in our laboratory using similar methodology have demonstrated a similar phenomenon in human fetal lung explants, cultured diploid lung fibroblasts (HFL-1), and cultured mouse fibroblasts (3T6). In addition, degradation of newly synthesized collagen has been described in a variety of experimental systems, including guinea pig carrageenin granulation tissue (33), intact chick embryos (34), rat costal cartilage (35), chick embryo skin (36), rat calvaria (37), embryonic chick lens (38), and embryonic mouse fibroblasts (39, 40). In those studies in which degradation was quantitated it was found to be of approximately the same magnitude as in the rabbit lung explants (36, 37, 39, 40).

2. Degradation of newly synthesized collagen within rabbit lung explants has in vivo correlates. When rabbits were given labeled proline intravenously and the lungs removed 4 h later, approximately 30% of the labeled hydroxyproline found within the lung was dialyzable. In vivo degradation of newly synthesized collagen has also been found by others. Laitinen (41) noted that, after injecting rats with [14C]proline, [14C]hydroxyproline appeared in the urine within 1 h; similar findings were reported by Kibrick and Singh (42). In addition, Krane et al. (43) found labeled hydroxyproline in the urine of patients with Paget's disease 1 h after they were given [14C]proline.

The data are not sufficient to determine exactly at what stage the degradation of newly synthesized collagen occurs, but several findings suggest that it occurs either intracellularly or very soon after secretion. 1) Approximately 30% of collagen synthesized by the explants is degraded within 8 min of the time a labeled amino acid precursor is added to the incubation; this is within the time required for synthesis and less than that required for secretion (27-29). 2) Degradation of newly synthesized collagen is invariant with time, suggesting that there is a very short time interval during which the maturing collagen molecule can be attacked; such a compartmentalized degradative process is more likely to be intracellular than extracellular. 3) Serum added to the explants had no effect on the magnitude of the process, suggesting it occurred within a compartment inaccessible to serum antiproteases.

There is no explanation in these studies as to why such a large percentage of collagen is destroyed in what appears to be a very wasteful process. Rapid degradation of newly synthesized collagen does not seem to be age-related; it occurs in lung of 1-week-old and 6-month-old rabbits at the same magnitude (Fig. 8). This finding is of interest because a previous study demonstrated that the rate of collagen synthesis in lung explants derived from younger animals was 3 to 4 times that in explants from older animals (16).

One explanation for this degradative phenomenon is that it
regulates the quality of proteins that are synthesized, such that abnormal molecules are recognized and destroyed (44-47). Collagen is a large protein (one pro-α chain has a molecular weight of 145,000) that is subject to many post-translational modifications during its passage from the polyribosome to the mature collagen fibril in the extracellular space. Thus, in some fashion, errors in the synthesis of collagen at the transcriptional, translational or post-translational levels may "flag" a newly synthesized collagen molecule for rapid destruction. Other possibilities include regulation of translational modifications during its passage from the polyribosome to the mature collagen fibril in the extracellular space. Thus, in some fashion, errors in the synthesis of interstitial collagens. We are currently investigating these hypotheses using fibroblasts in culture as a model system.

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