C: ethyl acetate, 1-propanol, 0.1 percent aqueous formic acid (4 : 1 : 2); D: 2-propanol, water, concentrated amonium hydroxide (7 : 2 : 1); and E: ethyl acetate, 1-propanol, water (4 : 1 : 2).
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Human Granulocyte Collagenase

Abstract. *A collagenase, operative at neutral and alkaline* p*H, has been extracted from the granule fraction of human granulocytic leukocytes. It digests reconstituted collagen fibrils and reduces the viscosity of collagen solutions. Cleavage of collagen in solution with purified enzyme produces the discrete products characteristic of other animal collagenases.*

The first animal enzyme known to lyse collagen at neutral *p*H was isolated by Gross, Lapiere, and their co-workers (*1*, *2*) from the culture fluid of tadpole skin. Collagenase has also been demonstrated in culture fluids of rat bone (*3*, *4*) and uterus (*5*). Extracts of rat bone have also been reported to contain collagenolytic activity (*6*). A similar activity of tissues in man was first demonstrated in culture fluids of gingivae by Fullmer and Gibson (*7*) and was later confirmed by other investigators (*8*, *9*). Recently collagenase has been found in culture fluids of human bone (*4*) and skin (*10–12*). Culture fluids of synovia obtained from patients with rheumatoid arthritis have also demonstrated collagenase activity (*13*, *14*). Most of these enzymes (*5*, *12–15*) have been shown to cleave the collagen molecule into two pieces representing three quarters and one quarter of the molecule (*15–17*). In view of this highly specific action, the finding of these products can be considered diagnostic of a true collagenase.

Digestion of collagen occurs on a large scale during inflammation and abscess formation at a time when large numbers of granulocytic leukocytes are present. However, the presence of collagenase has not been detected heretofore in these cells (*6*, *18*). This report describes investigations which demonstrate the presence of a collagenase in the granule fraction of human granulocytic leukocytes.

Preparations containing predominantly polymorphonuclear leukocytes were obtained by differential centrifugation and dextran sedimentation of the leukocyte-rich red blood cell interfaces pooled from six units of normal human blood. Red blood cell contamination was removed by hypotonic saline hemolysis (*19*). The granulocytes were washed three times with isotonic saline and collected by centrifugation at 400*g* for 10 minutes. After white blood cell counts and differential smears were made, a portion of each granulocyte preparation was frozen for use as a whole cell lysate. The remaining granulocytes were homogenized at a concentration of 1.8×10^8 cell/ml for 3 minutes in cold 0.34*M* sucrose solution in a ground glass homogenizer and were then fractionated into a nuclear, granule and post-granule supernatant fraction according to the method of Cohn and Hirsch (*20*). The granule fraction was examined by phase microscopy, and typical granules were seen.

Human lymphocytes were prepared by sedimenting the buffy coats from 4 units of fresh whole blood with dextran. The cells obtained were resuspended in heparinized fetal calf serum at 37°C. After 1 hour of incubation they were placed on a 45-cm glass bead column and the lymphocytes were eluted (*21*). Contaminating red blood cells were removed from the effluent by hypotonic saline hemolysis (*19*). The lymphocytes were then washed three times with isotonic saline and frozen after a white blood cell count and differential smear were obtained.

To prepare whole cell lysates, granulocyte and lymphocyte preparations were homogenized in a ground glass homogenizer for 3 minutes and then frozen and thawed ten times. The cell fractions from the granulocyte preparation were also disrupted by freezing and thawing ten times prior to their use. Immediately before assaying for collagenolytic activity all preparations were centrifuged at 25,000*g* for 15 minutes. The volume of the resultant supernatant was adjusted with Tyrode's solution to give a preparation derived from 1.8×10^8 cell/ml. Aliquots of the final adjusted supernatants were analyzed for protein according to the Miller modification of the Lowry technique (*22*).

Table 1. Collagenolytic activities of normal human white blood cells as determined by the reconstituted radioactive collagen fibril assay. The enzyme preparations were derived from 9×10^7 cells.

Preparation	Protein (mg)	Collagen gel lysed (%)
Human granulocyte homogenate*	2.7	21.0
Nuclear fraction	1.2	7.3
Granule fraction	0.5	21.0
Post-granule supernatant fraction	.9	5.0
Human lymphocyte homogenate†	2.4	5.5
Trypsin (25 μg)		5.0

* 81 percent granulocytes, 19 percent lymphocytes. †95 percent lymphocytes, 5 percent granulocytes.

Native acid-soluble rat skin collagen was prepared by a modification of the method of Kang *et al.* (*23*) after injecting the animals with glycine-^{14}C. The collagen prepared had a specific activity of 3750 disintegrations per minute per milligram, and when it was incubated with a number of different proteolytic enzymes it was found to be almost completely resistant to nonspecific digestion. Indeed, when a 1 : 2 weight ratio of trypsin to collagen was incubated in the C^{14}-labeled collagen fibril assay (described below) only 5 percent of the substrate was digested.

Reconstituted C^{14}-labeled collagen fibrils were prepared as a 0.2 percent collagen gel according to the method of Gross (*2*). To a 0.2-ml gel of fibrils (1500 disintegrations/min) in a 3-ml test tube were added 0.25 ml of 0.001*M* calcium chloride in 0.05*M* tris buffer, *p*H 7.6, and 0.5 ml of the sample to be assayed. The mixture was incubated for 18 hours at 37°C and then filtered through a 0.9-μ-pore-size, 13-mm-diameter Versapor filter (Gelman Instrument Company, Ann Arbor, Michigan) in a Swinney adapter. The filter retained undigested collagen fibrils but allowed the passage of soluble digestion products. A 0.5-ml aliquot of the filtered solution was added to 20 ml of Bray's solution (*24*) and counted in a liquid scintillation counter. All counts were corrected for quenching by the channels ratio method and adjusted to a 100 percent efficiency (*25*). In addition to buffer blanks, all experiments included trypsin controls (25 μg) in the incubation mixture in order to indicate the

extent of nonspecific proteolytic breakdown of the collagen gel.

Table 1 shows the data obtained by the radioactive fibril assay for various white blood cell elements. A crude preparation derived from 9×10^7 cells having 81 percent granulocytes and 19 percent lymphocytes solubilized 21 percent of the collagen gel. In contrast, similar numbers of lymphocytes demonstrated no greater activity than the trypsin control. Fractionation of the granulocytes revealed that nearly all the activity was confined to the granule fraction. The activity in the nuclear fraction may be related to sedimentation of granule clumps in the nuclear pellet (*20*). The post-granule supernatant had negligible collagenase activity. Enzyme activity as measured by the radioactive fibril assay was completely abolished by boiling the leukocyte preparation or by addition of sodium ethylenediaminetetraacetate to a final concentration of 0.01*M*.

Viscometry was performed to extend these observations to collagen in solution and to follow the reaction as a function of time. Into Ostwald viscometers (flow time of 90 seconds for water at 20°C) were added: 1 ml of collagen solution (4 mg/ml), 1 ml of 0.1*M* calcium chloride in 0.05*M* tris buffer, *p*H 8.5, 3 ml of 0.7*M* sodium chloride in 0.05*M* tris buffer, *p*H 8.5, and 1 ml of the solution to be assayed. All viscometry measurements were made in duplicate and all experiments included buffer blanks and trypsin controls (300 μg). Results were computed as the percent reduction in specific viscosity with time from the initial measurement.

Experiments done with the unpurified granulocyte enzyme preparations at 20°C revealed a 25 percent fall in viscosity after 20 hours in the *p*H 7.5 to 9 range. Below *p*H 6 the crude enzyme preparation was unable to reduce the viscosity more than 3.5 percent; this was identical to the reduction effected by the trypsin control at *p*H 8.5. After 20 hours of incubation at *p*H 8.5 and 20°C, the addition of more crude enzyme produced a second fall in viscosity. This suggested that the collagenase was unstable, perhaps reflecting digestion of the collagenase by other proteases. The enzyme was purified by chromatography on diethylaminoethylcellulose at *p*H 8.5 by utilizing a linear salt gradient (*26*). The protein content of the purified granulocyte collagenase used was 0.9 mg/ml. The results of a viscometry experiment with the partially purified product at 20° and 25°C can be see in Fig. 1. At *p*H 8.5 the purified granulocyte collagenase reduced the specific viscosity 35 percent in 24 hours at 20°C and 65 percent in 4 hours at 25°C.

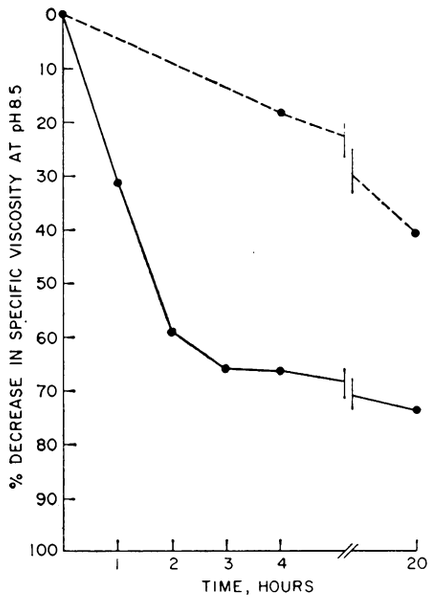

Fig. 1. Percent reduction in specific viscosity with time effected by purified granulocyte collagenase at *p*H 8.5 (dashed line, 20°C; solid line, 25°C).

The reaction products after 20 hours at 25°C from the experiment shown in Fig. 1 were examined by acrylamide gel electrophoresis. Collagen digestion products in the viscometry mixture were precipitated by increasing the sodium chloride concentration to 20 percent (wt/vol). The precipitate was then centrifuged and the pellet was dissolved in 8*M* urea titrated to *p*H 5.3 with HCl. Prior to electrophoresis, by the method of Sakai and Gross (*15*), the collagen solution was dialyzed against the *p*H 5.3 urea for 12 hours. The results of acrylamide gel electrophoresis are shown in Fig. 2. The control pattern consists of monomers (α), dimers (β), and the higher-molecular-weight species typically seen in denatured collagen (*15*). The products of the granulocyte collagenase digestion appear as discrete units of lower molecular weight. The front contains no stainable material. This pattern distinguishes granulocyte collagenase from nonspecific proteases and is conclusive evidence for the presence of a specific collagenase.

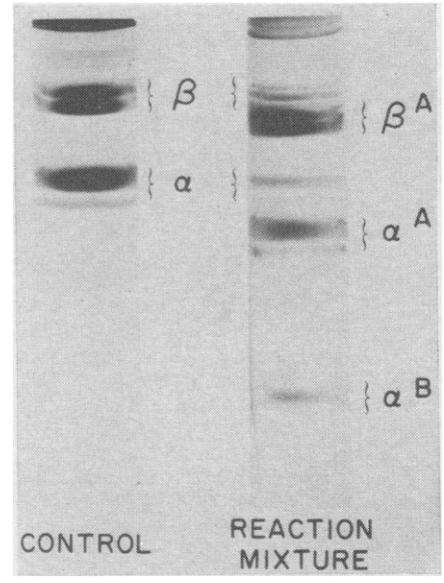

Fig. 2. Acrylamide gel electrophoresis of the products of collagen digestion (*p*H 8.5, 25°C, after 20 hours) by purified human granulocyte collagenase.

The foregoing data suggest a close similarity among the collagenases derived from human granulocytes, from other human sources, and from tadpoles (*5*, *12–15*). The cellular disposition of collagenolytic activity in granulocytes, however, appears to differ from other human collagenases in that culture is not required for detection. Instead, the collagenolytic activity is immediately available from the leukocyte granules. These granules have been defined as lysosomal structures (*27*) but the relationship of the collagenase described herein to other lysosomal enzymes is not known.

The finding of a collagenase in granulocytes active at physiological *p*H may have significant implications. The participation of the granulocyte in acute inflammation, abscess formation, and certain immunological phenomena such as Arthus vasculitis, serum sickness vasculitis, and nephrotoxic nephritis is well known (*28*). Inflammation of minor proportions evokes the exudation of large numbers of granulocytic leukocytes (*29*). The collagenase demonstrated within the granules of human granulocytic leukocytes, therefore, may well be involved in collagen destruction in a wide spectrum of pathologic states.

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Instrumental Learning of Vasomotor Responses by Rats: Learning to Respond Differentially in the Two Ears

Abstract. *Curarized and artificially respirated rats were rewarded by electrical stimulation of the brain for changes in the balance of vasomotor activity between the two ears. They learned vasomotor responses in one ear that were independent of those in the other ear, in either forepaw, or in the tail, or of changes in heart rate or temperature. In addition to implications for learning theory and psychosomatic medicine, these results indicate a greater specificity of action in the sympathetic nervous system than is usually attributed to it.*

In previous studies from this laboratory, we have discussed the importance of the instrumental learning of visceral responses for theories of learning and psychosomatic medicine, and have summarized the literature; we have presented evidence that the instrumental learning of cardiac, gastrointestinal, and renal responses can occur in curarized rats, with the use of either the onset of rewarding brain stimulation or the offset of mildly painful electric shock to the tail as reinforcement (*1*).

DiCara and Miller (*2*) have shown that vasomotor responses in the tail of the rat can be modified by instrumental learning. The purpose of the experiment we now report was to determine whether such vascular learning can be made specific to a given structure, such as one ear. In order to accomplish this, individual measures of vasomotor responses in the ears of the curarized rat were fed into a bridge circuit so that the differences in vasomotor activity between the two ears could be detected. The output of the bridge circuit was then fed in parallel into two pens of a Grass polygraph. One pen, the tip of which was constructed of a brass ballpoint, traced the between-ear difference over the surface of a plate constructed of 25 strips of brass, each 1.7 mm wide, inlaid into a flat piece of Plexiglas and separated from each other by 0.3 mm. When these strips were appropriately connected to programming equipment, reward consisting of electrical stimulation of the brain (ESB) could be delivered whenever the pen tracing the difference between the two ears was on or beyond a specified "reward criterion" strip. The second pen traced an ink record of the exact movements of the first pen. Other pens recorded the separate responses of each ear.

Subjects were 12 male rats of the Sprague-Dawley strain (394 to 558 g), implanted with permanent monopolar electrodes aimed at the medial forebrain bundle with Krieg stereotaxic coordinates of 1.5 mm posterior to bregma, 8.5 mm below the surface of the skull at 1.5 mm lateral to midline. Subsequent histologic examination showed that all of the electrode tips were located in the medial forebrain bundle at the level of the ventromedial nucleus [for details of general method, see (*3*)].

During vasomotor conditioning, the subject lay prone in a harness-supported cloth sling, placed in a soundproof, ventilated enclosure equipped with a loudspeaker delivering a 1000-hz tone at 82 db. The sling was cut so as to allow the subject's forepaws to hang down through the opening and rest on a small platform support. Grass photoelectric plethysmograph transducers were used to measure the vasomotor activity in the ears, forepaws, and tail. Recordings were taken at a sensitivity of 0.1 mv/cm for the ears, and 1.0 mv/cm for the forepaws and tail. The transducers were rigidly mounted on swivel arms attached to the metal frame of the harness so that the arms could be adjusted in all directions to place the photocells in homotopic positions on the ears (bisecting the central artery) and forepaws. The photocell used to measure vasomotor activity in the tail was placed at the base of the tail, and the tail was elevated about 3 cm to allow excreted boluses to pass without disturbing the position of the photocell. Heart rate was measured with previously implanted stainless steel electrodes, and temperature was measured by a thermistor probe inserted 4 cm into the rectum. A Grass Model 5 polygraph was used for all recordings.

Three to 4 days before vasomotor training, and approximately 2 weeks after electrode implantation, a current ranging from 30 to 100 μa was adjusted for each subject in order to elicit maximum rates of bar pressing for 0.5 second of 60-cycle a-c ESB. Current was then held constant, and on the next 2 days each subject was trained on fixed-interval schedules. During this training, a 1000-hz tone was turned on whenever bar pressing would secure ESB, and turned off during the time-out intervals (lengthened progressively: 5, 10, 20, 30 seconds), at which time the subject was in silence and ESB was not available.

The day after the above training, subjects were injected intraperitoneally with 3 mg of *d*-tubocurarine chloride per kilogram of body weight in a solution containing 3 mg/ml; the animals were fitted with a specially constructed face mask and artificially respirated, with the ratio of inhalation to exhalation being 1:1 (70 cycle/min, and a peak pressure of 20 cm-water). Addi-