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Identification of a Uterine Elastase in the Pregnant Rat Uterus (42788)

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Abstract. During development of the pregnant rat uterus there is a several fold increase in elastin content. Using Verhoeff's elastic fiber stain, we have shown that a significant proportion of these elastin fibers are in the extracellular matrix of the myometrium. They do not appear as an organized structure but rather in a variety of partially extended, random configurations. An elastase was identified in both the pregnant and the postpartum uterus. Partial characterization of the enzyme indicated that it is a serine protease with a molecular weight around 24,500 and a pH optimum of 8.5. In addition to the enzyme, relatively high levels on an elastase inhibitor were found in the uterine extracts. The inhibitor did not inhibit trypsin, indicating that it was not α-1-antiprotease. The data suggest that the elastase and inhibitor are uterine tissue derived and perhaps important in the normal remodeling process of uterine connective tissue.

Elastases are a heterogeneous class of enzymes, comprising both serine proteases and metalloproteases, that are capable of solubilizing fibrous elastin. These enzymes have been isolated from a wide variety of cells and tissues (1) and are postulated to perform important physiological and pathological functions, resulting in elastin destruction.

Elastin fibers comprise a significant portion of the extracellular matrix of tissues such as the major arteries, ligaments, and the lung, which undergo pronounced stretching and recoiling during normal function. Due to various factors, including the hydrophobicity and extreme crosslinking of the protein as well as protection from elastolytic enzymes by several protease inhibitors, elastin is one of the most stable proteins known, with a half-life best estimated in years (2–4). There is one organ, however, that experiences both rapid synthesis and degradation of elastin as part of its normal function. During pregnancy the mammalian uterus increases up to 10 times its virgin weight, which is accounted for by water, collagen, elastin, and muscle mass of the tissue (5–7). Following parturition, there is a dramatic involution of the uterus, returning it close to its initial size within 5–7 days postpartum (6–9).

The postpartum degradation of collagen in the involuting uterus has been carefully studied and represents perhaps the most rapid catabolism of collagen in mammalian physiology. After parturition, a specific neutral collagenase is synthesized and secreted. The synthesis and subsequent collagen breakdown is thought to be initiated by the drop in hormones following birth (10). However, very few studies have been conducted that pertain to elastin metabolism in the pregnant or involuting rat uterus. It is not known whether this marked elastin increase during pregnancy represents increased vascularity or extracellular accumulation of elastin fibers within the uterine wall. It is also not known whether the elastin catabolism which occurs during involution is due to an elastase released during an influx of neutrophils or macrophages, or is of tissue origin similar to collagenase. The following studies report on the existence of an elastolytic enzyme present in the postpartum rat uterus that may be responsible for the removal of the elastin that accumulates during pregnancy.

Methods and Materials. All animals used in the following studies were of Sprague–Dawley strain from Taconic Farms, Germantown, New York. First time pregnant rats and virgin nonpregnant rats were housed in an inverted light cycle room and given Purina rat chow and tap water *ad libitum.* The female rats were time bred for use in these experiments.

Litters were discovered between 2 and 12 hr after birth and uteri excised from the dams are designated as the time postpartum.

Uterine tissue samples, about 10 mm in length, were taken from a rat that was killed

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between the 19th to 20th day of gestation. The tissue was fixed in buffered neutral formalin overnight and then imbedded in paraffin. Several 6-μm sections were cut transversely. The specimens were stained with Verhoeff's elastic fiber stain as according to Mallory (11).

Elastin-rhodamine, 200 to 400 mesh (Elastin Products Co., St. Louis, MO), was prepared by suspending it in 0.2 *M* Tris buffer, pH 8.0, to a final concentration of 20 mg/ml. Samples were incubated with 200 μl elastin-rhodamine suspension at 37°C for the appropriate time as indicated in the figures. The residual substrate was then pelleted in a Beckman microfuge and the optical density of 100 μl of the supernatant measured at 550 nm.

In some experiments, a more sensitive synthetic substrate, succinyl-trialanyl-*p*-nitroanilide (SAPNA), (Peninsula Labs, Belmont, CA) was used. This substrate was prepared according to the method of Bieth *et al.* (12). A 0.25 m*M* stock solution was prepared in *N*-methylpyrrolidone. A 0.05 m*M* working solution was made by diluting the stock solution into 0.2 *M* Tris, pH 8.0. Samples were brought to a total volume of 140 μl with Tris buffer and incubated with 10 μl of substrate in microtiter plates for the appropriate time as indicated in the figures and the color development measured at 405 nm.

Tissue inhibitory capacity (TIC) was determined by adding to a microtiter plate 200 ng of porcine or rat pancreatic elastase and a 50 μl aliquot of the sample to be analyzed. The total volume was brought to 150 μl with 0.02 *M* Tris buffer, pH 8.8, and incubated at room temperature 30 min. Elastin-rhodamine or SAPNA was then added and the fractions incubated at 37°C. Color development was determined at either 545 or 405 nm. The inhibitory capacity of each fraction was expressed as a percentage of the control, i.e., the activity of the same amount of porcine pancreatic elastase that had not been preincubated with a uterine fraction.

Ceruloplasmin activity was measured using a modification of the procedure of Rice (13). *p*-Phenylenediamine was dissolved in 1 *M* sodium acetate buffer, pH 5.5, to give a 0.1% solution. Fifty microliters of substrate was mixed in a microtiter plate with 10–50 ul of serum or the uterus fraction and the total volume brought to 150 μl with acetate buffer. After incubation at room temperature, the difference in OD at 530 μm at zero time and 30 min was used as measure of enzyme activity. In some instances a faint precipitate appeared and it was necessary to spin the samples in a microfuge prior to reading.

SDS–polyacrylamide gel electrophoresis was performed according to the general methods of Laemmli (14). The running gel was 12.0% acrylamide, with a 4.0% stacking gel. Samples were reduced and denatured by heating in sample buffer containing 1.0% SDS and 0.05% 2-β-mercaptoethanol. Protein standards (BRL, Gaithersburg, MD) included myosin (200,000), phosphorylase B (97,400), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsin (25,700), β-lactoglobulin (18,400), and lysozyme (14,300). Gels were stained with Coomassie blue or silver stain.

In order to recover enzyme activity following SDS–PAGE, some gels were run with samples that were incubated for 15 min at room temperature in 1% SDS without β-mercaptoethanol. After the run, the gels were sliced in 2 mm sections and each section soaked in 100 μl of 2% Triton X-100 for 2 hr to remove the SDS. The Triton solution was removed and the slices incubated from 1 to 4 days at 37°C in 150 ul of 0.02 *M* Tris buffer, pH 8.8, containing SAPNA or elastin-rhodamine substrate. The optical density of 100 μl of the substrate solutions were read at 405 or 550 μm.

Rat pancreatic elastase was purified by the following procedure. The pancreas was obtained from 30 normal Sprague–Dawley rats about 6 months of age. The pooled pancreas (60 g) were homogenized in 3 vol of cold 0.1 *M* sodium acetate, pH 4.5. The homogenate was centrifuged at 20,000*g* for 20 min, the pellet reextracted twice with acetate buffer, and the supernatants combined. The combined supernatants were first dialyzed against three changes of distilled water and then against 0.02 *M* Tris buffer, pH 8.0. The slight precipitate that formed was removed by centrifugation and the extract applied to a DEAE cellulose column (1.5 cm × 15 cm) and eluted with the Tris buffer. Fractions (2.8 ml) were assayed for elastase activity,

using elastin-rhodamine as the substrate and for protein by absorbance at 280 nm. The fractions containing elastase activity were combined, dialyzed against 0.05 *M* sodium acetate buffer, pH 5.0, and loaded on a carboxymethyl cellulose column (1.5 cm × 23 cm) equilibrated with the same buffer. After washing the column with 45 ml of the starting buffer, a linear NaCl gradient to 0.75 *M* in the acetate buffer was initiated. The total volume of the gradient was 140 ml. Each fraction (2.8 ml) was again monitored for elastase activity with elastin-rhodamine and the OD at 280 nm recorded. The fractions containing the enzyme activity were pooled, dialyzed against distilled water, and freeze dried.

Further purification of the rat pancreatic elastase was performed using a Mono S 5/5 column on a Pharmacia FPLC System. Up to 10 mg of the freeze dried elastase preparation was dissolved in 1 ml of 0.05 *M* sodium phosphate buffer, pH 6.0, filtered through a 0.22 mm filter, and applied to the Mono S column equilibrated with the phosphate buffer. Flow rate was 1.0 ml/min and fraction size 1.0 ml. Six minutes later a linear gradient was initiated with 0.05 *M* sodium phosphate buffer, pH 6.0, containing 0.5 *M* sodium chloride. The gradient was programmed to reach 0.25 *M* after 30 min. Fractions were monitored for protein at 280 nm and enzymatic activity measured using the SAPNA substrate. Fractions containing the elastase activity were pooled, dialyzed against distilled water, freeze dried, and stored at −20°C. Analysis by SDS–acrylamide gels revealed a single protein band at a molecular weight of 24,000.

For isolation of the rat uterine elastase the excised rat uterus was cleaned of all fat, implantation nodes, blood vessels, and the cervix and washed extensively in saline. The uterus was then blotted dry and weighed prior to homogenizing on ice in 5 vol of either 0.05 *M* sodium phosphate buffer, pH 6.8, or 0.02 *M* Tris buffer, pH 8.8. The homogenate was centrifuged at 40,000*g* for 1 hr and the supernatant used for further fractionation.

Gel filtration was performed on an Ultrogel AcA 54 column (1.5 × 85 cm) equilibrated with 0.05 *M* sodium phosphate buffer, pH 6.8. Five milliliters of the uterus supernatant was applied and the column eluted with the same phosphate buffer at a flow rate of 0.6 ml/min. Fraction size was 1.3 ml. Elastase activity was determined in each fraction using SAPNA as the substrate. Protein was estimated by absorbance at 280 μm.

Ion exchange chromatography was employed using a DEAE cellulose column (1.5 × 25) or a Pharmacia Mono Q column both equilibrated with 0.02 *M* Tris buffer, pH 8.8. A linear NaCl gradient was conducted with the DEAE column up to 0.75 *M* NaCl in the Tris buffer, with a total volume of 70 ml. The Mono Q column was also equilibrated with the 0.02 *M* Tris buffer, pH 8.8, programmed to give a linear gradient starting in fraction 7 and going to 0.75 *M* NaCl in the Tris buffer after 30 ml. Fraction size was 1 ml. Both TIC and enzyme activity were determined in each fraction.

Results. All elastin in the uterus of the pregnant rat was located within the myometrium, the muscular layer that surrounds the endometrium or inner lining. No elastic fiber staining could be found in the endometrium (Fig. 1, Plate 1). Most of the elastin can be described as being located in four places in the myometrium of the uterus: (i) it was associated with collagen as long, individual fibers in the stratum vasculara (Plate 2); (ii) it could be seen as smaller fragments randomly, but not uniformly, distributed throughout the myometrium (Plate 3); (iii) it was located around the stratum subserosum, the outer edges of the uterus (Plate 4); and (iv) it was associated with the vasculature (Plate 5). Both long fibers and short coils were observed.

In our preliminary studies, we had found that the elastin-rhodamine substrate could readily measure 10 μg of elastase within 1 hr at 37°C. The SAPNA substrate was somewhat more sensitive detecting down to 10 ng of elastase within the same time frame. Taking into consideration the considerable amount of elastin that was degraded postpartum, we expected the sensitivity of either assay to be adequate for elastase measurements in the 40,000*g* supernatants from uterine homogenates. However, when elastin-rhodamine was used as the substrate, uterine homogenates showed no evidence of

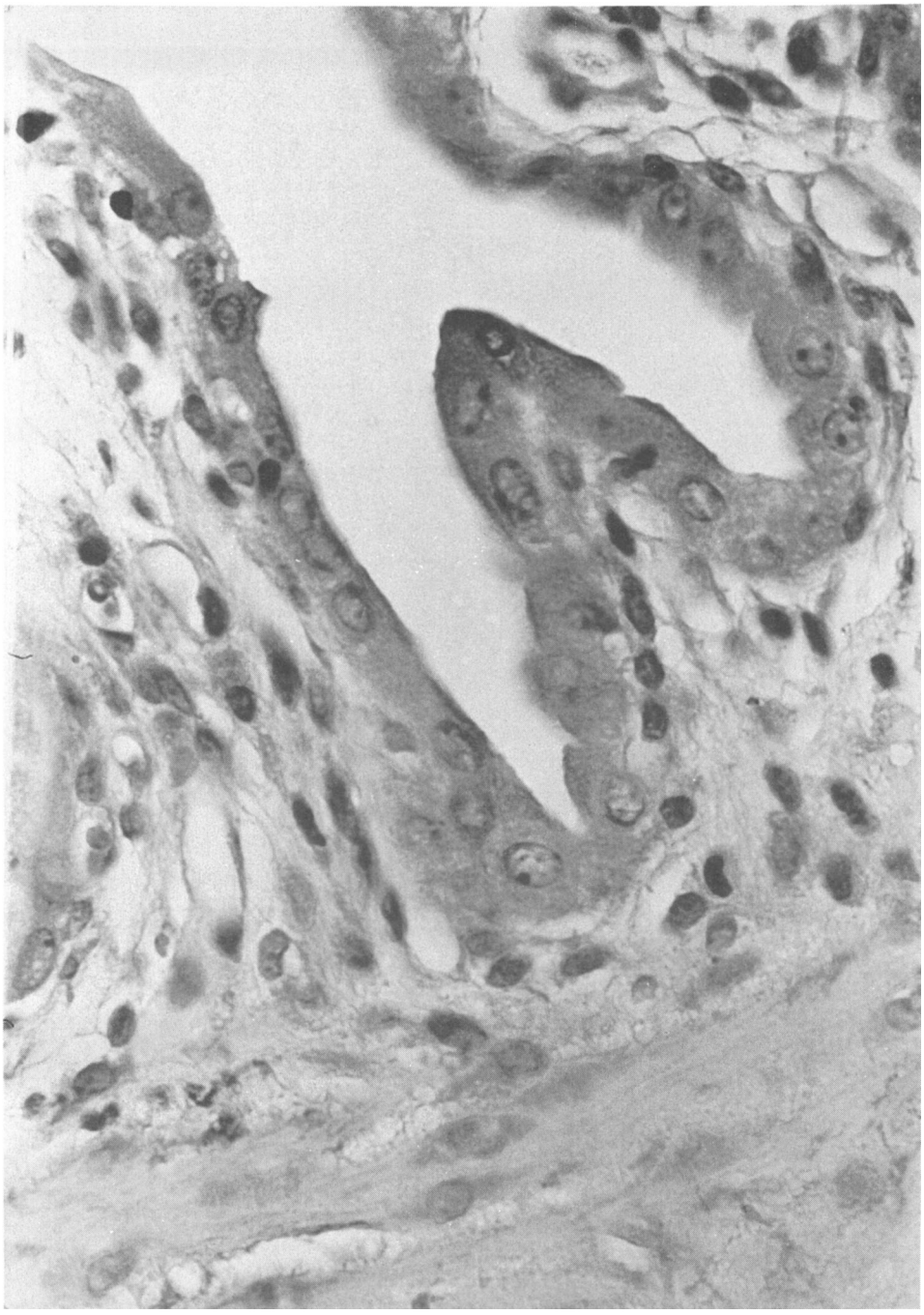

FIG. 1 (Plates 1–5). Transverse 6 μm histological sectons of a 17-day pregnant rat uterus stained with Verhoeff's elastic fiber stain. PLATE 1. Endometrium showing absence of elastin fibers (40× mag).

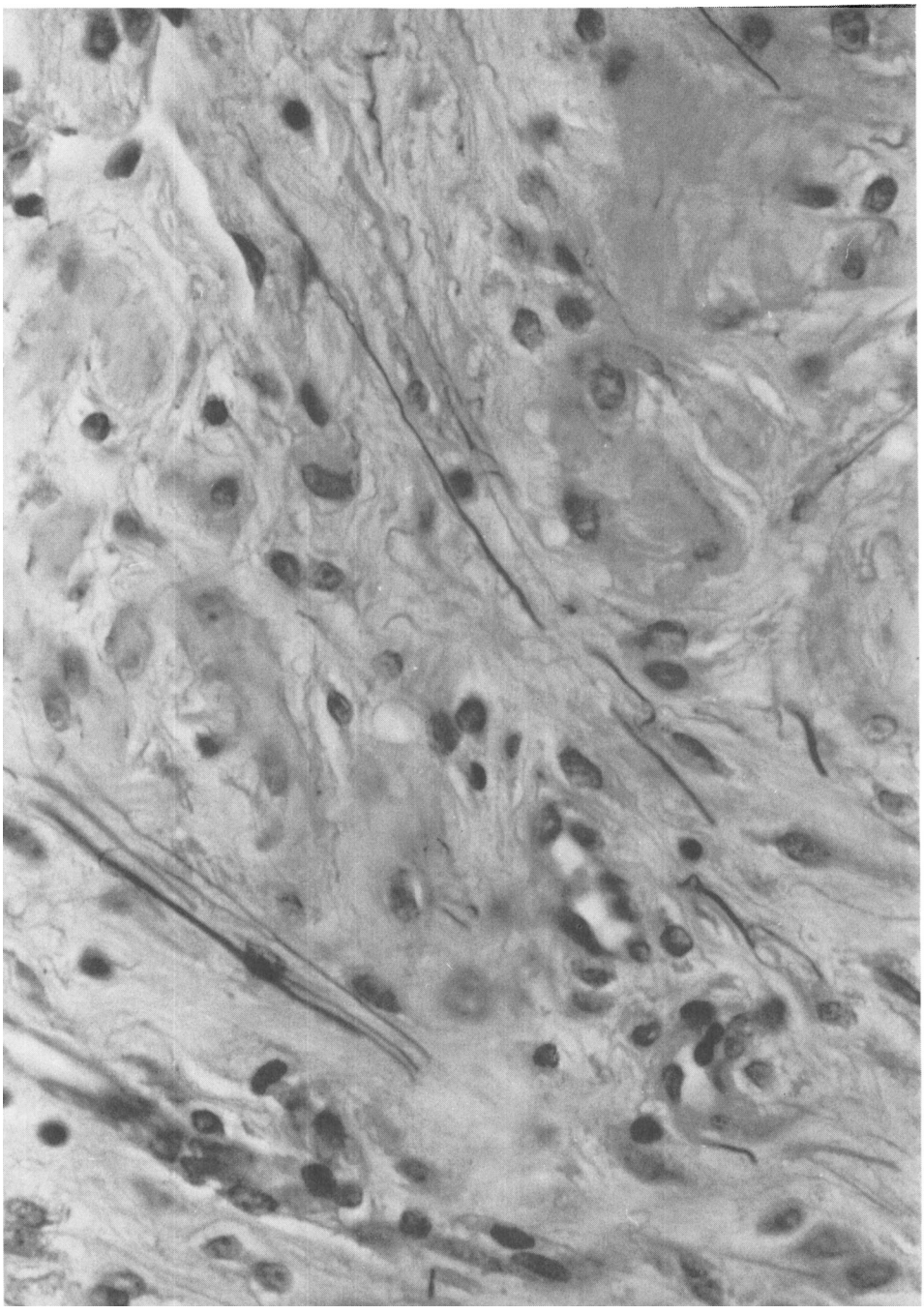

PLATE 2. Myometrium depicting elongated elastin fibers (40× mag).

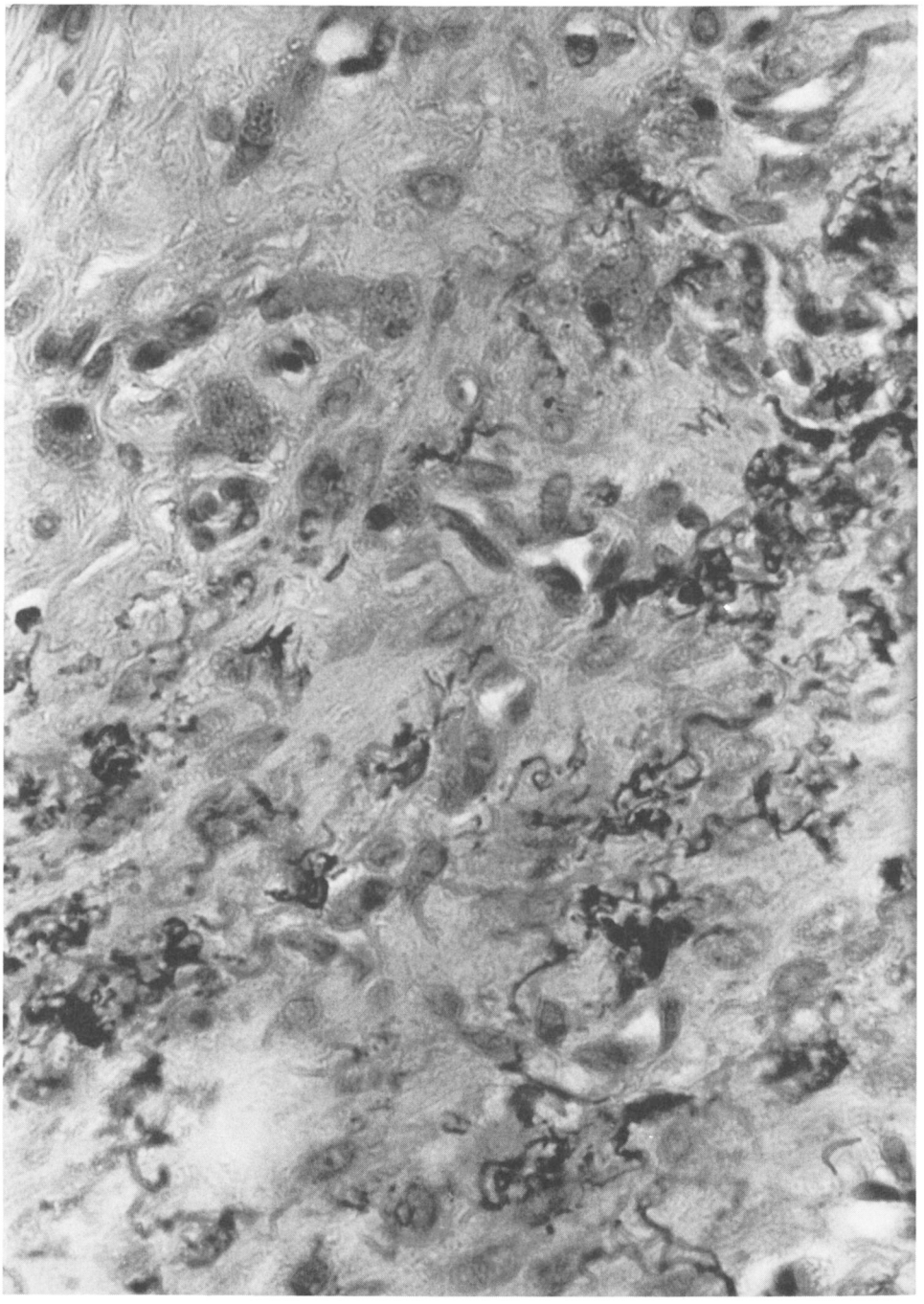

PLATE 3. Myometrium showing short, curly elastin fibers (40× mag).

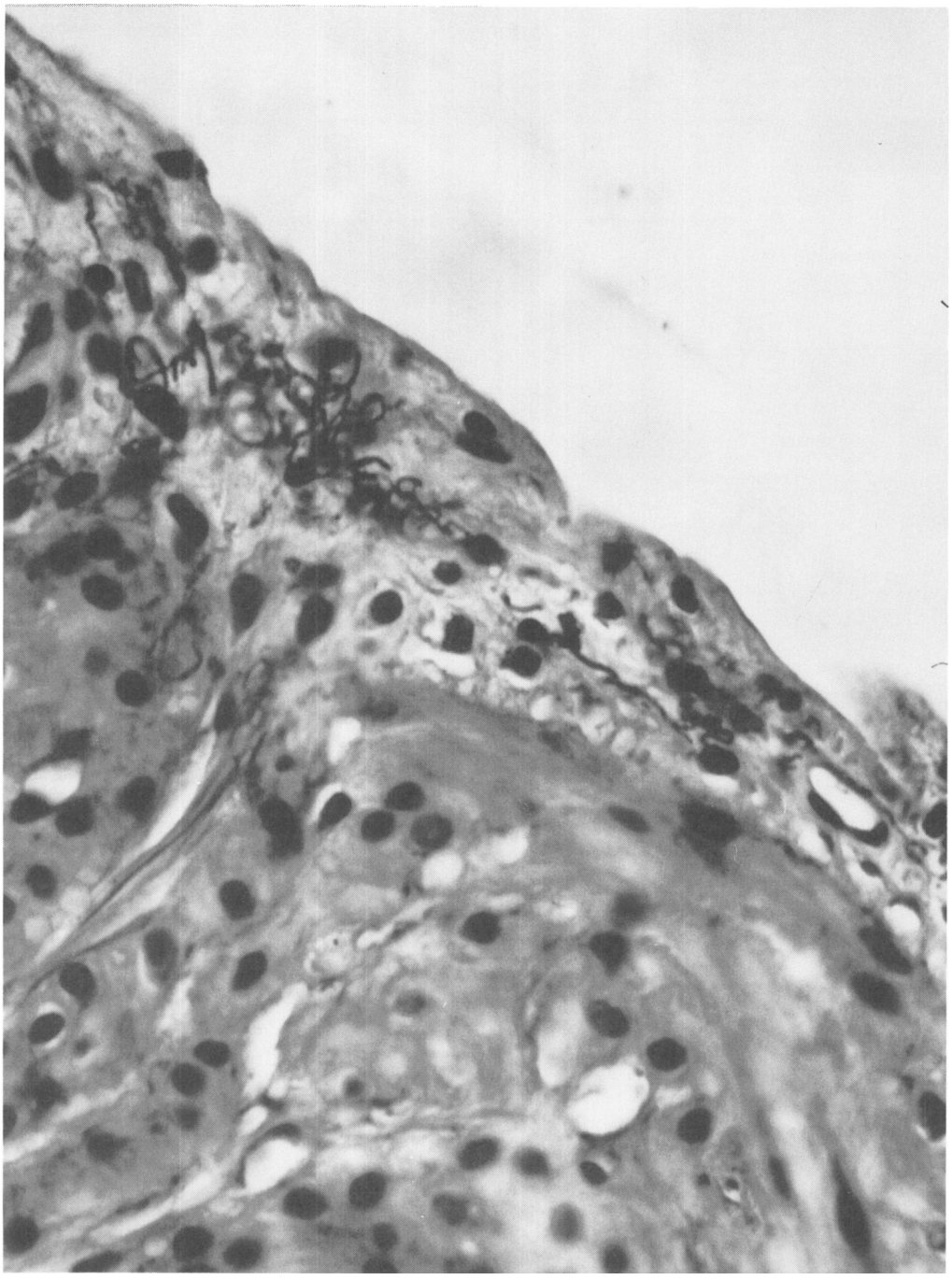

PLATE. 4. Stratum subserosum depicting elastin fibers (40× mag).

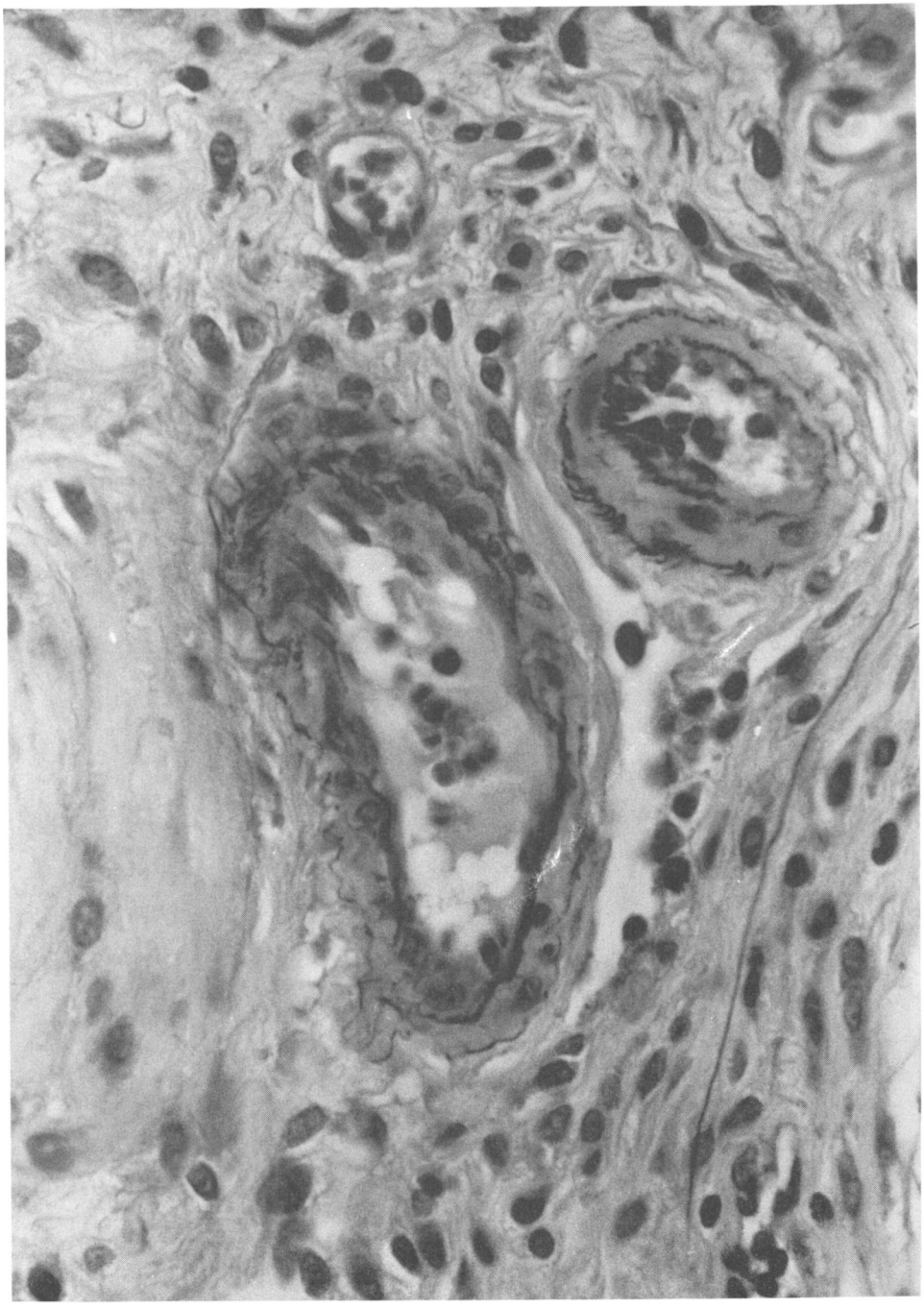

PLATE 5. Vasculature in the myometrium showing a rich network of elastin fibers in the vessel wall (40× mag).

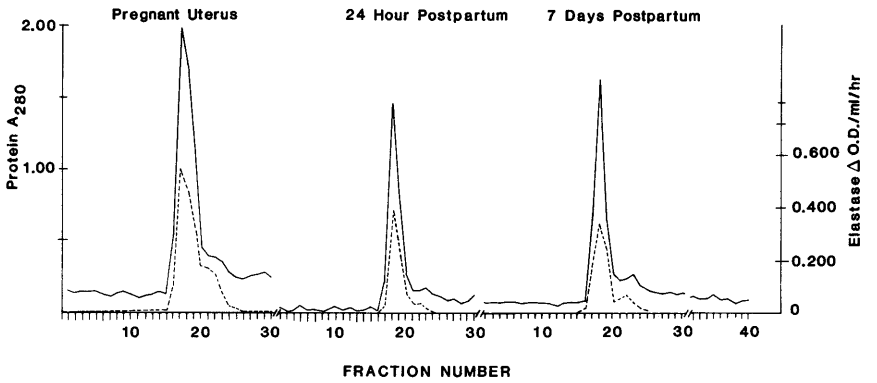

FIG. 2. Elastase activity of uterine extracts following chromatography on a Ultrogel AcA 54 column. Solid line represents OD 280 nm and the dotted line represents elastase activity using SAPNA (ΔOD/ml/hr).

elastase activity. This raised the possibility that either an elastase inhibitor was present or an enzyme was bound to native uterine elastin and was not available for the elastin-rhodamine substrate. Efforts to free the enzyme from endogenous elastin by raising the pH to 12 (15) were not successful. Several other attempts to measure elastase activity in whole homogenates, such as homogenizing the tissue in Triton X-100, freeze-thawing, trypsin activation, ammonium sulfate precipitation, and lowering the pH, were also ineffective. Similar negative results were obtained with the very sensitive SAPNA substrate, suggesting that one or more elastase inhibitors may be present or the elastase was present in quantities to low to detect.

Chromatography of the homogenates from a pregnant uterus, a 24 hr postpartum and 7 day postpartum uterus on the AcA 54 column is shown in Fig. 2. With all three homogenates elastolytic activity could be detected eluting close to the void volume, after a minimum of 24 hr incubation with SAPNA. Standard porcine pancreatic elastase eluted in fraction 31 when chromatographed on the same column. The elastase containing fractions from three 24-hr postpartum uteri were pooled, dialyzed against 0.02 *M* Tris, pH 8.8, and chromatographed on DEAE cellulose (Fig. 3). Elastase activity eluted about one third of the way through the gradient in fractions 11–14. When the column fractions were assayed for the presence of elastase inhibitor (TIC) against porcine pancreatic elastase, we were surprised to find a potent inhibitor in the same fractions (11–14) as the uterine enzyme. When the fractions containing the enzyme were pooled, dialyzed against 0.02 *M* Tris, pH 8.8, and rechromatographed on an FPLC Mono Q column, the elastase activity and inhibitor showed some separation but still overlapped (Fig. 4). However, when these fractions (14–17) were pooled, dialyzed against starting Tris buffer, and rechromatographed on the Mono Q column with a slower gradient, the elastase activity was clearly separated from the inhibitor (Fig. 5).

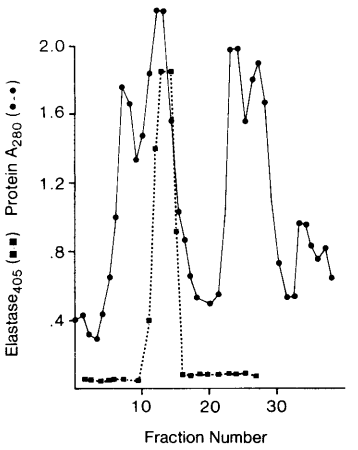

FIG. 3. Chromatography of the Ultrogel AcA 54 elastase fractions on a 1.5 × 25 cm column of DEAE cellulose. Solid line represents OD 280 nm and dotted line represents elastase activity using SAPNA.

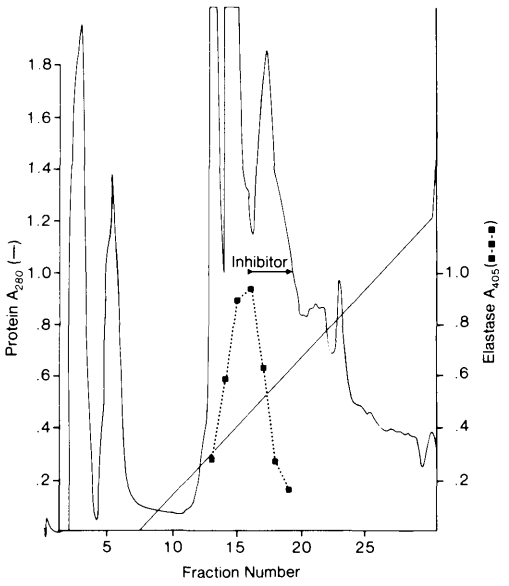

FIG. 4. Chromatography of the DEAE elastase fractions on a Pharmacia Mono Q column. The solid line represents OD 280 nm. The dotted line represents elastase activity (ΔOD/ml/hr) and the arrow includes the fractions containing an elastase inhibitor.

Separation of the inhibitor from the active uterine elastase enzyme was also seen following SDS–PAGE (Fig. 6). When pooled fractions containing the elastase from the Mono Q column in Fig. 4 were electrophoresed, the enzyme moved a distance corresponding to a molecular weight of 24,000–26,000, while the inhibitor was considerably larger with a molecular weight of approximately 45,000. Standards of porcine and rat pancreatic elastase were run on the same gel and gave molecular weights of 26,000 and 24,000 respectively. All three enzymes eluted from the gel were active against both SAPNA and elastin-rhodamine, indicating that the uterine enzyme can be considered a true elastase.

Due to the apparent low levels of enzyme (<1 μg/uterus) and the possible inhibitor complications, the specific activity of the elastase was not tabulated at each purification step, nor did we attempt to achieve absolute purity in quantities enough for amino analysis. All further characterization of the enzyme employed the partially purified enzyme following chromatography on Mono Q.

The results from adding various inhibitors of serine proteases and metalloproteases to the uterine elastase are shown in Table I. The profile was typical of serine proteases, with PMSF and SBTI giving good inhibition and metal chelating agents having no effect. Puri-

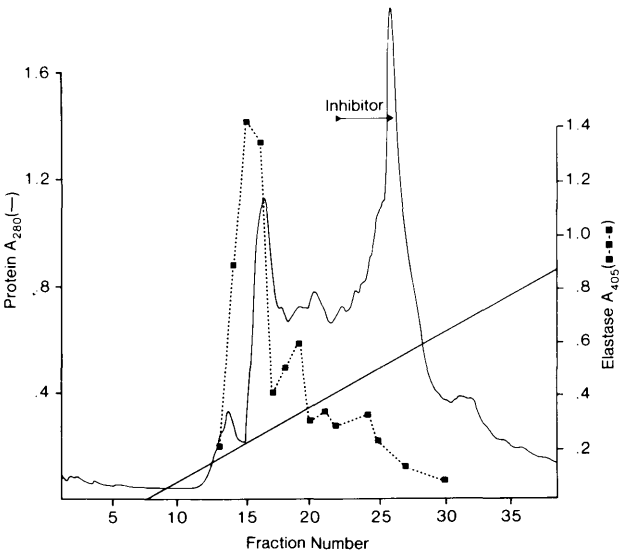

FIG. 5. Rechromatography of the elastase fraction from Fig. 4 on a Mono Q column with a reduced salt gradient. The solid line represents OD 280 nm and the dotted line represents elastase activity (ΔOD/ml/hr). Fractions containing the elastase inhibitor are included in the arrow.

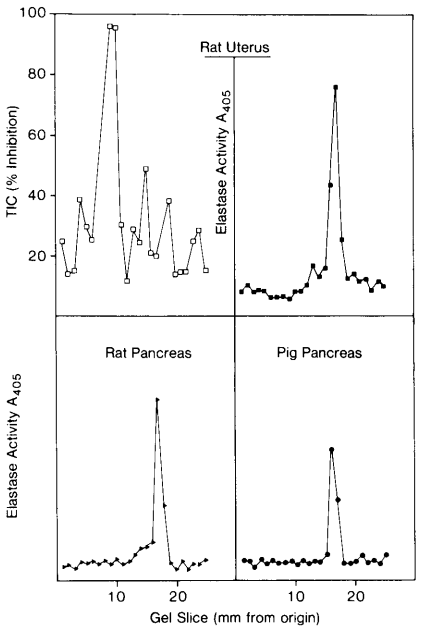

FIG. 6. SDS–acrylamide gel electrophoresis of the pooled elastase fractions from the Mono Q column in Fig. 5 compared with purified elastase standards. Each point represents a 2-mm gel slice. TIC (□), uterine elastase (■), rat pancreatic elastase (▲), and pig pancreatic elastase (●) were measured using SAPNA substrate.

fied human α-1-antiprotease was also an effective inhibitor of the uterine elastase (Fig. 7).

The pH optimum of the uterine elastase was rather broad with maximum activity around pH 8.5 (Fig. 8).

The amount of serum present in uterine homogenates was estimated by comparing ceruloplasmin activity in the 40,000*g* supernatant with serum activity from the same rat. Prior to and following parturition, when vascularization was maximum, the homogenate contained around 6% serum. This value rapidly declined and was to low to be measured 7 days postpartum.

Discussion. The extracellular elastin present in the pregnant rat uterus was confined to the myometrium layer as vascular elastin and in what appears to be a rather random and patchy network of fibers in the extracellular matrix. What role these elastin fibers have is unknown, but they may be important in allowing uterine elasticity in more than one direction during growth of the fetus. A separate histological study is being conducted to follow the metabolism of these elastin fibers during pregnancy and the postpartum period.

The rapid loss of elastin during the postpartum period is undoubtedly initiated by the action of an elastase. Such an enzyme was observed in uterine extracts that appears capable of elastin proteolysis. Inhibition by PMSF demonstrates that the rat uterine elastase can be classified as a serine protease and not a metalloprotease, aspartic, or cysteine protease as judged by its full activity in the presence of EDTA, pepstatin, and NEM, respectively. Interestingly, both the rat pancreatic and the uterine elastase were inhibited by soybean trypsin inhibitor while the porcine enzyme was not inhibited at all. All three elastases were strongly inhibited by the elastase inhibitor from the marama bean which in many other respects resembles the soybean (16). The uterine enzyme is clearly not of macrophage origin, since metal chelators have no effect on its activity and macrophage elastases of both the mouse and the rat have been shown to be metalloproteases and not serine proteases (17, 18). The enzyme reported here has many properties in common with polymorphonuclear leukocyte elastase (PMN or neutrophil elastase). Human and rat PMN elastase are both inhibited by PMSF, SBTI, and α-1-AT and have a ph optimum in the same range as the uterine

TABLE I. INHIBITION OF ELASTASES BY PROTEASE INHIBITORS[a]

	Percent inhibition		
	RUE	RPE	PPE
1 m*M* EDTA	6	0	18
1 m*M* EGTA	12	0	19
0.1 m*M* PMSF[b]	96	9	76
0.5 m*M* PMSF	—	31	—
1 m*M* NEM	0	0	23
PEP 0.1 mg/ml	0	0	22
SBTI 0.02 mg/ml	100	50	0
MBI 0.02 mg/ml	55	97	91

[a] Rat uterine elastase (RUE), rat pancreatic elastase (RPE), and porcine pancreatic elastase (PPE).

[b] PMSF; Phenylmethylsulphonyl fluoride, NEM; *N*-ethylmaleimide, SBTI; soybean trypsin inhibitor, MBI; marama bean inhibitor, and PEP; pepstatin.

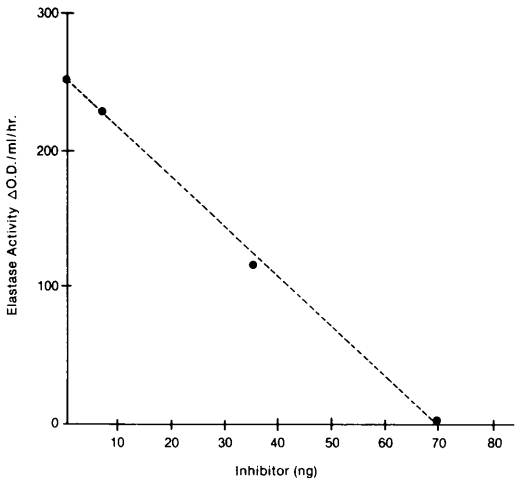

FIG. 7. Inhibition profile of uterine elastase taken from the Mono Q column against purified human α-1-antiprotease.

enzyme (18, 19). Rat PMN elastase also has a molecular weight in the same range as the uterine enzyme (24,000–26,000) whereas the human elastase is somewhat larger (30,000). However, our studies indicated that the uterine enzyme had several chromatographic properties that were distinct from rat neutrophil elastase. The rat neutrophil enzyme is similar to human neutrophil elastase and the pancreatic elastases, in that they bind to CM cellulose and do not stick to DEAE. The rat uterine enzyme behaved just the opposite, a fact that may reflect a difference in charge, or perhaps an indication that it was complexed to another protein. Several studies investigating leukocytes in the pre- and postpartum uterus have found that PMN leukocytes do not reside in the myometrium (20, 21). Although leukocytes may be present in the lumen and endometrium, these cells do not migrate into the myometrium and therefore PMN elastase probably would not exert its action on the myometrial elastin. In addition, the rat uterine elastase reported here is present prepartum and not just during involution, when the majority of phagocytes arrive at the uterine site. This does not eliminate the possibility of the elastase that we have isolated as being PMN elastase, since we homogenized the whole uterus and not just the myometrium. There could also be some contribution from blood PMN's.

This raises a perplexing question on not only the origin of the elastase, but what controls the level of activity so that we detected the enzyme during elastin accumulation in the uterus and postpartum. An inhibitor is definitely present that may be involved in regulation. The nature of the inhibitor is unknown. An obvious choice would be α-2-macroglubulin or α-1-antiprotease that could arise from residual serum in the homogenate. Ceruloplasmin assays comparing serum and homogenates confirm the presence of serum in the uterine homogenate. The highest level that we have seen represents about 6% serum in the homogenate. This would contribute close to 150 ug of α-1- or α-2-macroglobulin per milliliter of homogenate, certainly more than enough to inhibit all the elastase present. On the other hand, when we have looked at uteri taken several days postpartum, when most of the vascularization has receded, we cannot detect ceruloplasmin activity in the homogenate. This would indicate very low levels of serum contamination, yet the inhibitor was still present in quite high levels. We have also observed that the uterine inhibitor does not inhibit trypsin, which also suggests that it is

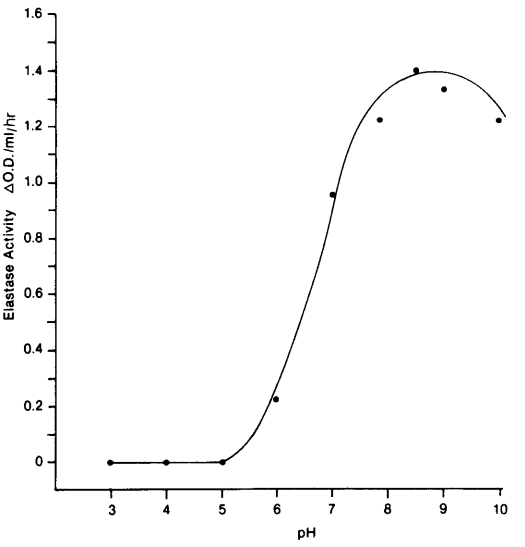

FIG. 8. Activity of uterine elastase at different pH levels. Buffers used were 0.1 *M* sodium acetate, pH 3.4 and 5; 0.1 M sodium phosphate, pH 6, 7, and 8; and 0.1 *M* Tris–HCl, pH 8.5, 9, and 10.

not α-1-antiprotease. Another question is why the inhibitor did not bind the uterine elastase and inhibit enzyme activity during chromatography on DEAE or Mono Q. In a similar study we have found that when pancreatic elastase is added to the uterine extract and subsequently chromatographed on Mono Q, the enzymatic activity was completely lost. A uterine inhibitor of an alkaline protease has been described by Afting (22), who concluded that the inhibitor was derived from the tissue, not the serum. Levels of this inhibitor were greater in the prepartum uterus, raising the possibility of a regulatory function. Further work is required before we can state whether the elastase inhibitor is of tissue origin or comes from residual serum and what role it may play in elastin metabolism.

The uterine elastase described here appears distinct from pancreatic, macrophage, and neutrophil elastase. An elastase of tissue origin, arising from fibroblasts or smooth muscle cells of the myometrium would provide an important means of coordinating elastin turnover with elastin synthesis. If tissue derived elastases are present in other organs such as the lung, they could also contribute to the development of certain pathological conditions such as emphysema.

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