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SOLID-PHASE RADIOIMMUNOASSAY OF RAT SKIN COLLAGENASE

COMPARATIVE STUDIES AND IN VIVO MEASUREMENT OF THE ENZYME

ARTHUR Z. EISEN, JAMES NEPUTE, GEORGE P. STRICKLIN, EUGENE A. BAUER and JOHN J. JEFFREY

Division of Dermatology, Department of Medicine, Washington University School of Medicine, St. Louis, Mo. 63110 (U.S.A.)

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SUMMARY

The solid-phase antibody method has been employed to develop a sensitive radioimmunoassay for the measurement of collagenase in rat skin. The solid-phase radioimmunoassay, in which approximately 1.0 μg of functionally monospecific anti-collagenase antibody globulin is bound to polypropylene tubes, determines 0.3–20 ng of enzyme. This assay permits the measurement of collagenase either in crude tissue extracts of rat skin, which contain no detectable enzyme activity, or in enzymatically active preparations obtained from tissue culture medium. The immunoassay is at least 1000 times more sensitive than the enzymatic assay.

The similarities between rat skin and rat uterus collagenases, as well as collagenases from a variety of other animal sources, were evaluated by assessing their relative binding capabilities in the solid-phase radioimmunoassay. Rat uterus collagenase was found to be identical in its cross-reactivity with rat skin collagenase in the immunoassay indicating that these rat collagenases are structurally closely related. Of particular interest was the observation that the cross-reactivity between the collagenase from mouse skin and the rat skin collagenase was one of complete immunologic identity although this was not the case for the skin collagenase from another rodent, the guinea pig. This indicates that the solid-phase radioimmunoassay can be used to measure collagenase levels in mouse skin and perhaps other mouse tissues as well. Collagenases from phylogenetically more distant species cross-react much less strongly, suggesting the existence of fewer similarities to the rat enzymes.

Quantitative determination of collagenase in rat skin using the solid-phase radioimmunoassay indicates that the enzyme content reaches a maximum during the first 24 h after birth and by one week of age has declined to a level found in the mature animal.

INTRODUCTION

Assessment of the significance of collagenase (EC 3.4.24.3) in the in vivo remodeling of collagen in vertebrate tissues has been hampered largely by the inability

to readily detect enzymatic activity in tissue extracts [1, 2]. The difficulties encountered in measuring enzyme directly in tissue have been attributed to the presence of enzyme inhibitors [2–5], to the existence of possible inactive forms of the enzyme [6, 7] as well as to the high affinity of the enzyme for its substrate [8, 9].

Under some conditions, however, enzymatically active forms of both human and animal collagenases have been detected in tissue extracts [2, 10–12], but the methods employed are laborious and the recovery of active collagenase is difficult to determine. To overcome these problems, we have used immunologic methods. For example, the use of functionally monospecific antisera against collagenases purified from tissue culture medium has made it possible to demonstrate the presence of collagenase in extracts of human and certain other vertebrate tissues [2, 10, 13] and to develop a specific radioimmunoassay for human skin collagenase, using a double antibody technique [13]. Since human synovial, gingival and granulocyte collagenases are identical in their cross reactivity with human skin collagenase [1, 13], the immunoassay has provided a quantitative method that is independent of enzymatic activity for measuring collagenase in vivo in a variety of human tissues. In addition, immunocytochemical techniques have now been employed to identify the fibroblast as the primary cell of origin of collagenase in human skin [14].

The demonstration of a specific collagenase in the medium from tissue cultures of rat skin [15], suggested that the use of immunologic techniques could also be useful in the in vivo detection of collagenase in an animal system. It also seemed likely that such an approach might not only apply to rat skin but also to the post-partum rat uterus, in which collagenase production is under hormonal control [16, 17]. This would indeed be so if the collagenases from various rat tissues were found to be immunologically identical, as is the case for human collagenases [13].

The present report describes a quantitative assay for collagenase in extracts of rat skin, by a highly sensitive, solid-phase radioimmunoassay. The solid-phase system [18], which has not been employed previously in the assay of enzymes, eliminates the need for a second antibody, is rapid and can also be used to quantitate collagenase from other rat tissues.

METHODS

Enzyme preparation

Rat skin collagenase was obtained from tissue culture medium and purified by the method of Tokoro et al. [15] with the addition of isoelectric focusing [19] as a final step in the procedure. Complete details of the purification and properties of rat skin collagenase will be presented in a subsequent communication. The electrophoretically pure collagenase obtained was used for iodination and as the unlabeled antigen in the radioimmunoassay.

Human, mouse and guinea pig skin, rat uterus and tadpole tailfin collagenases were harvested from tissue culture using techniques previously described [15–17]. Purified bacterial collagenase was obtained from Worthington Biochemical.

Preparation of antiserum

Functionally monospecific antiserum to rat skin collagenase was prepared in rabbits as previously described [20]. The antiserum was taken to 33% saturation with

$(NH_4)_2SO_4$ at 0 °C and pH 7 to obtain the γ-globulin fraction. The γ-globulin was dissolved in 0.05 M Tris–HCl (pH 7.4) containing 0.15 M NaCl and adjusted to a concentration of 35 mg of protein per ml.

Immunologic procedures

Microimmunoelectrophoresis was performed on 0.8% Ionagar (Colab) buffered with 0.02 M Tris–HCl. (pH 7.4). Enzyme preparations were electrophoresed at 4 °C for 90 min at 5.6 V/cm in the same buffer [20]. Following electrophoresis the enzyme was allowed to react with anti-rat skin collagenase antiserum at 25 °C for 72 h.

Quantitative precipitin reactions were carried out by the method of Bauer et al. [10, 20].

Radioiodination

Purified rat skin collagenase was iodinated and separated from free ^{125}I by the method of Bauer et al. [13]. The specific activity of the ^{125}I-labeled collagenase was approximately 1.2 Ci/g of protein. Following gel filtration, the labeled enzyme was diluted to the desired concentration of radioactivity with 1% buffered serum albumin (Sigma Chemical Co.) and stored in small aliquots at −20 °C until used. The iodinated enzyme was found to be relatively stable with a loss of less than 10% of its immunoprecipitability after 3 weeks at −20 °C.

After storage of the radioiodinated enzyme for more than 14–21 days, the ^{125}I-containing peptides having altered immunoreactivity were removed from the original preparation by gel filtration on a column (2 cm × 30 cm) of Sephadex G-150. This procedure for purifying ^{125}I-labeled proteins has been used to enhance the binding specificity in radioimmunoassays of human skin collagenases [13] and growth hormone [21].

Solid phase radioimmunoassay

For adsorption to the solid phase 1 μg of anti-rat skin collagenase gamma globulin in a volume of 300 μl was added to the bottom of a series of 12 mm × 75 mm polypropylene tubes (Falcon Plastics) and incubated at 25 °C for 16–24 h. The tubes were then rinsed 4 times with a total of 5 ml of 0.01 M Tris–HCl (pH 7.5), inverted to dry and stored at 4 °C until used. Storage usually did not exceed one week.

The radioimmunoassay was performed in triplicate in all cases. The standard inhibition curve was obtained by adding the following to a series of antibody coated tubes: 100 μg of 1% bovine serum albumin, 100 μl of purified unlabeled rat skin collagenase in various concentrations and 100 μl of ^{125}I-labeled rat skin collagenase (ranging from 10 000 to 20 000 cpm). The tubes were incubated for 24 h at room temperature, then washed 5 times with 1-ml aliquots of 0.01 M Tris–HCl (pH 7.5) and counted in a single channel automatic gamma scintillation counter (Nuclear Chicago). Unknowns, consisting of crude tissue extracts, or tissue culture medium, were assayed for collagenase in an identical manner using 100-μl aliquots of serial doubling dilutions of the extracts. Controls without antiserum or in which non-immune rabbit γ-globulin was used in place of anti-rat skin collagenase γ-globulin were included in all assays.

The effect of pH on the precipitability of ^{125}I-labeled rat skin collagenase was

assessed over a pH range of 4–9 as previously described for the human skin collagenase radioimmunoassay [13].

Tissue extracts

In adult animals the hair was removed prior to obtaining the tissue by means of a commercial depilatory (Neet, Whitehall Lab). This was more convenient than removing the hair by shaving and had no effect on the enzyme values. To assess collagenase levels in rat skin two specimens, approximately 6 mm in diameter, were excised and the wet weight determined in both pieces of skin. The mean variation in the wet weight between the two skin samples was approximately 5%. One specimen was used for the determination of dry weight after drying for 48 h in an Abderhalden pistol at 130 °C. The second piece of skin was minced finely with scissors and homogenized in an all glass homogenizer at 0 °C using two separate 1.0-ml aliquots of 0.05 M Tris–HCl (pH 7.4) containing 0.005 M $CaCl_2$. Following centrifugation at 27 000 × *g* the supernatant fractions from the two sequential extractions were pooled and the pellet resuspended in 1.0 ml of the same buffer. The resuspended tissue pellet was then sonicated for 30 s at 30 decibels using a Biosonic III sonicator (Bronwell Scientific) equipped with a microprobe. Following centrifugation the supernatant was combined with the initial two fractions and 150 μl assayed directly for collagenase activity on [^{14}C]glycine-labeled, native, reconstituted collagen fibrils for 16 h at 37 °C as previously described [13]. In the reaction mixture 50 μl of the collagen gel substrate used contained approximately 4800 cpm. In every case a trypsin control containing 10 μg of trypsin represented less than 10% of the total counts in the substrate gel indicating that no appreciable amount of denatured collagen was present. For use in the solid-phase radioimmunoassay, serial doubling dilutions of the combined tissue extracts ranging from 1:125 to 1:32 000 were prepared in 1% bovine serum albumin. The immunoreactivity of these crude tissue extracts was stable for at least 4 weeks at −20 °C.

Enzyme recovery was determined directly by the addition of known amounts of pure rat skin collagenase to the combined supernatants from crude tissue extracts and in every case was greater than 98%. In addition, to assess the efficiency of the extraction procedure, the pellet from a 6-mm piece of rat skin was homogenized (as described above) until no further immunologic collagenase could be detected. The pellet was resuspended in a solution containing 40 μg of enzymatically active rat skin collagenase and incubated at 4 °C for 4 h. Approximately 26% of the added enzyme was bound to the collagen in the pellet during the incubation. The pellet was then washed 3 times with ice-cold 0.05 M Tris–HCl (pH 7.5) containing 0.005 M $NaCl_2$ and re-extracted and sonicated as originally described. Immunoassay of the extract indicated that 96.8% of the bound enzyme was recovered, suggesting that the extraction procedure is capable of effectively removing collagenase present in the tissue. This was confirmed by the fact that when the insoluble, collagen containing pellets from tissue extracts were incubated in 0.05 M Tris–HCl, pH 7.5, containing 0.005 M $CaCl_2$ for 16 h, as described by Ryan and Woessner [8], virtually no hydroxyproline was released into the supernatant. Thus, little or no enzyme remains bound to endogenous collagen after extraction.

RESULTS

Immunologic properties

An immunoelectrophoretic comparison of enzymatically active crude collagen-

ase from tissue culture medium and rat skin collagenase purified from the culture medium with an enzymatically inactive crude tissue extract, which contains immunoreactive material, is shown in Fig. 1. Under these conditions the enzyme preparations migrate toward the cathode and give corresponding precipitin arcs when reacted

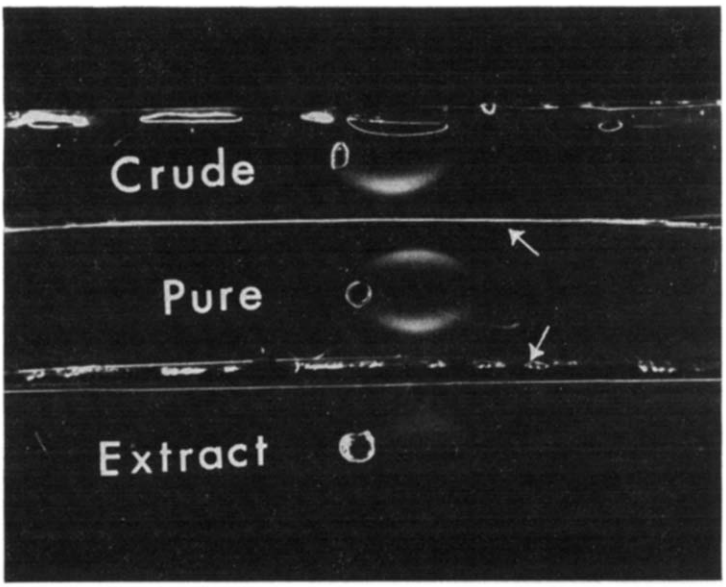

Fig. 1. Comparison of crude and pure rat skin collagenase from culture medium with immunoreactive collagenase from rat skin extracts by immunoelectrophoresis. Electrophoresis was carried out at 5.6 V/cm for 90 min after which the enzymes were reacted with anti-rat skin collagenase (arrows) for 72 h. Cathode is at the right.

with anti-rat skin collagenase antibody. No precipitin bands were seen when the antiserum was reacted with normal whole rat serum under identical conditions. On immunodiffusion, anti-rat skin collagenase antibody gave a single precipitin band with both enzymatically active collagenase and crude extracts of rat skin (not shown), which indicates that rat skin extracts contain material immunologically identical with the enzymatically active collagenase isolated from the medium of cultured rat skin explants.

To determine the specificity of anti-rat skin collagenase antibody for the enzyme, a quantitative precipitin reaction was performed by adding increasing amounts of anti-rat skin collagenase γ-globulin to a constant amount of enzyme. Fig. 2 shows that, after precipitation, collagenase activity in the supernatant decreases with increasing concentration of the functionally monospecific antibody. The fact that the level of maximum protein in the precipitate coincides with the minimum enzyme activity in the supernatant provides additional evidence that the antibody is specifically directed against rat skin collagenase.

Iodination of rat skin collagenase

At the time of iodination 78% of the radioactivity in the ^{125}I-labeled rat skin

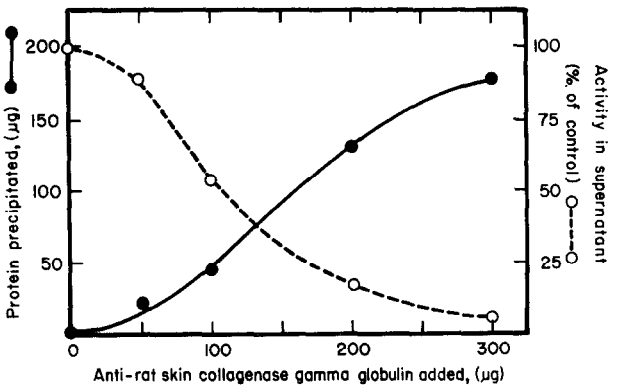

Fig. 2. Quantitative precipitin reaction between rat skin collagenase and anti-rat skin collagenase γ-globulin. Increasing amounts of γ-globulin, at a concentration of 25.0 mg protein per ml, was added to 0.1 ml of a crude enzyme solution from tissue culture medium containing approximately 0.18 mg of protein. Following incubation, the protein content of the precipitates was determined and collagenase activity in the supernatants assayed by incubating 0.2-ml aliquots on ^{14}C-labeled collagen fibrils for 14 h at 37 °C. A control reaction containing 0.18 mg of rat skin enzyme released 1157 cpm in the absence of non-immune rabbit γ-globulin. In the presence of 0.3 ml of non-immune rabbit γ-globulin (25 mg/ml) the same amount of enzyme released 1092 cpm. The substrate collagen gel contained 4800 cpm. ●—●, protein in the precipitate; ○—○, collagenase activity in the supernatant.

collagenase preparation was precipitable with 10% trichloroacetic acid. After 2 weeks at −20 °C the trichloroacetic acid-precipitable material decreased to 71%. ^{125}I-labeled rat skin collagenase was also subjected to gel filtration on a column (2 cm × 30 cm) of Sephadex G-150 at 4 °C at varying lengths of time following iodination, in order to separate ^{125}I-labeled rat skin collagenase having unaltered immunologic properties from radiation damaged ^{125}I-labeled material showing altered immunoreactivity [13]. At 4 days after iodination the ^{125}I-labeled rat skin collagenase specifically bound by anti-rat skin collagenase γ-globulin represented 73% of the total counts in the preparation. At 21 days the immunoprecipitable material decreased to only 64% indicating that little radiation damage to the enzyme had occurred from the ^{125}I. In practice, the radioiodinated enzyme was subjected to gel filtration after storage for 10–14 days to insure the removal from the original preparation of any ^{125}I-containing peptides having altered immunoreactivity.

Titration of anti-rat skin collagenase γ-globulin

To determine the optimum concentration of antiserum for use in the solid phase immunoassay plastic tubes were coated with increasingly dilute solutions of anti-rat skin collagenase γ-globulin. Fig. 3 shows that at low dilutions of anti-rat skin collagenase γ-globulin, maximum binding is approximately 90% and that at antibody dilutions of 1:10 000 to 1:20 000, binding decreased to a plateau of between 50 and 60%. Furthermore, the addition of 35 ng of unlabeled rat skin collagenase caused a significant displacement of bound ^{125}I-labeled rat skin collagenase at dilutions of antibody greater than 1:250. For routine use in the immunoassay, therefore, 300 μl of a 1:10 000 dilution of γ-globulin (approximately 1.0 μg per tube) was used.

The effect of pH on the binding of ^{125}I-labeled rat skin collagenase to antibody

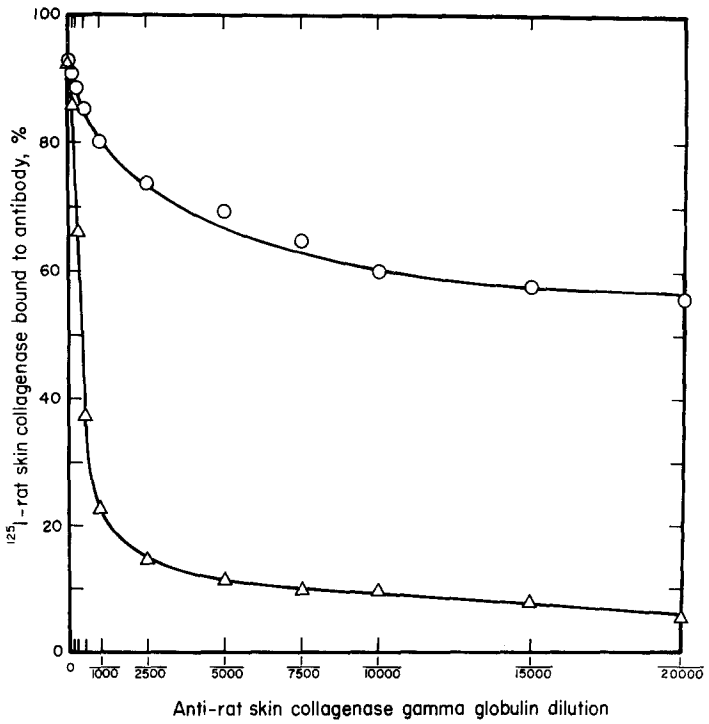

Fig. 3. Precipitation of ^{125}I-labeled rat skin collagenase at various concentrations of anti-rat skin collagenase γ-globulin. Aliquots of 300 μl of the indicated dilutions of anti-rat skin collagenase γ-globulin were bound to polypropylene tubes and subsequently reacted with ^{125}I-labeled rat skin collagenase (21 457 cpm) in the presence or absence of unlabeled purified rat skin collagenase. ○—○, ^{125}I-labeled rat skin collagenase bound to antibody (%); △—△, ^{125}I-labeled rat skin collagenase bound to antibody in the presence of 35 ng of rat skin collagenase (%).

was examined over a pH range of 4–9 using tubes coated with 1.0 μg of antibody. The pH optimum was between 7.0 and 7.4 where 87% of the ^{125}I-labeled rat skin collagenase was bound to antibody. Below pH 6.0 and above pH 8.0 there was a sharp decline in ^{125}I-labeled rat skin collagenase precipitability.

Standard curve

Fig. 4A shows that the addition of increasing amounts of purified unlabeled rat skin collagenase to antibody coated tubes containing a constant amount of anti-rat skin collagenase antibody and ^{125}I-labeled rat skin collagenase under standard assay conditions resulted in the progressive decrease in the amount of radioactivity bound to the tubes. On the linear portion of the curve, the amount of antibody-bound ^{125}I-labeled rat skin collagenase is proportional to the logarithm of the concentration of unlabeled rat skin collagenase added to each tube, providing a working range of approximately 0.3–20 ng for the determination of enzyme concentration.

A standard inhibition curve obtained by using serial dilutions (undiluted = 1 mg of protein per ml) of two different extracts of adult rat skin, and extracts from newborn rat skin or adult rat uterus is shown in Fig. 4B. The slopes of these curves are

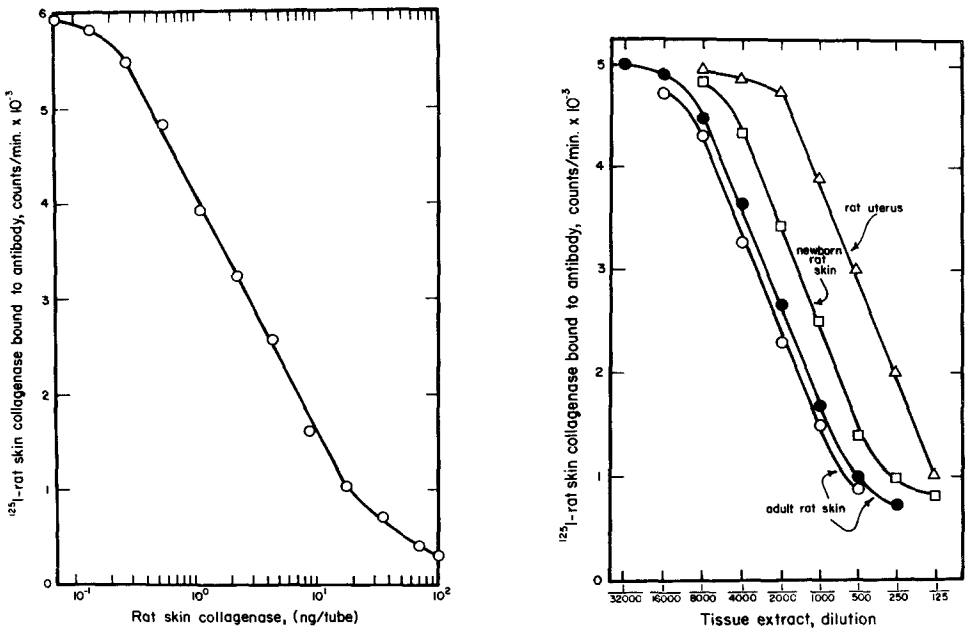

Fig. 4. Standard solid-phase radioimmunoassay curve for rat skin collagenase. Aliquots of 100 μl of pure rat skin collagenase containing from 0 to 100 ng of protein or crude tissue extracts of the indicated dilutions were reacted in the solid-phase radioimmunoassay with 1.0 μg of anti-rat skin collagenase gamma globulin and ^{125}I-labeled rat skin collagenase (14 000 cpm). In control tubes containing an equivalent concentration of non-immune rabbit γ-globulin less than 0.5% of the added radioactivity was bound. A, pure rat skin collagenase; B, crude tissue extracts from 2 different adult rats, from new born rat skin, and from rat uterus.

parallel with the standard curve of the purified enzyme. This indicates that identical antigenic determinants are being measured not only in extracts of newborn and adult rat skin but also in extracts from rat uterus. Thus, levels of immunoreactive collagenase in tissue extracts from rat skin and uterus can be expressed in terms of known quantities of the purified skin enzyme. Rat uterus enzyme obtained from crude tissue culture medium produces a curve identical to that depicted for the tissue extract.

The sensitivity of the solid phase radioimmunoassay was compared to the radioactive collagen fibril assay as previously described [13]. The fibril assay is widely used to quantitate collagenase activity by determining the lysis of ^{14}C-labeled native collagen fibrils at 37 °C [22]. Using preparations of crude rat skin collagenase obtained from tissue culture medium, the minimal amount of the enzyme protein readily detectable by enzymatic assay after a 4-h incubation period at 37 °C was approximately 70 μg. When identical enzyme preparations were serially diluted for use in the radioimmunoassay collagenase was still detectable at 1000- and 1250-fold dilutions respectively in two separate experiments. This indicates that the solid phase radioimmunoassay is at least 1000 times more sensitive in assessing enzyme protein in the same preparations.

Comparative analysis

The extent of immunologic cross-reactivity of collagenases from other animal

sources with rat skin collagenase was determined by assessing the percentage of inhibition of binding in the radioimmunoassay by each unlabeled collagenase relative to the amount of ^{125}I-labeled rat skin collagenase bound to anti-rat skin collagenase antibody in the absence of competing proteins [23, 13]. In these experiments the source of the collagenases was from crude tissue culture medium. Table I shows that collagenases

TABLE I

EFFECT OF VARIOUS COLLAGENASES ON THE IMMUNOPRECIPITATION OF ^{125}I-LABELED RAT SKIN COLLAGENASE

100-μl aliquots of the unlabeled collagenase preparations (0.1 mg/ml) were reacted with ^{125}I-labeled rat skin collagenase (16 200 cpm) and anti-rat rat skin collagenase γ-globulin (1 μg per tube). In the absence of competing unlabeled collagenase 46% of the total labeled rat skin collagenase was bound by anti-rat skin collagenase. The values presented are the percentages by which binding of ^{125}I-labeled rat skin collagenase to antibody were inhibited by unlabeled collagenase. Control tubes coated with non-immune rabbit γ-globulin bound less than 0.5% of the total labeled enzyme.

Unlabeled collagenases	Inhibition of binding (%)
Rat skin (adult)	97.3
Rat skin (newborn)	97.8
Rat uterus	89.9
Mouse skin	85.8
Guinea pig skin	8.8
Tadpole skin (tailfin)	5.1
Human skin	0.1
Clostridium histolyticum	2.6

from adult and newborn rat skin and post-partum rat uterus inhibit the binding of ^{125}I-labeled rat skin collagenase by antibody approximately 90–98% at a protein concentration of 0.1 mg/ml. It is of particular interest that a collagenase from another rodent source, mouse skin, cross-reacts very strongly with the rat enzymes. In contrast, collagenase from still another rodent, the guinea pig, or from amphibian, human and bacterial sources showed little cross reactivity with the rat skin enzyme, inhibiting binding in all instances by considerably less than 10%. Although it was necessary in most cases to use crude enzyme preparations, the findings suggest that fewer structural similarities to the rat skin enzyme will be found when the other enzymes are available in pure form.

From the standard inhibition curve obtained by using serial doubling dilutions of collagenase from mouse skin, it is apparent that the slope of the curve is parallel with the rat skin enzyme (Fig. 5) indicating that these two enzymes are closely related, if not immunologically identical. Comparison with guinea pig skin collagenase, on the other hand, shows that this enzyme is capable of moderate displacement of ^{125}I-labeled rat skin collagenase in the radioimmunoassay but cross reactivity occurs only at enzyme concentrations markedly higher than those used to measure rat skin collagenase (Fig. 5). Human skin collagenase shows only slight displacement of ^{125}I-labeled rat skin collagenase at the highest concentration of the enzyme used in this assay system.

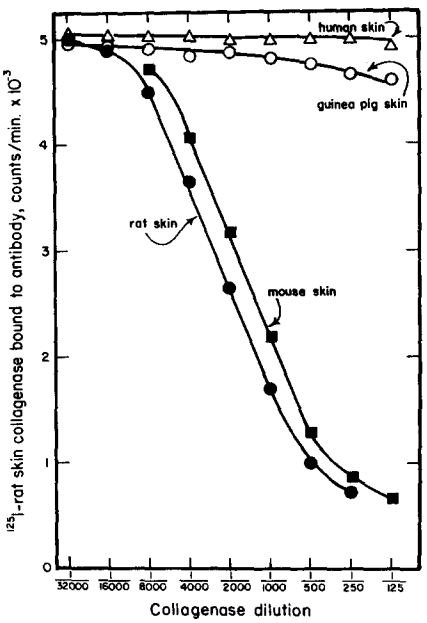

Fig. 5. Comparison of rat skin collagenase with collagenases from mouse, guinea pig and human skin by the solid-phase radioimmunoassay. Reaction conditions as for Fig. 4 except the ^{125}I-labeled rat skin collagenase contained 12 300 cpm. Non-immune rabbit γ-globulin bound less than 0.5% of the total radioactivity.

Collagenase levels in skin extracts

The fresh tissue extracts contained no measurable enzymatic activity as determined by assaying a 150-μl aliquot on ^{14}C-labeled collagen fibrils (4800 cpm per substrate gel) for 18 h at 37 °C. The mean value for lysis of the labeled collagen substrate was 0.8%. No age differences were noted.

Using the solid-phase radioimmunoassay, however, collagenase can be readily quantitated in supernatant fractions of crude extracts of rat skin. The values

TABLE II

COLLAGENASE CONTENT IN RAT SKIN DETERMINED BY SOLID-PHASE RADIOIMMUNOASSAY

Collagenase was determined by solid-phase radioimmunoassay using 100-μl aliquots of serial doubling dilutions of crude tissue extracts as described in Methods.

Age	Collagenase content (μg/mg dry wt)
Newborn*	4.4 ± 0.96**
1 day	9.5 ± 1.23
2 days	3.4 ± 1.67
5 days	2.6 ± 0.64
60 days	2.3 ± 0.09

* Newborn refers to animals 4–8 h of age.
** Mean ± S.D.

obtained by radioimmunoassay for collagenase in the rat skin of rats of varying ages are presented in Table II. The enzyme attained its highest level, 9.5 μg/mg dry weight, at approximately 24 h after parturition. By 48 h, the collagenase content had decreased significantly and by 5 days after birth the enzyme had reached a level of 2.6 μg/mg dry weight which is similar to that found in the skin of mature adult animals.

DISCUSSION

Although the double-antibody radioimmunoassay has been used extensively as a sensitive and specific method for quantitating a variety of protein hormones [24] its application to the assay of enzymes has been limited [25]. In a previous investigation [13] we have shown that human skin collagenase can be readily quantitated using a double antibody radioimmunoassay. This indicated that perhaps a similar approach might be successfully applied to collagenases from other mammalian species in which it is also difficult to detect enzymatic activity in crude tissue extracts [1, 26]. The major advantages of the radioimmunoassay over the enzymatic assay, not only for mammalian collagenase [13] but for other enzymes as well [26], are its high degree of sensitivity, the ability to measure the enzyme in terms of its total protein concentration and, of special importance for the determination of both animal and human collagenases [1–4, 11], the fact that the radioimmunoassay is usually independent of the presence of enzyme inhibitors [13, 25].

The introduction of solid-phase antibody methods as described by Catt and Tregear [18] in which fixed amounts of functionally monospecific antiserum are strongly bound to an unsubstituted polymer (polystyrene) provides a considerable simplification of the double antibody radioimmunoassay. The antibody coated on polystyrene tubes provides a convenient substrate for the binding of radioactively labeled antigen and the measurement by competitive inhibition of unlabeled antigen. This technique has been applied successfully to the assay of peptide hormones [27, 28], a variety of immunoglobulins [29, 30] and Australia antigen [31].

The present study demonstrates that the solid-phase radioimmunoassay provides a sensitive, specific and relatively simple approach to the measurement of rat skin collagenase which requires as little as 1 μg of antiserum globulin. In addition, the solid-phase radioimmunoassay is greater than 1000-fold more sensitive in assessing enzyme protein in the same preparation when compared to the enzymatic assay. Unlike iodinated human skin collagenase [13] iodinated rat skin collagenase does not have an increased sensitivity to radiation damage and, therefore, it is not necessary to purify the ^{125}I-labeled rat skin collagenase by gel filtration every 3–4 days prior to use.

The fact that the slopes of the curves obtained from tissue extracts are parallel with the standard curve of the pure enzyme indicates that identical antigenic determinants are, in fact, being measured. Further evidence for the close similarity of the immunoreactive protein from rat skin extracts and the enzymatically active collagenase obtained from tissue culture medium is demonstrated by their identical mobilities on immunoelectrophoresis (Fig. 1). Thus, the quantitative determination of collagenase from rat skin can be assessed by the solid-phase radioimmunoassay either in crude tissue extracts which contain no detectable enzyme activity or in enzymatically active preparations obtained from tissue culture medium. Furthermore, the identical cross reactivity in the solid-phase radioimmunoassay of the collagenase from rat uterus with

rat skin collagenase establishes the validity of using this method for measuring rat uterus collagenase under both normal and experimental conditions.

If, as is the case for a variety of human and animal collagenases, the inability to detect enzyme activity in crude rat skin extracts is due, at least in part, to known inhibitors of collagenase activity such as the α-globulins [2, 3, 11, 26], the presence of these serum proteins in tissue extracts does not interfere with the solid-phase radioimmunoassay. In addition, since it has been suggested that the collagenases from tadpole fin [6] and mouse bone [7] are present as proenzymes, the possibility exists that at least some of the rat skin enzyme is present in the form of a zymogen in vivo. It is likely, however, on the basis of immunologic investigations of established proenzyme–enzyme systems [25], that cross reactivity between the zymogen and the active enzyme will occur. Thus, the solid-phase radioimmunoassay should permit the quantitation of enzyme protein whether the collagenase is in an inhibited or proenzyme form.

The results obtained in this study confirm and extend our previous observations [20, 13] that immunologic species specificity exists among collagenases and indicates that the rat collagenases are immunologically closely related. When binding measurements were obtained by determining the percentage of inhibition of unlabeled rat skin collagenase relative to the amount of ^{125}I-labeled rat skin collagenase bound to anti-rat skin collagenase antibody (Table I) it was evident that the rat collagenase markedly inhibited the binding of labeled enzyme by antibody. In contrast, collagenases from a variety of other sources, except for the enzyme obtained from mouse skin, inhibited binding by less than 10%. The collagenase from mouse skin, another rodent, shows strong cross reactivity with the rat enzymes. A similar situation has been observed in the radioimmunoassay for human skin collagenase where collagenase from another primate cross reacts strongly with human skin collagenase [13]. A standard inhibition curve (Fig. 5) indicates that the cross reactivity with the mouse skin collagenase is one of complete immunologic identity which is not the case for another rodent, the guinea pig. This suggests that extensive homology to the rat collagenase will be found in collagenases from the mouse in contrast to collagenases from the guinea pig and other phylogenetically more distant species which cross react much less strongly. These other enzymes can be expected to have fewer structural similarities to the rat collagenases when sequence analysis becomes available. It is also evident that the rat solid-phase radioimmunoassay can be used successfully to measure collagenase levels in mouse skin.

By means of the solid-phase radioimmunoassay it has been possible to readily quantitate collagenase in rat skin (Table II). It is of interest that the collagenase content in rat skin reaches a maximum within the first 24 h after birth and by 5 days of age the skin enzyme has decreased significantly, attaining a level similar to that present in adult rat skin. This rapid decline in collagenase concentration occurs at a time when the animals are still rapidly growing and collagen remodeling is presumably still actively in progress. The significance of these observations remains to be determined and will be the subject of further investigations.

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