Enzymatic Proteolysis of Rat Sponge Biopsy Collagen Tissue. (23479)

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The appearance of excessive collagen in degenerative disease and aging in man focuses attention on factors influencing collagen formation and degradation. Thus far, no naturally occurring mammalian collagenase has been proven to exist; the means whereby collagen turnover takes place remains obscure. The rate of resorption of collagen from the involuting post-partum uterus is, however, exceptionally rapid(1) and this suggests the presence of a substance which has collagenolytic properties. Alternately, the rapid post-partum degradation might reflect an alteration in the uterus itself so that, under the influence of hormonal change, it acquires a new susceptibility to less specific proteolytic agents.

The present study had as its objective the evaluation of the effects of certain enzymes upon the saline-insoluble fraction of rat sponge-biopsy connective tissue(2) at pH 6.8-7.8. The degree of collagenolysis was compared in biopsy-connective tissue removed from untreated rats and from rats treated with estrogen and relaxin prior to removal of the tissue.

Method. Sponge-biopsy connective tissue was obtained from 5 male, 6 female and 2 castrate adult Sprague-Dawley rats. Polyvinyl alcohol sponges had been implanted in the rats from 3 to 8 months before biopsy. Premarin,® 0.025 mg, and relaxin,† 4 mg, were injected subcutaneously into 7 of the rats for 21 consecutive days prior to the removal of the sponge biopsies. The remaining 6 rats received no therapy and served as controls.

The biopsied sponges were sliced into distilled water and homogenized at medium speed in a VirTis homogenizer. Connective tissue was grossly separated from the sponge material after high speed centrifugation. It was then stirred vigorously with 0.1 N saline for two 10-minute periods to remove saline-soluble collagen and non-collagenous proteins. The insoluble residue served as the substrate for the enzymatic studies. Fresh rat-tail collagen, which served as a control collagen, was prepared by stripping out the tendons from an untreated rat, cutting them into short lengths, and treating them similarly to the sponge-biopsy material. All preparative work was done in a cold room or refrigerated centrifuge at 5°C.

200-300 mg aliquots of freshly prepared biopsy tissue were shaken at 37°C for 12 hours with 1 ml buffered solutions containing Trypsin 2 mg, Chymotrypsin 3 mg, Papain 1 mg, Elastase (pure) 1 mg, Hyaluronidase 3 mg,‡ or Elastase (impure) 3 mg.§ All enzymes were dissolved in 0.1 M phosphate buffer, pH 7.8, except Papain (Versene buffer, pH 6.0, containing 0.005 M of Cysteine) and Trypsin (0.1 M phosphate buffer, pH 6.8). Tissues incubated with each buffer but without enzyme served as controls for each animal. As further control, a sample of each enzyme was heated to 100°C for 10 minutes and incubated with the tissue of one rat.

After incubation, the tissues were centrifuged in the cold at approximately 70,000 x g for 40 minutes. The supernatants were passed through sintered glass filters to remove any remaining particulate collagen. Aliquots of each supernatant were evaporated to dryness and hydrolysed in 1 ml of 6 N HCl at 110°C for 16 hours. The residues were hydrolysed similarly. The collagen concentration of each fraction was determined by the

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† Relaxin was supplied through the courtesy of the Warner-Chilcott Laboratories.

‡ Trypsin, 2 x crystalline, Chymotrypsin, 2 x crystalline, Papain, 2 x crystalline, Hyaluronidase, 100-200 TRU/mg—Nutritional Biochemicals Co.; Elastase, 2 x—Worthington Biochemicals Corp.

§ Elastase, impure, was supplied through the courtesy of the Warner-Chilcott Laboratories.

TABLE I. Enzymatic Digestion of Collagen.

Enzyme	Collagen digested, % of total collagen: Untreated control	Treated	Hydroxyproline, mg/100 mg protein in residue
Buffer,			
pH 6.8	.37 ± .08	.60 ± .35*	6.54 ± .39
pH 7.8	.28 ± .09	.32 ± .12	6.36 ± .54
Chymotrypsin 2×	2.74 ± .71	2.29 ± .38	8.66 ± .37
Trypsin 2×	2.99 ± .87	3.85 ± .76	7.64 ±2.12
Papain 2×	3.76 ± .74	2.85 ± .84	8.30 ± .30
Hyaluronidase 100-200 TRU	.59 ± .23	.66 ± .30	—
Elastase 2×†	1.11	1.42 .99	—
Trypsin 2× & elastase 2×	3.69	4.18 3.34	—
Elastase (impure)	10.03 ±1.56	9.77 ±2.51	9.95 ± .54

* Mean and stand. dev.
† Only 3 samples assayed.

hydroxyproline technic of Neuman and Logan (3) and the digested collagen in the supernatant expressed as a percentage of total collagen of the sample. The total protein content of each residue was derived from the nitrogen content as determined by the Conway(4) method after Kjeldahl digestion.

Results. Enzyme incubation of the biopsy-connective tissue from the rats pretreated with estrogen and relaxin resulted in collagen digestion which was not statistically different from those obtained with tissues of the untreated animals (Table I). Because the connective tissues of the male, female and castrate rats were equally susceptible to all the enzymes, the results were calculated and compared on the basis of treatment and control.

Tissue incubated with buffer alone or with heat-inactivated enzyme gave virtually no collagen digestion. Hyaluronidase, a non-proteolytic enzyme, similarly released an insignificant amount of collagen and, in effect, constituted a further control. Trypsin, Chymotrypsin and Papain, on the other hand, gave small but consistent digestion of collagen in all samples. These enzymes resulted in from 2.29 to 3.85% digestion in the two groups of rats. There was no statistically significant difference among the results for these 3 enzymes. Pure Elastase gave approximately 1% digestion, and when combined with crystalline Trypsin gave a mean value of 3.74% digestion for 3 tissues which was not statistically different from that obtained with Trypsin alone. Impure Elastase, on the other hand, resulted in from 6.0 to 18.7% digestion (mean 9.9%) for the 2 groups of animals. Incubation of rat-tail tendon with impure Elastase resulted in the release of 8.9% of total collagen, which is an amount comparable to that obtained with the biopsy-tissue.

The concentrations of hydroxyproline per unit of protein in the connective tissue residues after digestion with Chymotrypsin, Papain or impure Elastase were statistically greater ($p<0.05$) than those in the tissues incubated without enzyme. The large spread of data obtained for the Trypsin-digested residues nullified any significant difference from the controls.

Comments and summary. Pretreatment of rats with estrogen and relaxin does not appear to alter the susceptibility of sponge-biopsy connective tissue to digestion by proteolytic enzymes at near physiologic pH. No difference was noted in the proteolysis of collagen obtained from either sex or from castrate rats. Trypsin, Chymotrypsin and Papain all have a slight proteolytic activity on pretreated or untreated tissue, while pure Elastase and Hyaluronidase effect little, if any, liberation of collagen. Impure Elastase dissolves 2 to 6 times the amount of collagen digested by the proteolytic enzymes or a combination of pure Elastase and Trypsin. It is postulated that the impure Elastase, prepared from pancreas, contains a collagenolytic enzyme distinct from the proteolytic enzymes of the pancreas. The removal of a portion of the scleroprotein of connective tissue by enzyme action results in a residue richer in hydroxyproline (per unit protein) than in the enzyme-free controls. This may occur either through the removal of a non-collagenous, saline-insoluble protein liberated by the enzyme, or through the selective removal of a type of collagen which has a lower hydroxyproline concentration than the tissue as a whole.

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Ovulation in Persistent-Estrous Rats after Electrical Stimulation of the Brain.*† (23480)

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The fact of neural regulation of ovulation in rabbits and certain other animals classed as "reflex ovulators" has been well known for many years. Direct evidence has been obtained by the induction of ovulation through electrical stimulation of the hypothalamus(1, 2). More recently, ovulation has been reported in rabbits and cats after stimulation of the amygdaloid complex(3,4). Indirect evidence indicates that *spontaneous* ovulation as exemplified by the rat is likewise regulated by the hypothalamus(5). However, until very recently there was no direct evidence for this from stimulation of the brain. Critchlow(6) is the first to demonstrate it, using proestrous rats in which the "spontaneous" mechanism was blocked out with pentobarbital. To be effective, his electrodes had to be near the median eminence. To produce oyulation in rats by stimulation elsewhere in the nervous system, other means are required to block the spontaneous intrinsic stimulus. One such method is to subject the rat to continuous illumination for a few weeks(7,8,9). In time such an animal usually enters a state of persistent estrus. She can ovulate in response to coitus(9) and in this respect is like the rabbit, cat, etc. For comparison with them she would theoretically serve as a more suitable preparation than the pentobarbital-blocked rat. We wish to record the fact that such preparations can be made to ovulate by electrical stimulation of the brain through electrodes implanted at a distance from the hypothalamus.

Materials and methods. Adult female albino rats over 6 months old were exposed to continuous illumination until a persistent vaginal estrus had become established. Of this group, 12 rats formed the eventual series of acceptable experiments. A bipolar electrode was placed in the brain of each animal by use of a Horsley-Clarke stereotaxic instrument and Krieg's coordinates. The electrodes were fashioned from 0.32 mm Nichrome wire, stretched, and insulated with baked enamel. The spacing of the two wires at the tip was <0.05 mm. In 11 rats the electrode was aimed for the amygdaloid complex; in the remaining rat it was inserted into the septal region. It was fixed to the skull by a bridge made of an 18 mm wound clip clamped to the temporal crests and filled-in beneath with zinc dental cement. The operation was performed under ether anesthesia to allow rapid recovery. Soon afterward the rat was placed in a shoulder harness to which wires from the electrode were anchored before leading off to the stimulator. She had free range of movement within the radius of the wires. Stimulation was carried out after recovery from the anesthetic. The source of stimulus was an electronic impulse generator with independently controlled frequency and intensity sec-

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