# Use of a Mixture of Proteinase-Free Collagenases for the Specific Assay of Radioactive Collagen in the Presence of Other Proteins*

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ABSTRACT: An assay procedure has been developed in which radioactive collagen may be quantitatively measured even in the presence of large amounts of other proteins. This procedure makes use of bacterial collagenase to cleave the collagen into acid-soluble peptides. Commercially purified collagenase was found to contain at least two noncollagen-degrading proteinase activities, the major one being an SH-containing enzyme and the other containing no essential SH groups. By gel filtration on Sephadex G-200, the latter enzyme was completely removed from the collagenase activity and only trace amounts of the SH-containing enzyme remained. These small amounts of activity could be completely suppressed by the use of *N*-ethylmaleimide. The procedure used to measure proteolysis is relatively simple and rapid. Radioactive protein is precipitated from a homogenate with trichloroacetic acid after degrading aminoacyl-tRNA with RNase; the precipitate is redissolved and digested with collagenase under optimal conditions for 90 min.

Noncollagenous protein is reprecipitated with acid and the radioactivity in peptides derived from collagen which remain in the supernatant solution is measured in a liquid scintillation spectrometer.

Previous methods for specifically measuring collagen have relied on the presence of hydroxyproline in this protein and therefore required hydrolysis of the protein before finally utilizing some method to separate hydroxyproline from other amino acids, particularly proline. This is a rather tedious and time-consuming procedure. Collagenase, an enzyme obtained from the culture filtrate of *Clostridium histolyticum*, possesses a specificity for an amino acid sequence of the type R-Pro-X-Gly-Pro-, cleaving between X and Gly. This sequence is found almost exclusively and with great frequency in collagen (Hanning and Nordwig, 1967). Thus, it seemed possible that this enzyme could be used as an analytical tool for measuring collagen without resorting to prior hydrolysis of the protein and subsequent analysis of hydroxyproline.

Commercial preparations of chromatographically purified collagenase have been used to identify intermediates of collagen synthesis in various systems, and in one instance at least, the enzyme was shown to be contaminated with other proteolytic enzymes (Goldberg and Green, 1967). Collagenase freed of other proteolytic enzymes has been used to identify a ribosomal intermediate in collagen synthesis in which proline residues were not hydroxylated (Gottlieb *et al.*, 1965). Under the conditions of those experiments, however, only 40–50% of the available collagen was digested. In the present studies, a chromatographically purified collagenase preparation from a commercial source was found to contain a large contaminant of nonspecific proteolytic activity and therefore was purified further on Sephadex G-200 gel. Six peaks of collagenase activity were obtained by this treatment and only small amounts of nonspecific proteolytic activity were found in some of the fractions.

This paper describes the use of a mixture of these enzymes to quantitatively measure radioactive collagen in the presence of total chick embryo protein by a simple, rapid procedure.

## Materials and Methods

Chromatographically purified collagenase was purchased from Worthington Biochemical Corp.; protease-free RNase, Type XI-A, bovine tendon collagen, tannic acid, Sephadex G-200 gel, and NEM[1] from Sigma Chemical Corp.; azocoll and Hepes buffer from Calbiochem and L-[U-$^{14}$C]proline and DL-[2,3-$^{3}$H]tryptophan from the New England Nuclear Corp. Fertile hen eggs were purchased from Truslow Farms and incubated at 38° in a humid atmosphere to obtain embryos of various ages.

*Radioactive Protein Substrates.* Radioactive chick embryo protein was prepared from embryos varying in age from 7 to 12 days. [$^{3}$H]Tryptophan-labeled protein was prepared in order to measure digestion of noncollagenous proteins, since collagen contains no detectable tryptophan (Piez, 1965). [$^{14}$C]Proline was used to label collagen proline and hydroxyproline. A 0.10-ml portion of either L-[$^{14}$C]proline (20 $\mu$Ci/ml) or DL-[$^{3}$H]tryptophan (100 or 250 $\mu$Ci per ml) was injected into the air sac of embryonated eggs through a small hole in the shell, the hole was sealed with Scotch tape and the eggs incubated at 38° for varying periods of time. The embryos were removed from the eggs, washed with cold 0.15 M NaCl, blotted dry, weighed, and then homogenized with two volumes of 0.05 M Tris-HCl buffer (pH 7.6). In order to degrade aminoacyl-tRNA, RNase was added to give a final concentration of 20 $\mu$g/ml and the homogenate was incubated at 37° for 5 min. Trichloroacetic acid was added to give a final concentration of 5%, the suspension kept at 0° for 5 min, and the resultant precipitate was collected by centrifugation. The precipitate was suspended in 5% trichloroacetic acid and centrifuged. This washing procedure was repeated twice more. The precipitate was then washed twice with ethanol–ether (3:1, v/v), once with absolute ether, and dried slowly, yielding a grey powder.

*Gel Filtration of Partially Purified Collagenase on Sephadex G-200.* The procedure used was similar to that of Keller and

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[1] Abbreviations used are: NEM, *N*-ethylmaleimide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

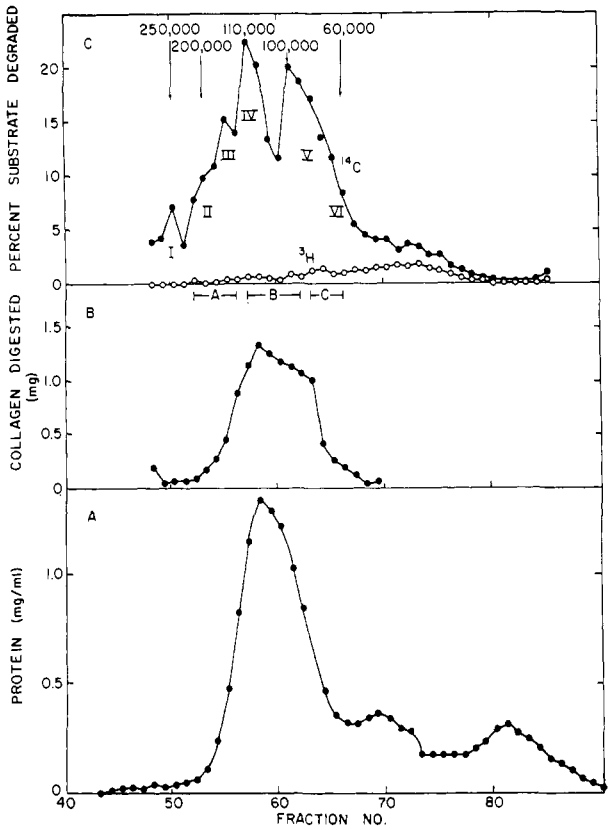


FIGURE 1: Gel filtration of commercially purified collagenase on a Sephadex G-200 column. Conditions are described under Materials and Methods. (A) Protein concentration. (B) Acitivity of 0.10 ml of each fraction for 1 hr against bovine tendon collagen. (C) Activity of 0.07 ml of each fraction against a mixture of 0.5 mg (4132 dpm) of [$^{14}$C]proline-labeled and 0.5 mg (51,537 dpm) of [$^{3}$H]tryptophan-labeled chick embryo protein in a 0.50-ml reaction mixture without NEM as described under Materials and Methods. Samples were counted by double-label techniques and results analyzed for each isotope by a computer program and expressed as the per cent of the substrate degraded in 25 min: (○-○) [$^{3}$H]tryptophan released, (●-●) [$^{14}$C]proline (and [$^{14}$C]hydroxyproline) released. Approximate molecular weights of the areas labeled I, II, IV, V, and VI are indicated at the top of the figure.

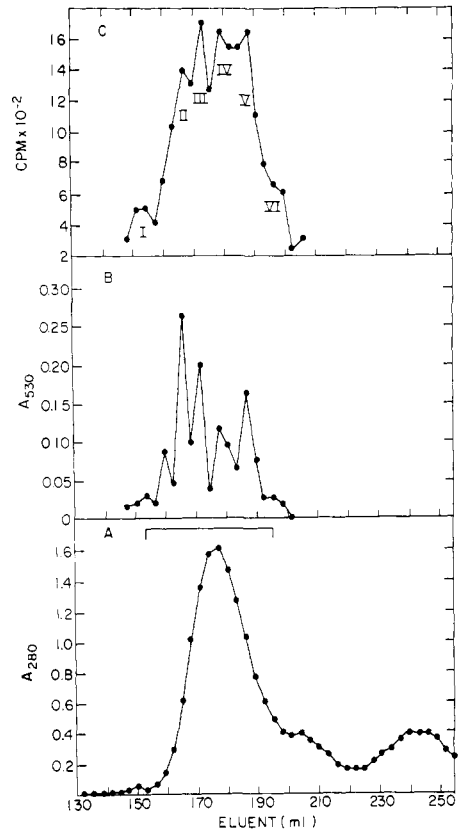


FIGURE 2: Coincidence of activities against azocoll and radioactive chick embryo protein. Commercially purified collagenase (46 mg) was chromatographed on Sephadex G-200 as described under Materials and Methods. (A) Protein concentration expressed as absorbance at 280 nm. (B) Acitivity against azocoll measured as described under Materials and Methods in the presence of NEM. Samples were incubated for 60 min with 10 μl of each fraction. (C) Activity against 1.0 mg (16,527 dpm) [$^{14}$C]proline-labeled chick embryo protein from 10-day-old embryos. Samples were incubated for 30 min with 10 μl of each fraction in the presence of NEM as described under Materials and Methods.

Mandl (1963) except that the elution buffer used was 0.05 M Tris-HCl (pH 7.6), containing 0.005 M $CaCl_2$. A 1.6 × 130 cm column was prepared from Sephadex G-200 which had been equilibrated with the above buffer and this same buffer was run through the column for several days before applying the sample. A 1.0-ml sample containing 30–50 mg of Worthington chromatographically purified collagenase was carefully layered on the top of the gel column and after it was adsorbed, 1 ml of buffer was added. When this had passed into the gel, a 5-ml head of buffer was added and elution started. The flow rate was 6.5 ml/hr and 2.7-ml fractions were collected at room temperature. The protein concentration of the fractions was determined by measuring absorbance at 260 and 280 nm according to the method of Warburg and Christian (1941). Fractions were assayed for proteolytic activities against various substrates as described in the legends to Figures 1 and 2. After initial assays, the fractions from the column described in Figure 1 were combined into three fractions designated A, B, and C. After further studies, these were subsequently combined in a 1:1:1 ratio by volume and this preparation is denoted in the tables as combined enzyme. The fractions from this area are denoted by the bracket in Figure 2. Enzyme fractions which had been stored at −20° retained their original activity for over 1 year.

*Assay of Enzyme Activity against Native Tendon Collagen.* Each tube contained bovine tendon collagen (2.0 mg), Tris-HCl (pH 7.6, 20 μmoles), $CaCl_2$ (0.25 μmole), and enzyme (15–26 μg) in a total volume of 0.50 ml. A control tube contained an equivalent amount of elution buffer in place of enzyme. The tubes were incubated at 37° with shaking and the reaction was stopped by adding 0.50 ml of ice-cold water and immediately placing the tubes in an ice bath. The reaction mixtures were then filtered through a Millipore filter (pore size 0.45 μ) and the filtrates were analyzed for protein using the method of Lowry *et al.* (1951). Specific activity is expressed as micrograms of protein solubilized per minute per milligram of enzyme protein.

*Assay of Enzyme Activity against Azocoll.* Reactions were carried out in 2-ml conical centrifuge tubes. Each tube contained azocoll (2.0 mg), Tris-HCl (pH 7.6, 30 μmoles), NEM (1.25 μmoles), $CaCl_2$ (0.25 μmole), and enzyme (12–26 μg) in a total volume of 0.50 ml. A control tube contained elution buffer in place of enzyme. The reaction was stopped by adding 0.50 ml of ice-cold water and immediately placing the tubes in an ice bath. The tubes were then centrifuged for 5 min at 400*g*, the supernatant solutions removed, and their absorbance

at 530 nm was measured. Specific activity is expressed as $A_{530}$ units per minute per milligram of enzyme protein.

*Assay of Enzyme Activity against Radioactive Chick Embryo Protein.* The dry protein was dissolved in 0.1 N NaOH at a concentration of 2.5–5.0 mg/ml by warming at 37° for 5 min with occasional shaking. If large particles still remained, the suspension was homogenized in a glass tissue homogenizer. The solution was then chilled to 0°. For each 0.5 ml of reaction mixture, 0.20 ml of this substrate solution was pipetted into 2-ml conical centrifuge tubes followed by the addition of 60 μmole of Hepes buffer (pH 7.2). In some of the experiments described, Tris-HCl buffer (pH 7.6) was used and if so, this is indicated in the tables and figures. However, during the course of this study, it was found that digestion proceeded about 10% further in the presence of Hepes buffer. The excess NaOH was partially neutralized by adding 0.20 ml of 0.08 N HCl. The other components of the reaction mixture were NEM (1.25 μmoles; unless otherwise indicated in Figures and tables), $CaCl_2$ (0.25 μmole), and Sephadex G-200 purified collagenase (12–26 μg) or an equivalent volume of elution buffer in the case of no enzyme controls. The tubes were incubated at 37° with shaking. The reaction was stopped by the addition of 0.50 ml of 10% trichloroacetic acid containing 0.5% tannic acid. The use of tannic acid was necessary for maximum precipitation of radioactive protein previously treated with NaOH. In the case of reaction mixtures greater than 0.50 ml, the incubations were carried out in appropriately sized test tubes and 0.50-ml portions were removed and added to 2-ml conical centrifuge tubes containing an equal volume of 10% trichloroacetic–0.5% tannic acid. The tubes were kept at 0° for 5 min and then were centrifuged at 400*g* for 5 min at 4°. The supernatant solutions were transferred to counting vials and the precipitates were resuspended in 0.50 ml of 5% trichloroacetic–0.25% tannic acid and recentrifuged. The resulting supernatant solutions were combined with their respective initial supernatant solutions in the counting vials, 10 ml of Triton–Omnifluor (2:1, v/v) solution was added, and the samples were counted in a liquid scintillation spectrometer. Counting efficiency was 80% for $^{14}C$ and 14% for tritium.

If required, the amount of radioactivity in the remaining precipitates which contain noncollagenous proteins may be determined. The precipitates are resuspended in 1 ml of 5% trichloroacetic–0.25% tannic acid and the suspensions are transferred to counting vials. The tubes are then rinsed with 0.5 ml of the trichloroacetic–tannic acid solution and these suspensions are added to the initial suspensions in the counting vials. The suspensions are completely dissolved in 10 ml of Triton–Omnifluor solution and counted in a liquid scintillation spectrometer.

*Analysis of [$^{14}C$]Proline and [$^{14}C$]Hydroxyproline in Collagenase Digestion Products.* Large-scale incubations containing [$^{14}C$]proline-labeled chick embryo protein were carried out in the presence or absence of G-200 gel-purified collagenase as described above. Duplicate portions containing the equivalent of 1 mg of substrate were removed after 90 min, added to an equal volume of 10% trichloroacetic acid–0.5% tannic acid and the supernatant solutions (peptide-containing fraction) obtained after centrifugation were collected as described above. An equal volume of concentrated HCl was added to the solutions and they were then hydrolyzed for 3 hr at 15 psi, 120°. A 1-mg portion of untreated protein from the NaOH solution used as substrate was also hydrolyzed with 3 ml of 6 N HCl in order to determine the total [$^{14}C$]-hydroxyproline content.

Recoveries of [$^{14}C$]proline and [$^{14}C$]hydroxyproline through all the steps of hydrolysis and analysis were determined. Known amounts of these compounds were added to the supernatant solutions from collagenase digestions carried out using equivalent amounts of unlabeled chick embryo protein or to duplicate samples of untreated radioactive protein.

After hydrolysis, the solutions were treated with Norit charcoal and the charcoal removed by filtration through a Millipore filter. HCl was removed by evaporation under vacuum. The samples were then dissolved in water and put on 2-ml Dowex 50 ($H^+$) columns. Amino acids were eluted with 4 ml of 1.5 N $NH_4OH$. The base was removed by evaporation under vacuum and the residues dissolved in $H_2O$. A small portion of each solution was counted to determine total radioactivity and duplicate portions of the remainder were analyzed for [$^{14}C$]proline and [$^{14}C$]hydroxyproline by a modification of a previously published method (Peterkofsky and Prockop, 1962). The modifications include reducing the volume of the reaction mixture, increasing the concentration of pyrophosphate buffer in the reaction mixture and increasing the ratio of toluene to aqueous phase during the extraction steps. The following components were present in 3.0 ml: sample (1.0 ml), L-proline (3.0 mg), L-hydroxyproline (0.30 mg), sodium pyrophosphate buffer (pH 8, 0.30 mmole), and Chloramine-T (51 μmoles). The reaction was stopped with 0.30 ml of 1 M sodium thiosulfate. The initial toluene extraction was carried out with 6 ml and a 5-ml portion of this was counted ([$^{14}C$]proline product). Three toluene washes were carried out using 10 ml of toluene for each and the blank and final pyrrole-containing fractions were obtained by extracting with 6 ml of toluene and 5-ml portions of these were counted. Recoveries of imino acids were determined using radioactive proline and hydroxyproline standards as described previously (Peterkofsky and Prockop, 1962).

## Results

*Gel Filtration of Collagenase on Sephadex G-200.* Assay of two different batches of commercially purified collagenase preparations against radioactive tryptophan-labeled chick embryo protein indicated that there was a substantial impurity of nonspecific proteolytic activity. In Table I (exp 2) it may be seen that one of these preparations degraded 21.6% of [$^{3}H$]tryptophan-labeled chick embryo protein. The preparation was therefore further purified by gel filtration on a column of Sephadex G-200. Keller and Mandl (1963) and Gottlieb *et al.* (1965) have used this gel to prepare collagenase substantially free of other proteolytic activities. In addition, Grassman *et al.* (1963) have shown that an amidase–esterase activity is separable from collagenase on G-200, its activity overlapping the end of the collagenase region. The amidase–esterase split the synthetic trypsin substrate *N*-α-benzoyl-arginine-β-naphthylamide although it was not sensitive to soybean trypsin inhibitor, and did not hydrolyze casein, hemoglobin, serum albumin, or ovalbumin. It was activated by cysteine and sensitive to reagents which bind to SH groups, such as *p*-mercuribenzoate, iodoacetate, and NEM (Nordwig and Strauch, 1963a).

Upon assaying fractions of the column against [$^{14}C$]proline-labeled chick embryo protein (Figure 1C), it was observed that the collagenase apparently had been fractionated into at least six activities. Assaying against tendon collagen (Figure 1B) also indicated that the preparation had been resolved into several activities and the results suggested that the

TABLE I: Effect of NEM on Noncollagen-Specific Proteolytic Activity in Various Collagenase Preparations.

| Expt[a] | Fraction | NEM Concn (mM) | [$^3$H]Trp Released (dpm) | Degradation (%) |
|---|---|---|---|---|
| 1 | A | 0 | 604 | 0.59 |
| | | 0.25 | 0 | 0 |
| | | 0.50 | 0 | 0 |
| | B | 0 | 2,110 | 2.05 |
| | | 0.25 | 144 | 0.14 |
| | | 0.50 | 0 | 0 |
| | C | 0 | 4,170 | 4.57 |
| | | 0.25 | 210 | 0.20 |
| | | 0.50 | 0 | 0 |
| 2 | Unfractionated | 0 | 18,896 | 21.61 |
| | | 0.50 | 3,943 | 4.51 |
| | | 2.50 | 2,892 | 3.30 |
| | Purified | 0 | 5,462 | 6.24 |
| | | 0.50 | 193 | 0.22 |
| | | 2.50 | 0 | 0 |

[a] In expt 1 the following amounts of enzyme, in micrograms were used per 0.5 ml of reaction mixture: A, 12.2; B, 38.8; C, 27.6. [$^3$H]Tryptophan-labeled protein, 1.0 mg, from 10-day-old embryos was used and contained 103,074 dpm. Samples were incubated for 75 min at 37°. In expt 2, 35 μg of unfractionated and 26 μg of purified enzyme were used and 0.50 mg of [$^3$H]tryptophan-labeled protein containing 87,442 dpm. Incubation was carried out for 90 min at 37°. All data are corrected for no enzyme control values.

TABLE II: Specific Activity of Purified Collagenases against Various Substrates.

| Fraction[b] | Substrate[a] Native Tendon Collagen (μg/min per mg) | Embryo Protein (dpm × $10^{-2}$/min per mg) |
|---|---|---|
| 50 (I) | 360 | 286 |
| 53 (II) | 297 | 99 |
| 58 (IV) | 165 | 20 |
| 62 (V) | 210 | 27 |
| 67 (VI) | 69 | 18 |
| Combined | 312 | 42 |

[a] Data are corrected for no enzyme control values in each assay. In the case of the radioactive substrate, this value was 2% of the total radioactivity in the protein which was 17,150 dpm/mg ([$^{14}$C]proline label). [b] These fractions are from the chromatogram described in Figure 1.

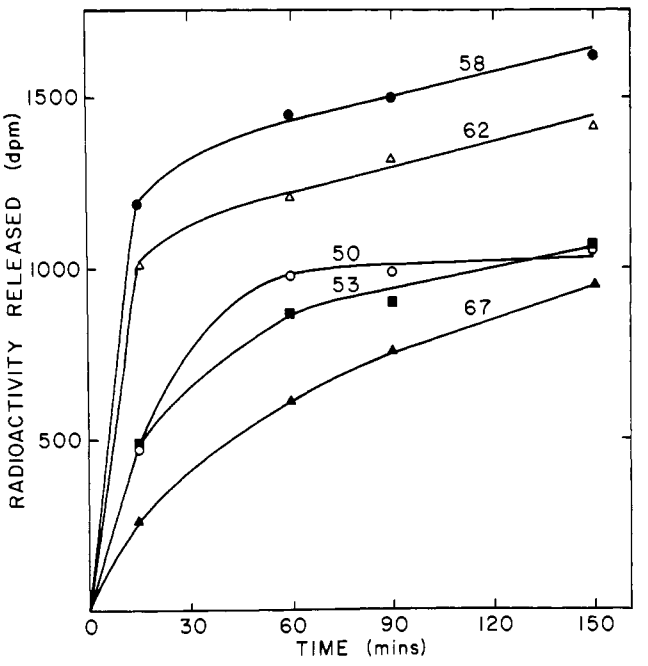


FIGURE 3: Kinetics of digestion of labeled chick embryo collagen by various fractions of collagenase. Incubation was carried out in a total volume of 2.5 ml containing 2.5 mg (42,388 dpm) of [$^{14}$C]-proline-labeled chick embryo protein and the following amounts of each fraction: 50, 5.6 μg; 53, 16.2 μg; 58, 203 μg; 62, 128 μg; 67, 48.2 μg; and 0.50-ml samples were removed at the times indicated in the figure. NEM was included in the reaction mixture and Tris-HCl buffer (pH 7.6) was used. Fractions were from the column described in Figure 1. The per cent digestion at the final time points were 60.3, 62.5, 95.0, 81.8, and 55.5 for fractions 50–67, respectively. These values were calculated on the basis of the theoretical amount of [$^{14}$C]proline and [$^{14}$C]hydroxyproline present in collagen in the radioactive protein mixture. This figure was determined by analyzing the protein for [$^{14}$C]hydroxyproline content as described in Materials and Methods and then mutliplying the value obtained by 1.14 to derive the amount of [$^{14}$C]proline in the collagen. The theoretical amount of [$^{14}$C]proline plus [$^{14}$C]hydroxyproline calculated to be present in the collagenase digests if there were 100% digestion was 1712 dpm/0.5 mg of protein.

enzymes in peaks I and II were more active against the radioactive collagen, which is probably denatured, than against native tendon collagen.

The nonspecific proteolytic activity detected with [$^3$H]-tryptophan-labeled chick embryo protein in the absence of NEM was present in an area overlapping the tail end of the collagenase-containing fractions (fraction C). On the basis of its chromatographic behavior and the finding that it could be completely inhibited by 0.50 mM NEM (Table I, expt 1), it was concluded that this activity was due to the amidase–esterase described above.

Further evidence that the major contaminating enzyme is the amidase–esterase, is the fact that the tryptophan-releasing activity in the unfractionated preparation was found to be almost completely heat stable at 60° for 10 min while the collagenase activity was relatively unstable. Nordwig and Strauch (1963b) found that the amidase–esterase was much more stable than either collagenase or another casein-splitting protease.

Not all of the noncollagen-specific proteolytic activity in the unfractionated preparation could be inhibited even by concentrations of NEM as high as 2.5 mM (Table I, expt 2). This suggests that there is yet a second type of proteolytic activity in the unfractionated preparation which is separated away from the collagenase activity during gel filtration. Since addition of NEM to reaction mixtures could completely suppress the amidase–esterase activity still present in some fractions, 2.5 mM of the inhibitor was routinely added to reaction mixtures to further increase the specificity of col-

TABLE III: Analysis of [$^{14}$C]Proline and [$^{14}$C]Hydroxyproline in Collagenase Digestion Products.

| Age of Embryo (Days)[a] | Total Radioactivity (dpm) (A) | Total Hydroxyproline (dpm) (B) | Radioactivity in Peptide Fraction (dpm) | | Digestion (D/B × 100), % | Pro/OH-Pro (C/D), % |
|---|---|---|---|---|---|---|
| | | | Proline[b] (C) | Hydroxyproline (D) | | |
| 7 | 24,919 | 1559 | 1377 | 1323 | 84.9 | 1.04 |
| 8 | 15,857 | 1690 | 1608 | 1467 | 86.8 | 1.09 |
| 9 | 55,774 | 3040 | 3210 | 2675 | 88.0 | 1.20 |
| 12 | 10,908 | 1791 | 2182 | 1951 | 109.0 | 1.12 |
| | | | | Average | 92.2 | 1.14 |

[a] Embryos were injected with 2 μCi of L-[$^{14}$C]proline and substrates prepared as described in Materials and Methods. The labeling periods used were as follows (in hours): 2, 2.5, 18, and 4 for the 7-, 8-, 9-, and 12-day-old embryos, respectively. A 1-mg sample was analyzed in each case. [b] These values have been corrected for the radioactivity from [$^{14}$C]proline in the no enzyme control sample in each case. No [$^{14}$C]hydroxyproline was found in these control samples. The no enzyme control values were 3.1, 1.4, 2.1, and 3.4% of the total radioactivity in the proteins in the order listed.

lagenase action. NEM did not inhibit the activity of collagenase when assayed against tendon collagen.

Peak fractions from five different areas of collagenase activity initially detected using [$^{14}$C]proline-labeled chick embryo protein as substrate in the absence of NEM were reassayed against native tendon collagen and radioactive embryo protein in the presence of the SH reagent. The specific activities of these fractions calculated from initial rates are compared in Table II. As already suggested by the results of the experiment in Figure 1, fractions 50 (peak I) and 53 (peak II) are relatively more active against the denatured substrate. The experiment shown in Figure 2 indicates that the six activities found using radioactive embryo collagen as substrate are coincident with six peaks of activity found using azocoll as substrate.

Although the enzymes in peaks I and II have the highest specific activities based on initial rates, they do not completely digest collagen. Figure 3 illustrates the kinetics of each of the peak fractions tested against [$^{14}$C]proline-labeled protein. Incubation with fractions 58 (peak IV) and 62 (peak V) resulted in almost complete digestion while digestion with fraction 50 (peak I) stopped at approximately 60%. Fraction 53 (peak II) also produced about 60% digestion. For routine

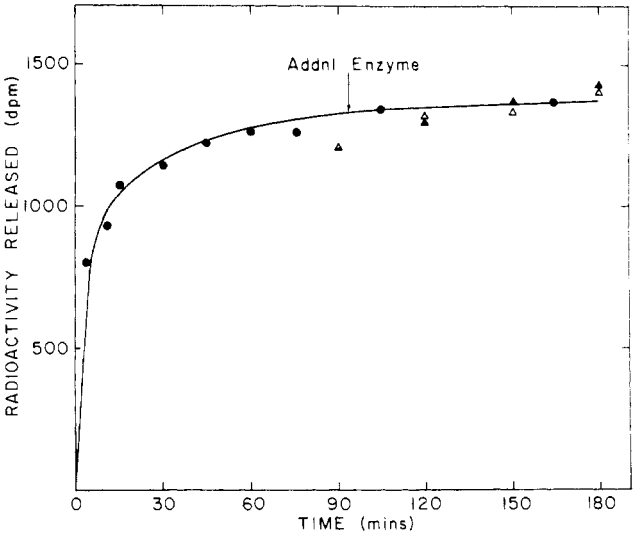


FIGURE 4: Kinetics of digestion of labeled chick embryo collagen by purified combined collagenase. In expt 1 (●-●), incubation was carried out in a total volume of 7 ml containing 367 μg of combined collagenase and 0.50-ml samples were removed at the times indicated. In expt 2, duplicate incubations were carried out in a total volume of 2.5 ml containing 131 μg of combined collagenase and at 90 min 0.50-ml samples were removed and 0.20 ml of Tris–$CaCl_2$ buffer was added to one (△-△) and 0.20 ml of collagenase (105 μg) was added to the other (▲-▲). Incubation was continued and 0.50-ml samples were removed at the times indicated. NEM was included in the reaction mixtures. Tris-HCl buffer (pH 7.6) was used.

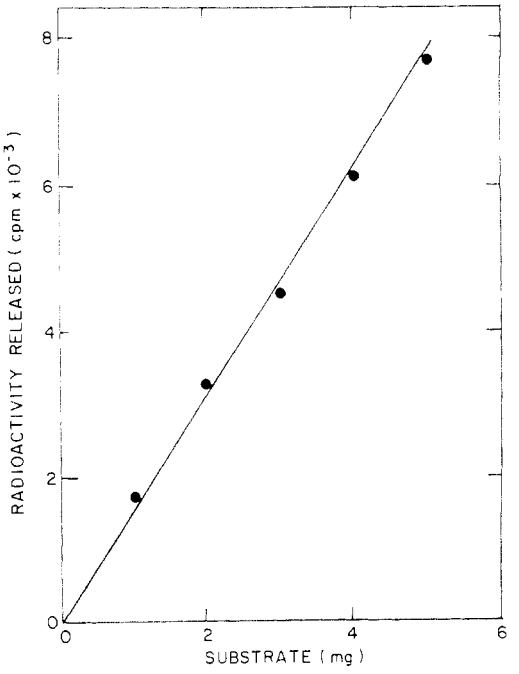


FIGURE 5: Digestion of varying amounts of [$^{14}$C]proline-labeled chick embryo collagen by purified collagenase. Incubations were carried out for 90 min in a total volume of 0.50 ml containing the amounts of [$^{14}$C]proline-labeled chick embryo protein indicated in the figure (7390 dpm/mg), 12 μg of combined collagenase, and 2.5 mM NEM.

assay of labeled collagen, fractions were combined to include peaks II–VI as shown in Figures 1 and 2. Figure 4 shows the kinetics with 52 μg/ml of combined enzyme. Maximal digestion was reached at approximately 90 min and the further addition of enzyme after 90 min did not increase the extent of digestion.

The extent of digestion of the [$^{14}$C]proline-labeled protein was determined by analyzing the solubilized peptides and untreated protein for [$^{14}$C]hydroxyproline after acid hydrolysis. Table III lists the results of analyses performed on four different preparations of substrate. The average extent of digestion based on the amount of [$^{14}$C]hydroxyproline found in the solubilized peptide fraction (D) compared to the total [$^{14}$C]hydroxyproline content of the substrate (B) was 92%. The data also show that the average ratio of [$^{14}$C]proline to [$^{14}$C]hydroxyproline in the peptide fraction is 1.14 which is almost identical with the values of 1.24 for total chick embryo collagen (Gottlieb *et al.*, 1965) and 1.11 for chick skin collagen (Kang *et al.*, 1969). This provides further evidence for the specificity of the collagenase preparation under the conditions used here.

The assay for collagen using purified collagenase is proportional to substrate concentrations up to at least 5 mg of protein (105 μg of collagen). In the experiment illustrated in Figure 5, several concentrations of [$^{14}$C]proline-labeled chick embryo protein were digested for 90 min with a constant amount of enzyme (combined).

## Discussion

In order to use collagenase as a reagent to measure the collagen present in a mixture of proteins, it is essential that there should be no contaminating proteolytic activity. The criterion used here to denote the presence of noncollagen-degrading enzymes was the release of radioactive tryptophan-labeled peptides from chick embryo protein. By this criterion, two different batches of commercially purified collagenase were found to contain at least two contaminating proteolytic activities. The major contaminant is probably the amidase–esterase activity studied by Nordwig and Strauch (1963a) and Grassman *et al.* (1963) who found the enzyme to be sensitive to SH group inhibiting reagents and close to but separable from collagenase on Sephadex G-200. This enzyme is inactive against many proteins commonly used to test the specificity of collagenase preparations, such as serum albumin and casein. About 80% of the nonspecific proteolytic activity in the commercially purified collagenase could be inhibited by 0.5 mM NEM while 15% was resistant to inhibition even at 2.5 mM NEM. A second enzyme present in crude collagenase and studied by this same group of investigators is one which contains an SS group and is active against casein. It is probable that the NEM-insensitive activity remaining in the commercial preparation is due to this enzyme.

Gel filtration of the commercially purified collagenase on Sephadex G-200 led to the separation of what appear to be six different collagenase activities. There have been several reports describing two or three different collagenase activities present in crude *Clostridium histolyticum* collagenase preparations (Grant and Alburn, 1959; Mandl *et al.*, 1964; Harper *et al.*, 1965; Yoshida and Noda, 1965). Most recently, Kono (1968) has reported the extensive purification of three such enzymes. The activities in peaks I and II exhibited a preference toward denatured collagen as substrate but digested only 60% of this substrate. It is possible that these enzymes hydrolyze only a limited number of peptide bonds, resulting in the production of very large peptides which are not soluble in 5% trichloroacetic acid–0.25% tannic acid. Limited hydrolysis may also be the reason why these enzymes appear to be relatively inactive against native collagen.

A small amount of amidase–esterase activity remained in the region of peaks V and VI. This activity, however, could be completely inhibited by including 2.5 mM NEM in the reaction mixture, so that the assay system measures only the reaction products of collagenase acting on collagen.

Using approximately 10–30 μg of the pooled peaks II–VI, an average of 92% digestion of collagen in the mixture of embryo proteins could be obtained in 90 min. This value is much higher than the 40–50% digestion obtained by Gottlieb *et al.* (1965) who also used a Sephadex G-200 treated enzyme preparation. In their studies, gelatinized collagen was used as substrate and it is possible that the enzymes which give incomplete digestion predominated in their enzyme preparation.

In the assay system described here, it was possible to measure specifically radioactive collagen of which the combined proline and hydroxyproline radioactivity comprised approximately 10–40% of the total labeled chick embryo protein. The average blank value of the assay, that is radioactivity in untreated substrate which is not precipitated by the trichloroacetic acid–tannic acid mixture, is approximately 2.5% of the total radioactivity in the protein. If significance is attached only to values greater than 50% of the blank value, then collagen of which the proline plus hydroxyproline radioactivity comprise as little as 2% of the total radioactivity in the protein can be measured accurately.

This assay procedure should be applicable in any case where tissue can be labeled with a radioactive amino acid, such as cell culture, tissue slices, or as used in these studies, intact animals. Because of its sensitivity, it should be particularly well suited for studying collagen synthesis in small samples of tissue such as skin biopsies.

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# Transfer Ribonucleic Acid Deficient in $N^6$-($\Delta^2$-Isopentenyl)adenosine Due to Mevalonic Acid Limitation*

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ABSTRACT: Mevalonic acid, a necessary growth factor for *Lactobacillus acidophilus* 4963, serves as a precursor of the isopentenyl group of the $N^6$-($\Delta^2$-isopentenyl)adenosine in the transfer ribonucleic acid of this organism. At certain concentrations of mevalonic acid, the requirement of mevalonic acid for growth could be uncoupled from the requirement of mevalonic acid as precursor to $N^6$-($\Delta^2$-isopentenyl)adenosine in tRNA. It was possible to select a concentration of mevalonic acid that fulfilled the requirement for maximum growth but satisfied only 50% of the requirement for maximum $N^6$-($\Delta^2$-isopentenyl)adenosine formation. In this way we prepared tRNA that was 50% deficient in $N^6$-($\Delta^2$-isopentenyl)-adenosine and compared its biological properties to those of fully modified tRNA. It was found that the species of tRNA that normally contain $N^6$-($\Delta^2$-isopentenyl)adenosine elute from reversed-phase columns later than most species of tRNA, but that their chromatographic properties are not changed by $N^6$-($\Delta^2$-isopentenyl)adenosine deficiency. Further, $N^6$-($\Delta^2$-isopentenyl)adenosine deficiency of tRNA does not change the isoacceptor pattern revealed by column chromatography of aminoacylated tRNA. Comparison of $N^6$-($\Delta^2$-isopentenyl)-adenosine-deficient and $N^6$-($\Delta^2$-isopentenyl)adenosine-saturated tRNA showed that they were indistinguishable in aminoacylation as well as in *in vitro* protein synthesis.

The function of the many minor nucleosides found in tRNA has remained elusive. Comparisons of the activity of undermethylated tRNA with normal tRNA from a relaxed strain of *Escherichia coli* have shown some differences with respect to aminoacylation and codon responses (Peterkofsky, 1964; Fleissner, 1967; Capra and Peterkofsky, 1968; Shugart *et al.*, 1968; Stern *et al.*, 1970). Most tRNAs contain more than one methylated nucleoside, thus the degree of undermethylation and the location of the unmethylated nucleosides might vary from one tRNA species to another, making it difficult to detect differences in activity.

In order to determine the function of a given minor nucleoside, it would be preferable to investigate one whose location was known to be the same in all the tRNA molecules in which it is found. Such a nucleoside is iPA,[1] which has been shown to be adjacent to the 3′ end of the anticodon of the tRNAs specific for serine and tyrosine (Zachau *et al.*, 1966; Madison *et al.*, 1967). *Lactobacillus acidophilus* 4963 is a mevalonic acid requiring mutant, whose tRNA can conveniently be labeled in the isopentenyl group of iPA by growing the cells in medium containing labeled mevalonic acid (Peterkofsky, 1968). In this study, we found that the content of iPA in tRNA was dependent on the level of mevalonic acid in the medium. We used tRNA isolated from this organism grown at different mevalonic acid concentrations to study the function of iPA. A study of the aminoacylation, codon recognition, and chromatographic properties of iPA-deficient and fully modified tRNA showed no detectable differences between the two types of tRNA.

## Materials

2-[$^{14}$C]DL-Mevalonic acid (DBED salt) (11.8 mCi/mmole) was purchased from New England Nuclear as were [$^{14}$C]-phenylalanine (409 mCi/mmole), [$^{14}$C]tyrosine (370 mCi/mmole, [$^{3}$H]tyrosine (8.3 Ci/mmole), [$^{14}$C]serine (125 mCi/mmole), [$^{14}$C]leucine (260 mCi/mmole), [$^{3}$H]leucine (59 Ci/mmole), [$^{14}$C]cystine (264 mCi/mmole), [$^{14}$C]valine (208 mCi/mmole), and [$^{14}$C]lysine (225 mCi/mmole). [$^{3}$H]Tryptophan (2.2 Ci/mmole) was purchased from Schwarz BioResearch. Sephadex LH-20 was a product of Pharmacia. Bacterial alkaline phosphatase was purchased from Worthington. Poly(U) was a product of Miles Laboratories, poly(UG) was prepared by the method of Basilio and Ochoa (1963) as modified by Capra and Peterkofsky (1968). Poly(UA) and poly(UC) were generous gifts of Dr. Marshall Nirenberg as was the *E. coli* S-30 material. iPA and msiPA were generous gifts of Dr. Nelson J. Leonard.

## Methods

Preparation of [$^{14}$C]iPA-tRNA from *L. acidophilus* 4963 was as previously described (Peterkofsky, 1968) except that

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[1] Abbreviations used are: iPA, 6-(3-methyl-2-butenylamino)-9β-D-ribofuranosylpurine; msiPA, 6-(3-methyl-2-butenylamino)-2-methylthio-9β-D-ribofuranosylpurine.