Table 2. The effect of a second sugar on the mutarotation of α-glucose by equine hemoglobin mutarotase, as determined by the dialysis-coupled enzyme method. The concentration of α-glucose was 0.02085 mole/liter; of all other sugars, 0.1 mole/liter.

Second sugar	Inhibition (%)	K_i (mole/liter)
None		.0502 (K_m)
L-Arabinose	100	
D-Galactose	100	
D-Arabinose	69	.0313
D-Ribose	66	.0365
D-Xylose	60	.0478
D-Maltose	44	.0906
D-Fructose	29	.1768
D-Lactose	26	.2020
Sucrose	0	

of inactivation was calculated from $t_{1/2}$ values, following Keston's method (*10*) for calculating fractional residual activity and percentage inhibition. The data indicate that this enzyme is quite sensitive to heat, since 92 percent of the activity could be abolished by heating the solution for 10 minutes at 55°C.

The dialysis–coupled enzyme method was also used in determining the effect of a second sugar on the mutarotase activity (Table 2). This was done by making a 0.2*M* solution of the sugar in EDTA buffer, letting it stand overnight to reach equilibrium, and using this solution to further dissolve the α-glucose for diluting the enzyme solution in the usual manner. The equine mutarotase was strongly inhibited by D-galactose, L-arabinose, D-ribose, and D-xylose but only slightly by D-fructose, lactose, and D-maltose, and not at all by sucrose. These results are similar to those reported by others when the enzyme was found in glucose oxidase preparations (*1*) and in mammalian tissues (*2*). The stereospecificity of the enzyme is demonstrated by the greater inhibition with L-arabinose (100 percent) than with D-arabinose (69 percent). In this respect, the equine hemoglobin enzyme differed somewhat from mutarotase of the eye lens which was not at all inhibited by D-arabinose, although strongly inhibited by L-arabinose (*10*). Inhibitor constants, K_i, were found by using Eq. 1 of Keston (*10*), which describes competitive inhibition in accordance with Michaelis-Menten theory.

Experiments yielding results very similar to the ones reported here with partially purified mutarotase were obtained by using lysed human erythrocytes and hemoglobin solutions with the dialysis–coupled enzyme method. Although they actually preceded the work reported here, details of these experiments cannot be given in this brief communication.

Studies with preparations of hemoglobin from five different species of mammals indicated considerable differences in mutarotase activity. Equine and porcine hemoglobin had far more activity than bovine, canine, and human hemoglobin. In those instances in which the mutarotase was separated from either hemoglobin (equine, bovine, and human) or lysed human erythrocytes and partially purified, the resulting enzyme activities were still in the same relative order.

The use of the dialysis–coupled enzyme method, which permitted assaying mutarotase activity in opaque and highly colored solutions, showed the presence of this enzyme in erythrocytes; its existence here had been suspected although not demonstrated by other investigators (*5, 6*). The separation of the enzyme from hemoglobin and its partial purification allowed the use of a conventional polarimetric method. It is thought that mutarotase participates directly in the transport of sugars (*5*) or that it may be a part of a mammalian "permease" system for sugars (*3*). The possible importance of finding mutarotase in erythrocytes is suggested by the specificity of some enzymes for anomers of glucose. The high specificity of glucose oxidase for β-D-glucopyranose was established by Keilin and Hartree (*11*). In a recent report (*12*) it was demonstrated that glucose-6-phosphate dehydrogenase from human erythrocytes is specific for β-D-glucopyranose-6-phosphate, and it was implied that mutarotase may have some metabolic function in erythrocytes.

WILLIAM SACKS

Research Center,
Rockland State Hospital,
Orangeburg, New York

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Human Collagenase: Identification and Characterization of an Enzyme from Rheumatoid Synovium in Culture

Abstract. *Synovial tissue from patients with rheumatoid arthritis produces lysis of gels of reconstituted collagen fibrils in culture and releases soluble collagenase when cultured in collagen-free medium. Collagen molecules in solution at neutral* pH *at 20° and 27°C are cleaved by the synovial enzyme into ¾ and ¼ length fragments. In this respect the action of synovial enzyme is similar to that of amphibian collagenase and distinct from that of bacterial collagenase. At 37°C reconstituted collagen fibrils and native fibers are attacked by the enzyme and further degraded to polypeptides of low molecular weight. These polypeptides are produced only after denaturation of the larger fragments, which occurs at temperatures near 37°C.*

The mechanism whereby collagen is degraded in human tissues under normal and pathological conditions has not been fully elucidated. In the native state collagen is relatively resistant to the action of the common proteolytic enzymes and there is no definite evidence that denaturation of the molecule precedes its breakdown in vivo. Since rheumatoid arthritis is associated with destruction of collagenous structures in and around joints we have examined

synovial tissue from subjects with this condition for the presence of a collagenase. We have demonstrated the presence of collagenolytic activity in such tissue by using the culture technique of Gross and Lapiere (*1*) and have partially characterized the enzyme responsible for this action (*2*). Prior to the pioneering studies of Gross and his colleagues of collagenases from amphibian tissues (*1, 3*) and, more recently, rat uterus (*4*), the only true collagenase characterized in detail was that derived from bacteria (*5*).

Synovial tissue was obtained at operation from patients with definite or classical rheumatoid arthritis (*6*). Fragments (2 to 5 mm in greatest dimension) were planted under sterile conditions onto ^{14}C-glycine–labeled reconstituted collagen gels in synthetic tissue culture medium (*1*) and incubated at 37°C for 3 to 5 days (*7*). Collagenolytic activity was assayed by measuring the radioactivity released into the medium. This was accomplished by centrifugation of the contents of the culture tubes at 45,000*g* at room temperature and counting an aliquot portion of the supernatant fluid in a modified Bray's solution (*8*) in a liquid scintillation spectrometer. Of ten specimens of tissue from patients with rheumatoid arthritis, eight produced lysis of gels greater than that produced by trypsin as a control for nonspecific protease. Sections of the cultured synovium which had produced enzyme were stained with hematoxylin and eosin and showed proliferating mesenchymal cells and synovial lining cells in addition to variable numbers of lymphocytes, plasma cells, and other mononuclear cells. In the two instances in which no collagenolytic activity was demonstrable, histological examination of the tissue after culture showed few cells, most of which had pyknotic nuclei. Collagenolysis did not occur when the tissue was frozen and thawed before planting.

The collagenolytic enzyme was obtained in solution by cultivating synovial tissue in Dulbecco's modified Eagle medium in the absence of collagen. The medium was harvested and replenished daily, and after 5 days the pooled medium was dialyzed exhaustively against water before lyophilization. The powder was taken up in 0.1*M* tris-HCl, *p*H 7.6, containing 0.001*M* $CaCl_2$. As judged by the ability to digest casein (*9*) different batches of crude enzyme solution prepared in this manner showed negligible nonspecific proteolytic activity.

Collagenolytic activity of the enzyme solution was assayed by measuring the radioactivity of soluble breakdown products released into the supernatant fluid obtained from ^{14}C-glycine–labeled reconstituted fibrils (*10*). The enzyme solution was added to approximately 100 to 200 μg of labeled fibrils suspended in 0.05 to 0.10*M* tris-HCl or tris-maleate at various *p*H values and incubated at 37°C. Collagenolytic activity was maximal in the *p*H range 7.0 to 8.5 and at *p*H 7.6 was linear with time and amount of enzyme solution added (Fig. 1). No activity was demonstrable at *p*H 5.0. Prior heating of the solution at 100°C for 2 minutes, or addition of ethylene diamine tetraacetic acid (EDTA) in a concentration sufficient to chelate all calcium present (0.001*M*), abolished the collagenolytic action. Normal serum and serum from patients with rheumatoid arthritis had a similar marked inhibitory effect, demonstrable at concentrations of serum as low as 0.1 percent. It is seen in Fig. 1 that as little as 5 μg of crude enzyme protein produced detectable collagenolytic effect. In all preparations where collagenolytic activity was pres-

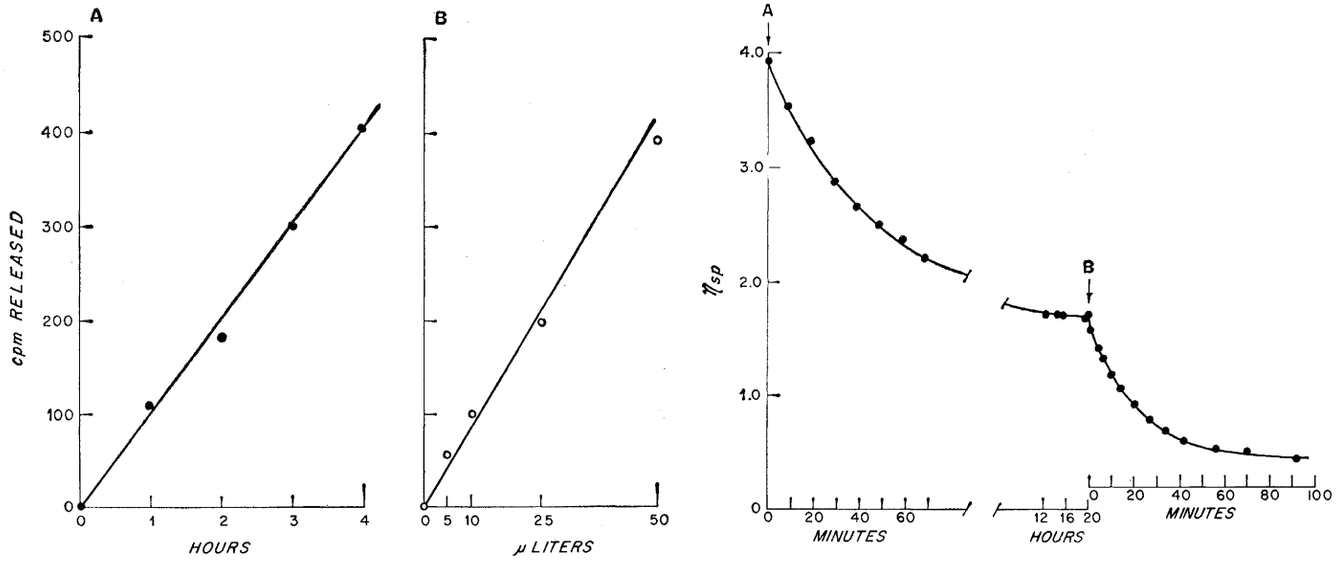

Fig. 1 (left). Collagenolytic activity of synovial enzyme as a function of time of incubation and amount of enzyme added. Reconstituted collagen fibrils from neutral salt extract of guinea pig skin labeled with ^{14}C-glycine were incubated at 37°C in 0.06*M* tris-HCl, *p*H 7.6, containing 0.16*M* NaCl and 0.0006*M* $CaCl_2$, in a total volume of 0.25 ml. Collagenolytic activity was measured by release of radioactivity (ordinate) into the supernatant fluid after centrifugation. Values for counts per minute (cpm) released are corrected for radioactivity released in the absence of enzyme. (A) Each tube contained approximately 100 μg of labeled collagen (660 count/min) and crude enzyme solution equivalent to 120 μg of protein (*14*). (B) Each tube contained approximately 170 μg of labeled collagen (1000 count/min). Crude enzyme solution contained protein equivalent to 948 μg/ml (*14*). Period of incubation was 18 hours. Fig. 2 (right). Effect of bacterial collagenase on residual specific viscosity after reaction of synovial collagenase with calf skin collagen (approximately 0.1 percent) in solution at 27°C, *p*H 7.6. Specific viscosity (η_{sp}) is shown on the ordinate and time after addition of enzyme on the abscissa. Point *A*: start of reaction with crude synovial enzyme equivalent to 1.5 mg of protein (*13*); point *B*: addition of purified bacterial enzyme (25 μg).

ent the enzyme solution contained nondialyzable hydroxyproline-containing peptides. For example, in Fig. 1A the solution having a total protein content of 4.7 mg per milliliter contained 0.32 mg of hydroxyproline (*11*) per milliliter, which presumably was derived from the enzymatic breakdown of collagen in the planted tissue. Therefore, the bulk of the protein in the crude solutions is not collagenolytic enzyme protein.

The action of the enzyme on collagen in solution at 20° and 27°C was studied by viscometry. The specific viscosity of approximately 0.1 percent solutions of calf skin collagen was reduced by 60 to 75 percent in the presence of the enzyme. The residual viscosity was considerably greater than that resulting from the action of an amount of bacterial collagenase (*12*) producing a similar initial rate of loss of viscosity. Bacterial enzyme, added at the end point of the reaction between collagen and synovial enzyme, produced a further loss of viscosity, whereas additional synovial enzyme had no effect (Fig. 2). In other experiments optical rotation of a reaction mixture of calf skin collagen and synovial enzyme in solution at 27°C was followed in a Cary 60 spectropolarimeter at 240 mμ; simultaneously, viscosity was followed on an identical reaction mixture. Loss of viscosity (60 percent) was accompanied by a decrease in negative optical rotation of only 7 percent, indicating that the decrease in viscosity was not associated with significant loss of helical content. When the reaction reached completion, the enzyme was inactivated by the addition of EDTA to a final concentration of 0.0025*M*, and the temperature was raised 1°C every 10 to 15 minutes. Denaturation occurred, as evidenced by an abrupt and simultaneous fall in optical rotation and viscosity, with a midpoint melting temperature of 35°C.

Since enzymatic cleavage was not accompanied by denaturation of the products at temperatures of 20° to 27°C, it was possible to determine the size of the reaction products by using electron microscopic techniques described by Olsen (*13*) and Gross and Nagai (*3*). Solutions of calf skin collagen were reacted with synovial enzyme at 20°C, *p*H 7.6, and dialyzed against 0.05*M* acetic acid, and the collagen fragments were precipitated as "segment long spacings" by the addition of adenosine triphosphate to a final concentration of 0.3 percent. Electron microscopy of these precipitates, positively stained with phosphotungstic acid (1 percent) at *p*H 3.5, revealed fragments similar to the ¾-length piece (TC^A) and ¼-length piece (TC^B) described by Gross and Nagai (*3*) as the products of the amphibian collagenase. The larger fragments (TC^A) were occasionally seen as dimers, whereas the smaller fragments (TC^B) were frequently found as polymers in the form of long ribbons. Confirmation of the approximate size of the fragments produced at 20°C was obtained by electrophoresis of the thermally denatured reaction products in polyacrylamide gels (Fig. 3). However, when collagen fibrils were reacted with the synovial enzyme at physiological temperature (37°C) at *p*H 7.6, fragments of high molecular weight could not be identified by gel electrophoresis, but the intensity of staining of the solvent front was increased, which suggested that the molecular weight of the reaction products was less than that of TC^B.

We therefore incubated ^{14}C-glycine–labeled collagen, either in solution or as fibrils, with synovial enzyme at various temperatures. We estimated the size of the products by gel filtration of the reaction mixtures on columns (1 × 35 cm) of Sephadex G-75 equilibrated with 5*M* LiCl containing 0.01*M* tris-HCl, *p*H 7.4, at room temperature, using human γ-globulin, cytochrome *c*, and ^{3}H-proline as column markers. Labeled reaction products obtained at 20° or 27°C emerged with the void volume coinciding with the γ-globulin (molecular weight, 160,000) and well in advance of the cytochrome *c* (molecular weight, 13,000) (Fig. 4). In contrast, the products of enzymatic digestion of collagen fibrils at 37°C were retarded on these columns and emerged between the cytochrome *c* and ^{3}H-proline markers. When ^{14}C-labeled collagen was denatured to gelatin prior to incubation with synovial enzyme at 27°C, products of low molecular weight were obtained, emerging in a volume similar to that of the products of digestion of fibrils at 37°C (Fig. 4). The elution pattern of these products on columns of Bio-Gel P-10 suggested that the molecular weight was in the range 5,000 to 10,000.

Since the enzymatic products obtained at 20° to 27°C have a midpoint denaturation temperature of approximately 35°C, enzymatic cleavage of the larger TC^A and TC^B fragments to these smaller peptides probably takes place only after denaturation. Such denaturation would readily occur at physiological temperatures near 37°C. Both the initial enzymatic cleavage of the collagen molecule at 20° or 27°C and the further degradation of the denatured products at 37°C are completely inhibited in 0.001*M* EDTA.

As further support for a possible function of the synovial enzyme in degradation of collagen in vivo, we demonstrated in preliminary experiments that the enzyme could digest native collagen fibers as well as collagen in solution or as reconstituted fibrils. Rat tail tendon and fragments of guinea pig skin labeled in vivo with ^{14}C-glycine were attacked by synovial enzyme, as shown by visible fragmentation of the tendon and release of soluble hydroxyproline, and by radioactivity from the fragments of guinea pig skin.

Synovial tissue from patients with rheumatoid arthritis will thus survive in culture, releasing into the medium a soluble enzyme which can digest na-

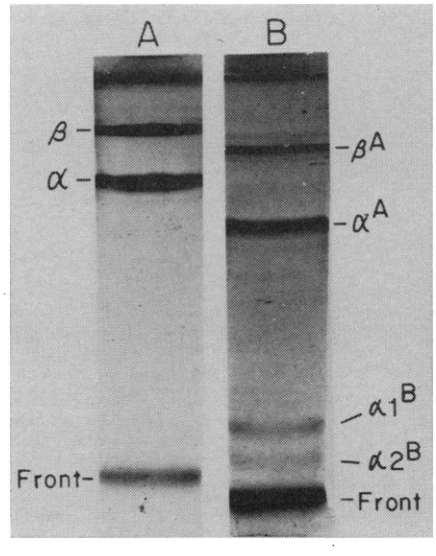

Fig. 3. Patterns of disc electrophoresis in polyacrylamide gels (*15*) of (A) control collagen after thermal denaturation, and (B) reaction products of collagen and synovial enzyme after 24-hour incubation at 20°C, *p*H 7.6, followed by thermal denaturation. Collagen components and products of enzymatic digestion are labeled according to Sakai and Gross (*16*). The bands labeled β^A and α^A represent the denatured ¾-length fragments; those labeled $\alpha 1^B$ and $\alpha 2^B$, the denatured ¼-length fragments resulting from enzymatic cleavage.

tive collagen with a *p*H optimum near neutrality. We have so far been unable to demonstrate activity greater than in trypsin controls with cultures of synovia from three subjects with degenerative joint disease. At temperatures of 20° to 27°C the enzyme appears to split the collagen molecule across the three chains to produce ¾- and ¼-length fragments similar to those resulting from the action of the collagenase from tadpole tissues. We did not identify a further split of the larger fragment to a 62-percent fragment recently observed by Jeffrey and Gross (*4*) using an enzyme from postpartum rat uterus. However, the products of the initial cleavage by synovial enzyme are denatured at 37°C, and the denaturation products are then further degraded to polypeptides of low molecular weight. This latter action is also inhibited by EDTA and is probably ascribable to the same enzyme that causes the initial cleavage, although purification of the enzyme will be necessary to establish this point.

The observations that activity is maximal at neutral *p*H and negligible at *p*H 5.0 and that frozen-thawed tissue does not produce enzyme suggest that lysosomal enzymes are not responsible for the effects observed. It is possible that synovial collagenase plays a role in destruction of collagenous structures which occurs in rheumatoid arthritis.

JOHN M. EVANSON*
JOHN J. JEFFREY
STEPHEN M. KRANE
Departments of Medicine, Harvard Medical School and *Massachusetts General Hospital, Boston 02114*

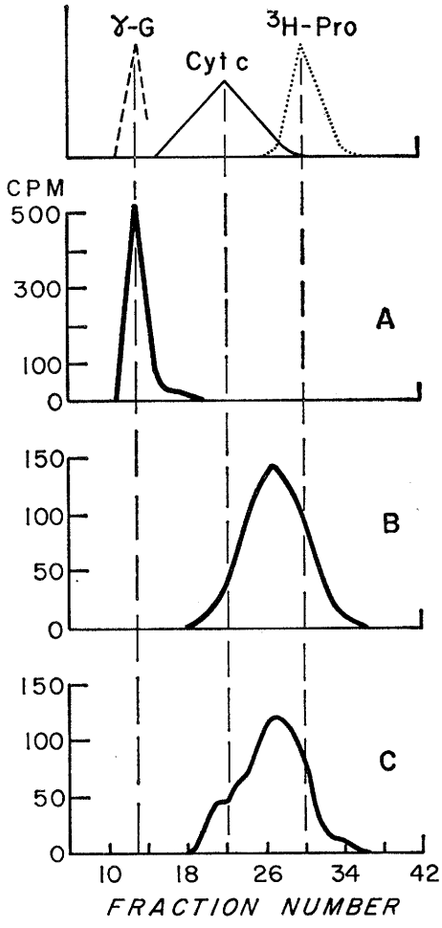

Fig. 4. Elution patterns of enzymatic degradation products of ^{14}C-labeled collagen on columns of Sephadex G-75. Columns (1 × 35 cm) were equilibrated with 5*M* LiCl containing 0.01*M* tris-HCl, *p*H 7.4, at room temperature. The positions where markers were eluted are shown at the top of the diagram. Reaction mixtures consisted of synovial enzyme and: (A) native collagen in solution at 20° or 27°C; (B) thermally denatured collagen (gelatin) in solution at 27°C; (C) reconstituted collagen fibrils at 37°C.

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* Travelling Fellow of the Medical Research Council of Great Britain.

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Slow Virus Kidney Disease of Mice

Abstract. *Preliminary observations based on organ weight differential, renal function, and glomerular lesions in mice infected neonatally with lymphocytic choriomeningitis indicate the presence of a slow virus-induced kidney disease of mice. This condition is accompanied by a marked decrease in the size of the kidneys, with progressive diminution of renal function, as shown by measurements of creatinine and urea clearance.*

In spite of increasing interest in recent years in the occurrence of viruria in man and animals, no clear-cut transmissible kidney disease of viral origin has been described (*1*). The closest candidates in this respect appear to be the temporary impairments of renal function reported by clinicians in such human infections as mumps (*2*), measles (*3*), infectious mononucleosis (*4*), acute lymphocytic choriomeningitis (LCM) (*5*), Coxsackie B3 infection (*6*), and vaccinia (*7*).

In the case of acute LCM infection of mice, their ability to excrete virus in urine is well known (*8*). This excretion continues during persistent tolerant infection (PTI) (*9*) which has also been shown to cause a late onset disease of the glomeruli (*10, 11*). This communication reports data which indicate that the kidney is the main target of damage in PTI infection of mice with LCM virus, resulting finally in a shrinkage of the kidneys and impaired renal function.

Eighty-four mice (NYLAR-A inbred strain) (*10*) were divided into two groups. One was inoculated intracerebrally at birth with 0.02 ml of a 10^{-3} dilution of a 20 percent mouse-liver suspension infected with LCM virus (PTI mice); the other group was inoculated intracerebrally at birth with 0.02 ml of a 10^{-3} dilution of a 20 percent suspension of normal mouse liver (control mice). The latter was used in order to eliminate the possibility that the disease was due to an autoimmune response to the liver tissue contained in the inoculum, rather than to the LCM virus.

The mice were killed at ages ranging from 10 to 16 months and their kidneys were weighed. Results can be seen in Table 1. The weights for PTI females and males were significantly less than those for the controls. The difference between mean kidney weights