Biochimica et Biophysica Acta, 309 (1973) 397–405
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

BBA 66899

COLLAGENASE ACTIVITY IN HOMOGENATES OF THE INVOLUTING RAT UTERUS

J. FREDERICK WOESSNER, JR AND JANET N. RYAN

Departments of Biochemistry and Medicine and Program in Cellular and Molecular Biology, University of Miami School of Medicine, Miami, Fla. 33152 (U.S.A.)

(Received November 22nd, 1972)

SUMMARY

Collagenase activity can be detected in the 6000 × *g* pellets of homogenates of the involuting uterus of the rat; it digests the collagen present in the homogenates. This enzyme appears to be identical to the collagenase obtained from cultures of uterine tissue. The enzyme is optimally active at pH 7.5, requires Ca^{2+} (0.01 M) for activity and is inhibited by EDTA and other chelating agents. EDTA inhibition cannot be reversed by adding excess Ca^{2+} or other divalent cations. The activity is inhibited by the tissue supernatant and by rat serum in 1:100 dilution. The enzyme is insensitive to 1 mM cysteine but is inhibited by other reducing agents such as 1 mM mercaptoethanol. The enzyme does not digest the peptide phenylazobenzyloxycarbonyl-Pro–Leu–Gly–Pro–D-Arg which is used for the assay of bacterial collagenase. The enzyme attacks insoluble collagen at 30 °C but does not release digestion products unless the temperature is raised to 37 °C. The digestion products at 37 °C are smaller than 100 000 daltons and about one-third is diffusible.

INTRODUCTION

While collagen is normally thought to be rather inert metabolically due to its extracellular location[1], there are a number of situations in which this fibrous protein can be catabolized with surprising speed. The most rapid rate of collagen breakdown is found in the involuting rat uterus, where the half-life may approach 30 h (ref. 2). This observation has prompted many studies of the uterus in the hope of finding collagenolytic enzymes. However, no one has succeeded in demonstrating collagen digestion by uterine homogenates or extracts, except at quite acid pH values[3–5]. Other workers have looked at other resorbing connective tissues with similar results. However, in 1962 Gross and Lapiere[6] detected collagenase activity in cultures of tissue grown on collagen gels. In this situation collagenolytic enzymes could diffuse away from the tissues and from possible tissue inhibitors. This approach resulted in the first demonstration of an animal collagenase, derived from tadpole tail fins, which could digest native collagen fibers at physiological pH. In the intervening years,

collagenase has been obtained from many different tissues by this method (see review by Eisen *et al.*[7]). Tissue from the involuting rat uterus has also been shown to produce collagenase when grown in culture[6,8]. However, except for two special cases[9,10], no one has succeded until recently in demonstrating collagenase directly in tissue homogenates.

This failure has raised several important questions. Is collagenase actually present in normal tissue *in vivo*, and if it is present *in vivo*, do the levels of activity correspond to the amounts produced in cultures? If it is present what factors prevent its expression and do these factors play a regulatory role?

Recently, two advances have been made in this area. We have shown that collagenase is present in uterine homogenates in the insoluble fraction, possibly bound to the collagen fibers[11,12]. Eisen *et al.*[13], working with homogenates of human skin, have shown that collagenase is present in the supernatant but is inactive until separated from inhibitory substances by chromatography on Sephadex G-150. These reports open the way for studies of the tissue levels of collagenase in various physiological situations. As a prelude to such studies, we would like to present further evidence confirming the collagenase nature of the activity found in uterine homogenates, comparing it to the collagenase found in uterus cultures by Jeffrey and Gross[8], and extending our knowledge of its properties.

The present study takes as its starting point several important findings presented in a previous publication[11]. There it was shown that homogenates of uteri removed 2 days after parturition contained both collagen and collagenase in a 6000 $\times$ *g* pellet. The uterine collagen served as substrate for the enzyme which was either bound to it or to some other sedimentable particle. The evidence that the enzyme was a true collagenase was that it attacked native collagen fibers at 37 °C and pH 7.5, producing digestion products smaller than α chains. About 10% of the uterine collagen may exist in a partly-denatured state since it is digested by trypsin; however, the collagenase activity digested 25% or more of the collagen upon prolonged digestion (40 h). The present paper provides further evidence that the enzyme is a true collagenase and that it is, in fact, indistinguishable from the uterine collagenase described by Jeffrey and Gross[8].

MATERIALS AND METHODS

Collagenase assay procedure

Pregnant female rats were purchased from Sprague–Dawley Farms, Madison, Wisc. They were maintained on Purina rat chow until 2 days after parturition. The uteri were removed, dissected free of mesentery and placental sites, and weighed. The tissue was minced and homogenized in 10 vol. of ice-cold 0.9% NaCl containing 0.05 M calcium acetate. Ten-Broeck all-glass homogenizers (A. H. Thomas Co., Philadelphia, Pa.) were turned by hand until the tissue fragments were dispersed; and then the pestles were attached to a motor for 5 passes through the suspension. The homogenates were centrifuged at 6000 $\times$ *g*, 20 min, 2 °C. The supernatants were discarded. The pellets were suspended in the original volume of 0.9% NaCl (no calcium) and centrifuged as before. The rinsed pellets were then homogenized in 12.5 vol. 0.9% NaCl containing 0.04 M Tris and 0.01 or 0.05 M calcium acetate, pH 7.5. Penicillin (200 units/ml) and streptomycin (250 μg/ml) were added to prevent

bacterial growth. The rehomogenized pellets were distributed to celluloid centrifuge tubes (4 ml/tube), stoppered, and incubated for 20 h, 37 °C in a Dubnoff metabolic shaker. The uterine collagen present in the pellets served as the substrate for the associated collagenase activity; each tube contained about 600 μg hydroxyproline (4 mg collagen). Controls included tubes shaken at 2 °C, tubes with 1 mM EDTA added, and tubes with EDTA *plus* 0.01% trypsin (Worthington Biochemicals, Freehold, N.J.).

After incubation, the final pH was checked in each tube. Bacterial contamination was monitored by the use of blood agar culture plates. The samples were cooled and centrifuged 30 min at 30 000 × g. The supernatants (solubilized and digested collagen) were evaporated on the steam table under air flow. Pellets (insoluble collagen) and dried supernatants were first hydrolyzed for 18 h at 110 °C in 6 M HCl and then assayed for hydroxyproline. Method I of Woessner[14] was used for assay of the pellet. Method II[14] was required for the supernatants due to the high level of soluble non-collagen protein present.

Linearity of the assay

Collagen digestion was expressed as the percentage of the total collagen solubilized after 20 h, 37 °C. A blank correction was made for the solubilization measured at 2 °C (usually less than 0.4%). While the parameter measured was solubilization rather than digestion, it was shown earlier[11] that 90% of the material in the solubilized fraction was smaller than collagen α chains. It was also shown that the release of collagen in this system is linear up to 48–72 h (ref. 11). Doubling or halving the incubation volume while keeping the collagen content constant has only a small effect on the rate of collagen digestion. We interpret this to mean that the collagenase remains in close proximity to its substrate, so that the law of mass action is not applicable to the bulk concentrations. In any event, the amount of collagen in each tube was held fairly constant in the various experiments.

Size of digestion products

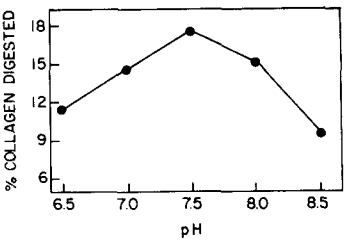
Digests were centrifuged as above, and the supernatants were passed through 0.22-μm Millipore filters to remove any fragments that had been dislodged in decanting. The supernatants were then passed through Diaflow membranes XM-100 and XM-50 (Amicon Corp., Lexington, Mass.). These filters have nominal retention sizes of 100 000 and 50 000 daltons, respectively. The final filtrates were dialyzed in Visking tubing overnight against water. Material passing through each size of membrane was hydrolyzed and assayed for hydroxyproline. Soluble guinea-pig-skin collagen was labeled *in vivo* with [^{3}H]glycine and purified by the method of Gross and Lapiere[6]. This soluble collagen was then heat-denatured and passed through the same membranes to provide a control.

RESULTS

As has been brought out in preliminary publications[11,12], the involuting rat uterus contains an active collagenolytic enzyme and this activity is found in the 6000 × g pellets of uterine homogenates. About 16% of the uterine collagen is digested in 20 h under the present assay conditions when the tissue is collected at

2 days after parturition. The present results are concerned with the detailed characterization of the collagenolytic activity found in the uterine pellets.

Attention was first turned to the pH optimum. If Ca^{2+} is present at a concentration of 0.01–0.05 M, the pH curve is as shown in Fig. 1. The optimum pH is at 7.5 and the activity falls to about one-half if the pH is shifted 1 unit in either direction. With low calcium (1 mM) the digestion is no longer maximal and the pH optimum is shifted close to pH 7.



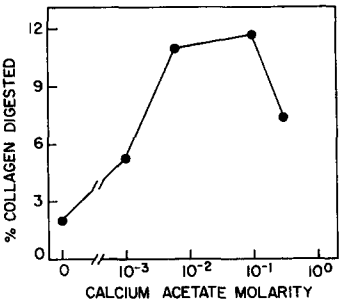


Fig. 1. pH optimum of collagen digestion. The assay system is described under Materials and Methods. The Ca^{2+} concentration was 0.05 M.

Fig. 2. Effect of calcium concentration on the extent of collagen digestion in 20 h. Calcium was not added during the initial homogenization or washing of pellet.

The dependence of enzyme activity on calcium concentration at pH 7.5 is shown in Fig. 2. Only slight activity is found in the absence of added calcium; this activity may be due to some calcium that remained bound during the homogenization step. Digestion is maximal between 0.01 and 0.1 M Ca^{2+}. It is important to note that high calcium concentrations become inhibitory. In preparing homogenates for the experiments illustrated in Fig. 2 it was necessary to omit calcium in the homogenizing fluid. This probably accounts for the lower maximum digestion (12% compared to the usual 16%). It suggests that there may be some free collagenase which does not bind to the collagen unless calcium is present during the homogenization step.

Ca^{2+} appears to be a specific requirement for collagenase activity. We noted before[11] that EDTA effectively abolished the activity, pointing to a need for a metal cofactor. A variety of metal ions were tried as possible replacements for Ca^{2+} in dialyzed preparations. Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} not only failed to substitute for Ca^{2+}, but in our particular system each gave less digestion than was found in the absence of any addition. Again the system was not completely calcium-free due to the complex nature of the pellet.

A peculiar feature of uterine collagenase is the irreversibility of the inhibition by EDTA[8]. This is also found in our experiments as illustrated in the upper half of Table I. If EDTA and enzyme are allowed to react for 10 min in the absence of added calcium and then a 50-fold excess of calcium over EDTA is added and incubation is continued for 20 h, there is almost no restoration of activity. It might be argued that two metals are involved, *i.e.* one for binding to the substrate and one for the catalytic function. However, a number of likely pairs of metals were added without any significant restoration of activity.

TABLE I

EDTA INHIBITION OF COLLAGENASE

Ca^{2+} was 0.05 M, other metal ions were 0.01 M. Fe^{2+} underwent oxidation during the experiment. In the first group of experiments, EDTA was added to resuspended pellets to a final concentration of 1 mM, and the mixture was held in an ice bath for 10 min. Metal ions were then added and incubation continued for 20 h at 37 °C. In the second group of experiments, Ca^{2+} or EDTA was added and the storage or dialysis was for 18 h at 4 °C. The samples were then incubated for 20 h at 37 °C to measure collagen digestion.

Addition	*Treatment*	*% Collagen digestion*
Ca^{2+}		16.4
EDTA		1.5
EDTA + Ca^{2+}		2.4
EDTA + Ca^{2+} + Mn^{2+}		2.9
EDTA + Ca^{2+} + Co^{2+}		2.9
EDTA + Ca^{2+} + Mg^{2+}		1.5
EDTA + Ca^{2+} + Cd^{2+}		1.0
EDTA + Ca^{2+} + Cu^{2+}		0.7
EDTA + Ca^{2+} + Zn^{2+}		0.5
EDTA + Ca^{2+} + Fe^{2+}		0.4
Ca^{2+}	Store in cold	16.4
Ca^{2+}	Dialyze *versus* Ca^{2+}	17.3
EDTA	Store in cold	1.5
EDTA	Dialyze *versus* EDTA, add Ca^{2+} before incubation	1.9
EDTA	Dialyze *versus* Ca^{2+}	12.4
EDTA + Ca^{2+}	Store in cold	11.2

On the other hand, the irreversibility is not complete as is illustrated by the experiments in the lower half of Table I. If the pellets with associated enzyme are kept in the cold overnight about 75% of the activity can be restored either by dialyzing EDTA out and calcium in or merely by letting the mixture of EDTA and excess calcium stand. This suggests a very slow exchange in which the Ca^{2+} eventually gets to its proper site for activity. When the same experiment is done at 37 °C, it must be presumed that the exchange is not sufficient to restore much activity in the early part of the 20-h period or that the enzyme is susceptible to denaturation under the conditions of Ca^{2+} absence.

It was noted in the initial experiments[11] that the uterine supernatant did not display any collagenase activity, and when it was added back to the pellet, it inhibited the pellet activity about 40%. Additional properties of this tissue inhibitory activity are described in Table II. Heating abolished most of the inhibitory effect and freezing for several weeks abolished all of it. Experiments with membrane filters showed that the active substance is larger than 50 000 daltons. This tissue inhibitor may be the same as the inhibitory activity found in rat serum; serum added to the pellet in a final dilution of 1:100 inhibited the collagenase activity by 65%.

A study of other possible inhibitors showed that cysteine (1 mM) was not inhibitory. However, similar concentrations of other reducing agents were inhibitory: 1 mM mercaptoethanol, dithiothreitol or glutathione inhibited the digestion 49, 32 or 18%, respectively. EDTA was the most effective chelating agent, but 1 mM 1,10-*o*-phenanthroline, α,α'-dipyridyl, or 8-hydroxyquiholine were only slightly less effective.

TABLE II

PROPERTIES OF THE TISSUE INHIBITOR OF COLLAGENASE

Collagenase activity was determined in the 6000 × *g* pellets resuspended in buffer and compared with activity in pellets resuspended in the 6000 × *g* supernatant. In the last two experiments the supernatant was filtered through Centriflo membrane cones which retain molecules greater than 50 000 molecular weight and the pellets were resuspended with each fraction.

Incubation mixture	*% Inhibition*
Pellet + NaCl	0
Pellet + supernatant	38
Pellet + heated supernatant (100 °C, 10 min)	5
Pellet + frozen supernatant (−20 °C, 2 weeks)	0
Pellet + supernatant (<50 000 molecular weight)	3
Pellet + supernatant (>50 000 molecular weight)	38

An important property of collagenases is their ability to attack collagen at temperatures well below the point where any denaturation of the substrate might occur. In the uterus homogenates the substrate is mature collagen fibers, so that 37 °C is still well below the shrinkage temperature of 59 °C[15]. However, we also have shown that uterine collagenase is effective below body temperature, *i.e.* at 30 °C (Table III). In this case, trypsin must be used as a tool because when mature collagen is digested at 30 °C it does not leave the structure of the fiber. Crosslinks of various sorts probably hold the digestion products to the neighboring fiber structure.

In the present experiment, incubating 22 h with EDTA produced no change in the collagen, so that subsequent digestion with trypsin released only 10% of the collagen (Table III, Expts 6 and 7). That is, trypsin released no more collagen products from pellets preincubated with EDTA than from pellets that were not

TABLE III

PELLET COLLAGENASE ACTIVITY AT 30 °C

In Step A, the uterine pellets were resuspended in buffer with the addition of 0.05 M Ca^{2+}, 0.01% trypsin or 1 mM EDTA and incubated for 22 h. "Collagenase" refers to the action of endogenous enzyme in the pellet. EDTA completely inhibits this activity but may still permit the activity of other endogenous proteases. In Step B, 0.05 M EDTA was added to block any further collagenase activity. Incubation was continued for a further 22 h in the 3 cases noted.

I. Experimental procedure		
Step A (22 h)	*Step B (22 h)*	*% Digestion*
1. Collagenase, 30 °C		2.5
2. Trypsin + EDTA, 37 °C		10.8
3. Collagenase, 30 °C	Trypsin + EDTA, 37 °C	30.3
4. EDTA, 37 °C		1.1
5. Collagenase, 30 °C	EDTA, 37 °C	17.3
6. EDTA, 30 °C	Trypsin + EDTA, 37 °C	9.8
7. EDTA, 30 °C		0.8
II. Calculation of the extent of collagenase attack at 30 °C		
Sequential digestion	*Parallel digestion*	*Difference**
8. Expt 3 30.3%	Expts (1 + 2) 13.3%	17.0%
9. Expt 5 17.3%	Expts (1 + 4) 3.6%	13.7%

* Corresponds to collagen partially digested but not solubilized at 30 °C. Upon raising the temperature to 37 °C, this fraction is now susceptible to trypsin[9] and nonspecific tissue proteases[10].

preincubated (expt 2). Incubating with endogenous collagenase at 30 °C released very little collagen (Expt 1) but subsequent treatment with trypsin released almost 3 times as much collagen as did trypsin alone (Expts 3 and 2). Even when no trypsin was added, the endogenous tissue proteases were able (in the presence of EDTA, Expt 5), to release 17% of the collagen. These experiments indicate that collagen molecules were cleaved at 30 °C but not released from the fiber structure. When the temperature was brought to 37 °C the cleaved portions could undergo denaturation[16] and digestion by nonspecific endogenous proteases or added trypsin.

The size of the collagen digestion products was examined by the use of membranes of varying porosity (Table IV). Almost all of the material released from native mature collagen fibers at 37 °C consists of pieces smaller than α chains (100 000) and over 80% is smaller than 50 000. This accords with the idea that multiple points of cleavage occur at a temperature of 37 °C.

TABLE IV

SIZE DISTRIBUTION OF THE PRODUCTS OF COLLAGENASE ACTION

Samples in which 25% of the collagen had been digested (48 h) were centrifuged as usual and passed through a Millipore 0.22-μm filter to remove any particles. The soluble digestion products were then passed through Diaflo membranes XM-100 and XM-50 and dialyzed in Visking tubing. Acid-soluble collagen from guinea pig skin was denatured by heating; it did not pass any of the membranes (< 1%).

Size	*% of initial sample*
Greater than 100 000	4
50 000–100 000	16
Less than 50 000 and not diffusible	47
Diffusible	33

Finally, it should be stressed that the uterine collagenase does not resemble the *Clostridium histolyticum* collagenase in its specificity. Extracts of involuting rat uterus will digest the synthetic substrate 4-phenylazobenzyloxycarbonyl-Pro–Leu–Gly–Pro–D-Arg at neutral pH[17,18]. However, the pellets which contain the collagenase activity have no effect on this synthetic substrate at pH 7–8. It might be argued that the synthetic substrate cannot gain access to the enzyme since the latter is bound to its natural substrate. However, it is possible to show the digestion of labeled soluble collagen when this is added to the pellets (unpublished results). Therefore, the enzyme must occasionally come free from the uterine collagen and presumably could bind to the peptide substrate just as it appears to bind to certain inhibitors.

DISCUSSION

Two points are of major concern in this discussion, *i.e.* to establish that the enzyme activity of the uterine pellets is due to a collagenase and to show that it is the same collagenase obtained by Jeffrey and Gross[8] from cultured rat uterus. A true collagenase should digest native collagen molecules at physiological pH and at temperatures below the thermal denaturation temperature of collagen. It should also produce collagen fragments smaller than α chains.

It was shown previously[11] that the collagenase activity in the uterine pellets digested native collagen that was completely inaccessible to the action of trypsin. After 48 h of incubation more than 25% of the collagen had been digested, whereas trypsin never digested more than 10–12% of the collagen. Trypsin, of course, is employed only as a test for the native condition of the collagen. There is no appreciable trypsin-like activity in the uterine pellets, since almost all release of collagen is blocked by EDTA (Table I). Studies with various metal ions indicate that the only enzymes having any appreciable effect on collagen in either the native or denatured state are those requiring Ca^{2+}. Furthermore, we have found that if pellets are incubated with calcium to allow collagen digestion and then EDTA and trypsin are added, the trypsin still digests about 10% of the collagen. Thus, there is no evidence that any enzyme in the uterus attacks the trypsin-susceptible collagen, and there is no evidence for any enzyme other than collagenase which can release hydroxyproline-containing material from the pellets.

Also in keeping with the definition of collagenases, it is seen that the uterine enzyme has an optimum pH at 7.5 (Fig. 1) and that it acts on collagen at 30 °C to produce partial degradation products (Table III). The substrate in this case is mature (*i.e.* insoluble) native collagen fibers. Many demonstrations of collagenase activity have been limited to attack on soluble collagen or on reconstituted soluble collagen, but in the present case natural fibers are digested. Only 3–4% of the uterine collagen can be extracted by neutral salts and dilute acids[19].

The second important question is whether the activity measured in uterine pellets corresponds to the collagenase isolated from cultures of involuting uterus tissue[8]. There is every indication that this is the case. Both activities digest native collagen fibers at 37 °C; both have a pH optimum of 7.5 with a similar decrease in rate at pH 6.5 and 8.5; both produce large fragments of collagen at 30 °C and small fragments at 37 °C; both are inhibited by 1 mM EDTA and the inhibition is not reversed by adding excess Ca^{2+}; both require Ca^{2+}; and neither is inhibited by 1 mM cysteine.

The present studies reveal some additional properties of the uterine collagenase. It has been possible to reverse the EDTA inhibition, at least in part. The uterine collagenase was not susceptible to inhibition by 1 mM cysteine, but it was inhibited by 1 mM mercaptoethanol to the extent of 50% and to a lesser extent by glutathione and dithiothreitol. Therefore, there is some susceptibility to reducing agents. Tokoro *et al.*[20] have recently noted that uterine collagenase is inhibited by 0.01 M cysteine.

It is not surprising that collagenases have rarely been detected in tissue homogenates. If the enzyme is largely in the pellet fraction, it will generally have been discarded because most experimenters have been looking for a soluble fraction that can be applied to standardized collagen substrates. Any enzyme that is free in the supernatant will probably be inhibited by other substances from the tissue or serum.

It is still not known why collagenase is found in the pellet. A very attractive hypothesis is that it is bound to its substrate, the collagen fibers. However, it might also be in a particulate fraction or membrane-bound form. If so, it is quite difficult to disrupt or free the enzyme from such a fraction. Freeze-thaw treatment, Triton X-100, and washing with 1 M NaCl do not remove more than a small fraction of the collagenase from the pellet.

We believe that the activity in the pellet correlates directly with the phy-

siological action of the enzyme. The activity is maximal (16%) at the peak of uterine involution at 2–3 days *postpartum*. It is low in the nongravid uterus (4%) and in *postpartum* uteri in which estrogen treatment has been used to inhibit involution (9%)[12].

ACKNOWLEDGEMENTS

This work was supported by U.S. Public Health Service Grants HD 06773 from the National Institute of Child Health and Human Development and HE 11035 from the National Heart and Lung Institute. J.N.R. received fellowship support from U.S. Public Health Service Training Grants HE 05463 (National Heart and Lung Institute) and GM 00649 (General Medical Sciences). Part of the material in this paper was submitted in the dissertation for the Ph.D. degree (J.N.R.) at the University of Miami, January, 1971. We would like to thank Mrs Carolyn Taplin for her excellent technical assistance.

REFERENCES

1 Neuberger, A., Perrone, J. C. and Slack, H. G. B. (1951) *Biochem. J.* 49, 199–204
2 Harkness, R. D. and Moralee, B. E. (1956) *J. Physiol.* 132, 502–508
3 Woessner, Jr, J. F. and Brewer, T. H. (1963) *Biochem. J.* 89, 75–82
4 Morrione, T. G. and Seifter, S. (1962) *J. Exp. Med.* 115, 357–365
5 Schaub, M. C. (1963) *Helv. Physiol. Pharm. Acta* 21, 227–238
6 Gross, J. and Lapiere, C. M. (1962) *Proc. Natl. Acad. Sci. U.S.* 48, 1014–1022
7 Eisen, A. Z., Bauer, E. A. and Jeffrey, J. J. (1970) *J. Invest. Dermatol.* 55, 359–373
8 Jeffrey, J. J. and Gross, J. (1970) *Biochemistry* 9, 268–273
9 Lazarus, G. S., Brown, R. S., Daniels, J. R. and Fullmer, H. M. (1968) *Science* 159, 1483–1485
10 Aer, J. and Kivirriko, K. I. (1969) *Z. Physiol. Chem.* 350, 87–90
11 Ryan, J. N. and Woessner, J. F., Jr. (1971) *Biochem. Biophys. Res. Commun.* 44, 144–149
12 Ryan, J. N. and Woessner, J. F., Jr. (1971) *Fed. Proc.* 30, 1249A
13 Eisen, A. Z., Bauer, E. A. and Jeffrey, J. J. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 248–251
14 Woessner, J. F., Jr. (1961) *Arch. Biochem. Biophys.* 93, 440–447
15 Brown, P. C., Consden, R. and Glynn, L. E. (1958) *Ann. Rheum. Dis.* 17, 176–196
16 Sakai, T. and Gross, J. (1967) *Biochemistry* 6, 518–528
17 Strauch, L. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), Vol. 3, pp. 1675–1682, Academic Press, New York
18 Ryan, J. N. and Woessner, J. F., Jr. (1972) *Biochem. J.* 127, 705–713
19 Woessner, J. F., Jr. (1962) *Biochem. J.* 83, 304–314
20 Tokoro, Y., Eisen, A. Z. and Jeffrey, J. J. (1972) *Biochim. Biophys. Acta* 258, 289–302
21 Evanson, J. M., Jeffrey, J. J. and Krane, S. M. (1968) *J. Clin. Invest.* 47, 2639–2651