Biosynthesis of Collagenase by Human Skin Fibroblasts in Monolayer Culture*

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The biosynthesis of human skin collagenase was studied in fibroblast cultures incubated with [³H]leucine under serum-free conditions. The radioactively labeled enzyme was isolated and quantitated directly from culture medium using a highly specific immunoprecipitation method. Sodium dodecyl sulfate gel electrophoresis of the immunoprecipitates showed two radioactive bands which co-migrated with the two proenzyme forms of purified human skin collagenase. Furthermore, the immunoreactive enzyme coeluted with collagenase activity in ion exchange chromatography. The qualitative nature of the intracellular ³H-labeled enzyme protein was examined by precipitation from cell lysates after partial purification. The results indicated that some of the intracellular enzyme protein exists in a form electrophoretically identical to the two proenzyme species of collagenase found in the culture medium.

The labeled human skin collagenase represented 3 to 6% of the trichloroacetic acid-precipitable protein in the culture medium and 0.2 to 1.0% of the total newly synthesized ³H-labeled protein (medium + cells) produced under these serum-free conditions. Intracellular labeled enzyme protein could be detected after a 15-min labeling period and reached a constant level at 45 to 60 min, after which there was no substantial increase in the intracellular storage of collagenase for up to 20 h. Secretion was linear once the intracellular labeled enzyme reached a constant level, a finding which suggests that the labeled enzyme had become equilibrated with the pre-existing intracellular enzyme pool and that the rate of secretion of the extracellular enzyme was largely reflective of de novo synthesis.

Vertebrate collagenases are of critical importance to the study of connective tissue metabolism because of their unique capacity to initiate collagen degradation (for review, see Refs. 1-4). In particular, the essential role of human collagenases is further emphasized by their being involved in the pathophysiology of certain diseases (5-10). Some of the mechanisms by which the activity of this class of enzymes is regulated have been defined: proenzyme synthesis (11-15), zymogen activation by proteolysis (16-20), interaction with essential metal cofactors (21, 22), inhibition by various serum- and/or tissue-derived inhibitors (23-37), and binding to the collagenous substrate (38-44). Nevertheless, a detailed system for examining the biosynthesis of collagenase has not been described.

Human skin collagenase is synthesized by normal human skin fibroblasts (13) and recent large scale culture of these cells has permitted isolation (15) and characterization (42) of the enzyme in its pure form. This collagenase is secreted as two proenzyme species of approximate molecular weights of 60,000 and 55,000 daltons (15) and a specific antisera has been elicited in rabbits which reacts equally with each of these proenzyme species as well as with their respective active enzyme forms (42). In the present communication we have utilized this antisera to develop a highly specific, sensitive system to study the biosynthesis of collagenase by normal human skin fibroblasts in culture. The results indicate that collagenase represents a major extracellular gene product of these cells.

METHODS

Cell Culture—Human skin fibroblast cultures were initiated from healthy volunteers or were purchased from the American Type Culture Collection, Rockville, Md. Cells were subcultivated in disposable plastic culture dishes or flasks (Coming) in Dulbecco’s modified Eagle’s medium-high glucose + glutamine (Microbiological Associates) with 0.03 M Hepes’ buffer (pH 7.6), 20% fetal calf serum, and 200 units of penicillin and 200 µg of streptomycin per ml at 37°C in a humidified atmosphere containing 5% CO₂.

Labeling Procedures—Replicate fibroblast cultures in early confluence (20,000 to 40,000 cells/cm²) were incubated in leucine-free Dulbecco’s modified Eagle’s medium-high glucose + glutamine containing antibiotics and further modified to contain 30 mM Hepes buffer, vitamins, and a 40 µM concentration of each of the following amino acids: L-aspartic acid, L-proline, L-asparagine, L-glutamic acid, and L-alanine. [³H]Leucine (NET-460, 70 to 100 Ci/mmol, New England Nuclear) was added to a final concentration of 0.4 µM (50 µCi/ml). Using cultures incubated at 37°C in an air/CO₂ atmosphere with 0.1 to 0.2 µl of the labeling medium per cm² of growth area, no significant change of culture pH was encountered during the usual 12- to 24-h duration of the experiments. After the desired incubation period, the medium was harvested, bovine serum albumin was added to a final concentration of 0.5 to 1 mg/ml, and the entire mixture was dialyzed at 4°C against several changes of 0.01 M Tris-HCl (pH 7.5) containing 1 mM CaCl₂ prior to immunoprecipitation. The cell layer was washed three times with Hepes’ balanced salt solution at 4°C, lyzed by sonication, and assayed for total protein and incorporation of [³H]leucine into protein.

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In experiments to determine the intracellular content of labeled enzyme protein, the cultures were labeled for the desired length of time with \(^{14}C\)leucine after which a 1000-fold excess of nonradioactive leucine was added to the medium. Aliquots of the culture medium were harvested at the beginning of the chase period and at hourly intervals thereafter to measure the intracellular labeled enzyme protein which was chased into the culture medium. The amount of labeled enzyme protein taken as that amount precipitated from the medium over what was present at the beginning of the chase period. In every case all of the intracellular enzyme was chased into the culture medium within 2 h, after which no additional accumulation of labeled enzyme protein was seen for a period of up to 6 h.

In some experiments, in order to assess the qualitative nature of the enzyme protein, the medium was decanted from labeled confluent cultures and the cells were washed five times with buffer (three times on the plate and two additional times with suspension followed by low speed centrifugation). After sonication, the lysate was centrifuged at 13,000 \(\times g\) for 15 min after which the supernatant was subjected to ultracentrifugation at 105,000 \(\times g\) for 1 h. This 105,000 \(\times g\) supernatant was then applied to a column (0.9 x 4-cm) of carboxymethylcellulose as described below except that bound proteins were eluted in a single stepwise fashion with 0.5 M NaCl.

Preparation of Antiserum to Human Skin Collagenase—Human skin procollagenase for use as the immunogen was purified to homogeneity from the medium of human skin fibroblast cultures as described by Stricklin et al. (15). This preparation was used to prepare functionally monospecific antiserum to the enzyme as given in detail previously (42). The antiserum gave a single immunoprecipitin band in Ouchterlony analysis with either the crude culture medium or with the antigen which had been purified to homogeneity (42). Furthermore, a \(\gamma\) globulin preparation of the antiserum produced >95\% inhibition of the collagenase activity after reacting 8 h at 37\(^\circ\)C compared to <5\% inhibition by a nonimmune \(\gamma\) globulin preparation (42).

To determine the amount of antiserum required to achieve quantitative precipitation of human skin collagenase, a constant amount of antiserum was titrated against increasing concentrations of electrophoretically homogeneous carrier enzyme. The reaction was monitored by the addition of tracer amounts of purified labeled enzyme. A typical reaction contained 10 \(\mu\)l of antiserum to human skin collagenase, a variable amount of electrophoretically pure human skin collagenase (0.1 to 5.0 \(\mu\)g), and 5 to 70 ng of purified \(^{3}H\)leucinated enzyme. The reaction was carried out in 0.01 M Tris-HCl (pH 7.5)-0.15 M NaCl buffer containing 1 mg/ml bovine serum albumin. After incubation for 3 h at 37\(^\circ\)C and 18 h at 4\(^\circ\)C, the resultant precipitates were harvested by centrifugation, washed three to four times with cold Tris/NaCl buffer, and solubilized in 0.1 M NaOH for counting.

Isolation of Human Skin Collagenase by Immunoprecipitation—For direct precipitation of labeled enzyme protein, dialyzed labeled cell-free supernatant or labeled enzyme preparation was used. The reaction mixture was incubated in 0.01 M Tris-HCl (pH 7.5) buffer, 5\% glycerol containing 100 \(\mu\)g/ml bovine serum albumin, and 1 \(\mu\)l of anti-human skin collagenase serum. After incubation for 3 h at 37\(^\circ\)C and 18 h at 4\(^\circ\)C, the resultant precipitates were harvested by centrifugation and washed four times with Tris/NaCl buffer. The precipitates were dissolved in 250 \(\mu\)l of 0.1 M NaOH, added to 8 ml of ACS scintillant solution (Amersham/Searle), and counted in a liquid scintillation spectrometer with correction for quenching.

As a control for the background radioactivity caused by nonspecific precipitation and trapping of labeled proteins in the immunoprecipitate, the same volume of labeled medium was incubated with 3 to 10 \(\mu\)l of antiserum to ovalbumin and a sufficient amount of ovalbumin soluble in the buffer to give an excess of 3000 in this system. The amounts of protein immunologically precipitated in the ovalbumin-antiovalbumin and collagenase-anti-collagenase systems were equal. The difference between radioactivity precipitated by specific anti-human skin collagenase serum and anti-ovalbumin serum was taken as a measure of the labeled collagenase.

The antisera capable antibody was also utilized for quantitation of labeled collagenase protein. For these experiments specific anti-human skin collagenase serum was used as was the fast antibody and goat antirabbit IgG as the second (precipitating) antibody. A typical reaction contained 300 to 400 \(\mu\)l of labeled enzyme protein, 0.25 to 6 \(\mu\)l of antiserum to human skin collagenase, or an equivalent amount of nonimmunized whole rabbit serum as a control, in a total volume of 500 \(\mu\)l of 0.01 M Tris-HCl (pH 7.5)-0.15 M NaCl buffer containing 1 mg/ml bovine serum albumin. After reacting for 4 h at 37\(^\circ\)C and 18 h at 4\(^\circ\)C, a 50-fold excess of goat antirabbit IgG was added and the resultant precipitates were harvested by centrifugation after an additional 20 h at 4\(^\circ\)C. The precipitates were washed and solubilized for counting as described above. The indirect method was found to be most useful when precipitating partially purified enzyme protein. In addition, it had the advantage of not requiring the large amounts of the electrophoretically pure carrier human skin collagenase that was needed in the direct system. The enzyme needed in the direct system was solubilized in a boiling water bath for 5 min prior to electrophoresis. Immediately before electrophoresis, one drop of glycerol containing bromphenol blue was added and the samples were applied to a discontinuous SDS slab gel (1 mm in thickness) made up according to King and Laemmli (46) with 10% (w/v) acrylamide and 0.27% (w/v) \(N\)-methylenebis-acrylamide. Electrophoresis was performed at a constant current of 70 mA per mm of slab thickness until the dye front approached the bottom of the gel. After the gel run, the positions of the top of the gel and the dye front were noted and the different gel slabs were either stained with Coomassie brilliant blue, sliced for quantitation of the radioactivity, or processed for fluorography. In the case of gel slicing, 2-mm slices of the slab gel were incubated with 2 ml of a 9:1 mixture of NCS tissue solubilizer (Amersham/Searle) and water in a glass counting vials for 18 h at 37\(^\circ\)C with stirring. After cooling to room temperature, 10 ml of ACS scintillation cocktail (Amersham/Searle) was added and the samples were counted in a liquid scintillation spectrometer. For fluorography, the slab gels were equilibrated with dimethyl sulfoxide, immersed in 20% 2,5-diphenyloxazole in dimethyl sulfoxide, and dried in a vacuum chamber. The dried gels were exposed to Kodak XR-5 X-Omat R film (Eastman Kodak) at -70\(^\circ\)C (47). Densitometric scans of the fluorographs were done with a Zeiss PM6 spectrophotometer.

Total Protein Synthesis—Total protein synthesis was determined at varying time points by quantitating the \(^{14}C\)leucine incorporated into 10% trichloroacetic acid-insoluble material for these studies. An equal volume of 20% trichloroacetic acid was added to 100 \(\mu\)l of labeled medium containing bovine serum albumin. The precipitates were harvested after 1 h at 0\(^\circ\)C, washed three times with 10% trichloroacetic acid, and dissolved in 0.1 M NaOH for addition to ACS scintillant and counting with quench correction. Cells were washed twice with 0.01 M Tris-HCl (pH 7.5) buffer, suspended in 0.01 M Tris-HCl (pH 7.5) containing 0.15 M NaCl, and lysed by sonication. Trichloroacetic acid precipitation was then carried out as described above.

Purification of Labeled Human Skin Collagenase—Crude labeled culture medium was dialyzed against 0.01 M Tris-HCl containing 0.1 M CaCl\(_2\) and applied to a column (0.9 x 10 cm) of carboxymethylcellulose (Whatman, CM52) equilibrated with the same buffer as described by Stricklin et al. (15). Following application to the column, sample buffer was used to elute unbound protein until the radioactivity returned to base-line. The bound proteins were eluted with a linear gradient established from 0 to 0.3 M NaCl in a volume of 320 ml (15). All fractions were filtered with 0.45 \(\mu\)m membrane filters, added to final concentrations of 0.5 mg/ml and 1 mm, respectively. Purified preparations maintained in this buffer were found to be stable for 2 mo at -20\(^\circ\)C.

Collagenase Assay—Purified human skin procollagenase to be used for carrier protein or for comparison of electrophoretic mobility was assayed for irreversible hydrolysis of collagens by the method of Stricklin et al. (16). The enzyme was assayed after proteolytic activation as previously described (13). For each enzyme preparation, a range of trypsin concentrations (0.1 to 2.0 \(\mu\)g of trypsin per 50-\(\mu\)l enzyme sample) was employed to ensure that maximal collagenase activity was measured. After preincubation with trypsin for 10 min at least a 5-fold molar excess of soybean trypsin inhibitor was added to inhibit further trypsin activity. Each mixture was then assayed for collagenase activity at 37\(^\circ\)C in 0.05 M Tris-HCl (pH 7.5)
in the presence of 10 mM CaCl₂ using native, reconstituted [¹³C]-glycine-labeled collagen fibrils containing approximately 3000 cpm per substrate gel (48). Protein was determined using established methods (49).

**RESULTS**

Titration of Anti-human Skin Collagenase Serum—The equivalence point of the anti-human skin collagenase serum was determined by titrating a constant amount of antiserum against an increasing amount of the electrophoretically homogeneous carrier enzyme. For these studies the efficiency of the precipitation was monitored by the addition of a constant tracer amount of purified [¹³C]-labeled human skin collagenase. As shown in Fig. 1, 10 μl of the antiserum quantitatively precipitated up to 1.2 μg of human skin collagenase (Fig. 1). Furthermore, the zone of equivalence was the same both in the presence of low levels (5 ng) or high levels (70 ng) of labeled enzyme.

A variety of factors influenced both the recovery and the specificity of this immunoprecipitation system (Table I). Although the addition of bovine serum albumin slowed the formation of the immunoprecipitates, a minimum concentration of 1 mg/ml was required to minimize the loss of labeled human skin collagenase and to reduce the background radioactivity due to absorption of unrelated labeled proteins to the tubes. Furthermore, as expected by the kinetic nature of the antigen-antibody reaction, higher concentrations of carrier enzyme and antiserum decreased the time required for maximal precipitation (Table I). With the addition of 10 μl of antiserum the recovery of labeled immunoreactive material was 90% after reacting for 2 h at 37°C and 4 h at 4°C. This value remained constant for up to a 24-h incubation at 4°C. The finding that approximately 90% of the total labeled human skin collagenase was precipitated under these conditions was confirmed by the addition of a marked excess of goat anti-rabbit IgG for precipitation in a double antibody system (not shown). As shown in Table I and by the double antibody precipitation, raising the concentration of anti-human skin collagenase above 3 μg increased the rate of the reaction but had no additional effect on the quantity of labeled enzyme precipitated. Thus, for the biosynthetic studies reported be-

**TABLE I**

Effect of varying incubation conditions on the immunoprecipitation of [¹³C]-labeled human skin collagenase

<table>
<thead>
<tr>
<th>Volume of anti-human skin collagenase serum</th>
<th>Bovine serum albumin concentration</th>
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<th>Incubation at 4°C (h)</th>
<th>Recovery</th>
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<td>mg/ml</td>
<td>1000</td>
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**Fig. 2.** Fluorographs of the SDS-gel electrophoresis patterns after immunoprecipitation from fibroblast culture medium. A, anti-human skin collagenase precipitate; B, anti-ovalbumin precipitate. The arrows mark the electrophoretic mobilities of the two purified unlabeled human skin collagenase proenzyme standards.

low, the use of 3 to 10 μl of antiserum ensured complete precipitation of the labeled collagenase in the presence of carrier enzyme.

Specificity of the Immunoprecipitation of Collagenase—Evidence for the specificity of precipitation was sought by subjecting the immune precipitates to SDS-acrylamide gel electrophoresis followed by scintillation autoradiography (47). Crude dialyzed medium containing [¹³C]-labeled proteins was reacted with either anti-human skin collagenase or antiovalbumin. Fig. 2 depicts the fluorographs of the SDS-gel electrophoresis patterns of the specific and control immunoprecipitates. In the case of the antiserum to collagenase, the only labeled proteins precipitated were those which co-migrated with the two proenzyme species of purified human skin col-
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Collagenase which exist as a characteristic doublet (15). Compared to the precipitate found with anti-human skin collagenase, the antiserum to ovalbumin gave no distinguishable bands, suggesting that the radioactivity precipitated in this system was due to nonspecific trapping.

Further evidence for the specificity of the precipitation of 3H-labeled collagenase was obtained by ion exchange chromatography (Fig. 3). For these experiments, three 150-cm² culture flasks of cells were labeled and the medium was dialyzed and applied to a carboxymethylcellulose column as described by Stricklin et al. (15) for quantitative purification of the enzyme. Immunoprecipitable enzyme protein was eluted from the column in the same position as collagenase activity. In every case 90 to 100% of the radioactivity applied to the column was recovered with 87% being found in the fall-through and 13% eluted with the gradient. When the fractions containing immunoprecipitable material were pooled as shown in Fig. 3B (Fractions 16 to 28), a mean value of 43% (range, 27 to 59%) of the total radioactivity in the pooled fractions was precipitated with the antiserum to human skin collagenase.

In separate experiments the nature of the precipitated protein was also examined by slicing the gels after electrophoresis (Fig. 4). The only 3H-labeled protein precipitated from the crude medium with the specific anticollagenase antiserum co-migrated with pure human skin collagenase (Fig. 4A). Furthermore, the ovalbumin-antiovalbumin control precipitates displayed no major peaks of radioactivity but instead showed the diffuse low level background radioactivity characteristic of nonspecific trapping. With this method about two-thirds of the total radioactivity was found to be due to specific precipitation of enzyme protein by anti-human skin collagenase or nonimmune rabbit serum as the first antibody, and an excess of goat antirabbit IgG as the second antibody. Gels were sliced after electrophoresis as noted in A.

The specificity of the indirect method for precipitation of labeled protein was also assessed with an SDS-gel electrophoresis (Fig. 4B). In this experiment all of the radioactivity which bound to a carboxymethylcellulose column was eluted stepwise with 0.5 M NaCl (see Fig. 3) and used as a source of labeled enzyme. This material was then reacted with either anti-human skin collagenase or nonimmune rabbit serum after which an excess of goat antirabbit IgG was added for precipitation and subsequent SDS gel electrophoresis.
electrophoresis (Fig. 4D). Again, the only labeled protein specifically precipitated with anti-human skin collagenase co-migrated with unlabeled purified enzyme.

Since the resolving capacity of the gel-slicing method was relatively low (as manifested by the failure to resolve the two proenzyme species), we routinely used the scintillation autoradiographic method (47) for screening the specificity of the precipitates. The high degree of specificity of the anti-human skin collagenase serum is further illustrated in Fig. 5. In this densitometric scan of the fluorograph of the SDS electrophoresis of the immunoprecipitate, the dominant doublet co-densitometric scan of the fluorograph of the SDS electrophoresis of the immunoprecipitate, the dominant doublet co-migrated with the two proenzyme forms of collagenase. The apparent molecular weights of these two proenzyme species were 56,000 and 53,000 (Fig. 5, inset), values which closely correspond to those previously reported for the two electrophoretically pure proenzymes (15). In addition, in this experiment the culture medium contained a small amount of the two lower molecular weight species corresponding to activated enzyme forms (15).

**Intracellular Collagenase**—Numerous attempts were made to precipitate intracellular [3H]labeled enzyme protein directly from crude cell lysates. In each case, we encountered substantial co-precipitation of contaminating intracellular proteins as has been reported in other such systems (50–52). Nonspecific trapping of labeled intracellular proteins could be reduced, but not completely eliminated, by using siliconized tubes for the reaction, by the addition of 0.5% of Triton X-100, and by ultracentrifugation of the lysate prior to precipitation. In addition, we found it necessary to include protease inhibitors (0.3 mM phenylmethylsulfonylfluoride and 5 mM N-ethylmaleimide) since, in their absence, a doublet of lower molecular weight (approximately 37,000 and 40,000) was found after incubation of the lysate with antiserum, suggesting the possibility that partially degraded enzyme protein was being precipitated. Finally, precipitation from the crude lysates followed by solubilization of the precipitates in NaOH, neutralization with HCl, and reprecipitation resulted in losses which were unacceptable either for qualitative or quantitative studies.

In order to examine the nature of the labeled immunoreactive collagenase protein in the cell lysate, we took advantage of the ion exchange behavior of the enzyme (15) to achieve a partial purification prior to precipitation. Of the total radioactivity from the cell lysates applied to a carboxymethylcellulose column, approximately 94% appeared in the fall-through peak and 6% was retained. As shown in Fig. 6A, a radioactive doublet which co-migrated with an extracellular, purified collagenase standard (Fig. 6B) was precipitated from the retained fraction of the cell lysate. This finding suggests that at least some of the intracellular enzyme exists in a form similar to or identical with the extracellular enzyme.

**Stability of Human Skin Collagenase in the Culture Medium**—Prior to examining the synthesis and secretion of collagenase, we first determined the stability of the extracellular labeled enzyme protein under our culture conditions. For these studies, the [3H]leucine was chased with an excess of nonradioactive leucine (400 μM final concentration) and both

![Fig. 5. Densitometric scan of a fluorograph of the SDS-gel electrophoretic pattern of immunoprecipitates. Crude dialyzed culture medium was reacted with anti-human skin collagenase serum as described under "Methods."](image)

![Fig. 6. Comparison of intracellular and extracellular immunoprecipitated [3H]-collagenase protein by densitometric scan of the precipitates after SDS gel electrophoresis. A, scan of enzyme protein precipitated from partially purified cell lysate (see text for details). B, scan of purified [3H]-labeled extracellular collagenase precipitated and subjected to electrophoresis at the same time.](image)
trichloroacetic acid-precipitable protein and antibody-precipitable enzyme protein were quantitated at varying time points (Table II). Once secreted, the ³H-labeled human skin collagenase remained stable in the serum-free medium as measured immunochemically. Furthermore, it is noteworthy that both the direct and indirect methods for quantitating enzyme protein gave essentially identical results.

**Synthesis and Secretion of Collagenase**—Synthesis and secretion of collagenase was determined by quantitating both intra- and extracellular labeled enzyme protein at varying time points. Replicate flasks were labeled with [³H]leucine for 2, 7, 12, and 22 h (Fig. 7). Since direct precipitation of intracellular enzyme protein resulted either in co-precipitation of noncollagenase protein or in unacceptable losses in reprecipitation (see above), a pulse-chase method was used to measure intracellular enzyme. Labeled intracellular collagenase reached a constant level within 2 h and remained in this steady state for a labeling period of up to 22 h without showing a substantial increase in the storage of intracellular enzyme. Secretion of extracellular enzyme protein was easily seen at 2 h (the earliest time point examined in this experiment) and remained linear for approximately 12 h after which the rate slowed considerably. At 12 h 80 to 90% of the total labeled enzyme protein was found in the medium.

The time required for synthesis and secretion of ³H-labeled enzyme protein was examined in greater detail by labeling the fibroblast cultures for periods of up to 2 h (Fig. 8). The time required for equilibration of the label, synthesis, and secretion of extracellular labeled trichloroacetic acid-precipitable proteins was found to be approximately 15 min (Fig. 8A). Intracellular labeled collagenase was first detected at 15 min, a value most likely representing the minimum time required for equilibration of the label and enzyme synthesis (Fig. 8B). Secretion of a measurable amount of labeled collagenase was not seen at 15 min but was clearly detectable 30 min after the beginning of the labeling period. Secretion was linear once the intracellular pool of labeled enzyme reached a constant level, approximately 40 to 45 min after the initiation of labeling.

Since our studies showed that no further accumulation of

![Graph](image)

**Fig. 7. Synthesis and secretion of ³H-labeled human skin collagenase.** Four identical confluent 75-cm² cultures were labeled for 2, 7, 12, and 22 h. At the end of each respective labeling period, a portion of the medium was harvested to determine the [³H]collagenase content. A 1000-fold excess of unlabeled leucine was then added to the same culture and the intracellular enzyme was chased into the medium. The intracellular enzyme represents the net increase in immunoprecipitable material during a 2-h chase period over that found at the beginning of the chase period. •—•, total (medium + cells) ³H-labeled human skin collagenase; ○—○, extracellular ³H-labeled human skin collagenase; □—□, intracellular ³H-labeled human skin collagenase.

### Table III

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<tr>
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- Time elapsed after [³H]leucine was added to the cultures.
- Total radioactivity precipitated with antiserum to human skin collagenase (specific) or in the ovalbumin antiovalbumin control (nonspecific).
- Data are expressed as the net counts per min (specific minus nonspecific) and as the per cent of the trichloroacetic acid-precipitable ³H-protein in the medium and in the medium + cells (% of total).
- Replicate 10-cm² culture plates were labeled with 50 µCi/ml of [³H]leucine.
- Replicate 10-cm² culture plates were labeled with 50 µCi/ml of [³H]leucine.
- Replicate 75-cm² culture flasks were labeled with 11 µCi/ml of [³H]leucine.
- Replicate 75-cm² culture flasks were labeled with 50 µCi/ml of [³H]leucine.

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in intracellular material occurred after the first 45 to 60 min of labeling (Fig. 8), a finding indicative of no substantial storage of the newly synthesized enzyme, we used the extracellular labeled enzyme to approximate the relative synthesis of collagenase by normal human skin fibroblasts (Table III). In these experiments, depending upon the cell line employed, collagenase in the medium represented 0.2 to 1.0% of the total trichloroacetic acid-precipitable leucine-labeled proteins (cells + medium) synthesized by these cultures under serum-free conditions. This resulted in a concentration in the medium of 3 to 6% of the total labeled extracellular proteins under serum-free conditions. It is noteworthy again that this value correlates well with the quantitative amounts of collagenase found in human skin fibroblast lines (15).

DISCUSSION

In this paper we have presented a system which has been used to initiate a detailed examination of the biosynthesis of human skin collagenase by fibroblasts. The immunoprecipitation methods employed have a high degree of specificity. The enzyme protein precipitated from crude culture medium co-migrated identically with purified standard human skin collagenase by fibroblasts. The immunoprecipitation method also permitted us to examine the qualitative nature of the intracellular enzyme protein. Although direct precipitation of labeled enzyme was complicated by nonspecific co-precipitation, as has been described in other such systems (50–52), we were able to obtain clean precipitations by a preliminary partial purification of the cell lysates. The results indicated that at least some of the intracellular labeled enzyme protein exists in a form similar to or identical with the extracellular proenzyme species (Fig. 6). Since we have not yet been able to achieve direct quantitative precipitation of the intracellular enzyme protein, however, we cannot be certain whether the two proenzyme species represent the direct products of translation or instead are representative of substantial post-translational modifications of either one or two initial gene products. In addition, it is possible that human skin collagenase may be synthesized in a pre-proenzyme form. The answers to these questions probably will require definition in a system of cell-free synthesis, but should be possible to approach by selecting, as a source for mRNA, a cell line in which 1% or more of the total proteins synthesized represent collagenase.

On the basis of the finding that latent collagenase can be activated by organic mercurials and chaotropic agents, it has been postulated that the latency observed in some of the collagenases is the result, not of the synthesis of a proenzyme, but rather represents the interaction of active enzyme with a low molecular weight inhibitor (53–55). Although it is possible that such a mechanism may be operative in some systems, our results with human skin collagenase indicate that there is intracellular enzyme produced which is electrophoretically identical to the extracellular purified latent enzyme species (Fig. 6), strongly supporting the suggestion that human skin collagenase is synthesized as a true proenzyme.

The synthesis and secretion data indicate that collagenase is a major extracellular enzyme product of normal human skin fibroblasts. Indeed, since collagenase represented as much as 1% of the total leucine-labeled protein in the cultured fibroblasts, synthesis of this enzyme appears to be a major function of these cells. Because little intracellular storage of collagenase occurred (Fig. 7), the enzyme represented an even greater percentage of the extracellular proteins and comprised 3 to 6% of the total labeled extracellular proteins under serum-free conditions. It is noteworthy again that this value correlates well with the amounts of collagenase obtained from fibroblast cultures using either quantitative purification of the enzyme (15) or measurement of enzyme protein by radioimmunoassay (45).

There is sufficient sensitivity in our immunoprecipitation system to estimate that in normal fibroblast cultures the time required for equilibration of the label and synthesis of enzyme protein is approximately 15 min (Fig. 8). Secretion of the enzyme was easily detected at 30 min and, once secreted, the collagenase is remarkably stable in the culture medium, at least as determined immunochemically (Table II). This finding is reflective of that seen previously when 125I-labeled purified human skin collagenase was added to conditioned fibroblast culture medium (56) and suggests that specific degradation of collagenase in these cultures is quite low. Thus,

Stricklin and his associates (42) using quantitative amounts of the pure enzymes. It should also be noted that under our biosynthetic conditions the relative quantities of the two proenzyme species varied, ranging from an approximately equal amount to a 2- to 3-fold predominance of the 53,000-dalton proenzyme form (Figs. 3, 5, 6). Such a finding suggests the possibility that either there was unequal synthesis of the two species or that the larger form was converted to the lower molecular weight form.
biosynthesis of collagenase by human skin fibroblasts

quantitation of the extracellular collagenase, which is largely representative of the state of collagenase synthesis in any given circumstance, should permit us to study a variety of normal biosynthetic parameters, such as modulation by steroid hormones (23). Nevertheless, determining the intracellular enzyme content is essential for a complete understanding of collagenase biosynthesis, particularly in disease (8–10).

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