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Research Article

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Role of α-Macroglobulin-Elastase Complexes in the Pathogenesis of Elastase-induced Emphysema in Hamsters

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ABSTRACT Radiolabeled, enzymatically active or chloromethyl ketone-inactivated porcine pancreatic elastase was endotracheally instilled into hamsters. Gel filtration of the bronchopulmonary lavage fluid revealed two major radioactive fractions: one, eluting at 780,000 daltons, corresponding to an α-macroglobulin-pancreatic elastase complex, and another, at 68,000 daltons, corresponding to an α-1-protease inhibitor-pancreatic elastase complex. Elastolytic activity was recovered in the bronchopulmonary lavage fluid up to 4 d after elastase instillation and was associated with the α-macroglobulin-pancreatic elastase complex. Small amounts of this complex were recovered 15 d after instillation. When <1% (1.5–1.7 μg) of the usual dose of elastase was instilled into hamsters, the major radioactive complex was α-1-protease inhibitor-pancreatic elastase complex, and little or no elastolytic activity was found in the lavage fluid. In contrast to the instillation of 220 μg of elastase, no disease or hemorrhagic reaction was detected with this low dose, and without hemorrhage only insignificant amounts of α-macroglobulin-pancreatic elastase complexes were recovered from the lungs. To study the interaction of circulating antiproteases with elastase, hamster plasma was allowed to interact directly with the radiolabeled elastase; α-macroglobulin bound much more of the elastase than α-1-protease inhibitor, confirming the findings in the lung lavage experiments. The hamster's susceptibility to pancreatic elastase-induced emphysema may depend on the preferential binding of elastase to α-macroglobulin, which protects the elastolytic potential, rather than to α-1-protease inhibitor, which inactivates elastase. We speculate that if even a fraction of the residual radioactivity found in the hamster lungs as long as 144 d after instillation of elastase represents enzymatically active α-macroglobulin-pancreatic elastase complex, this could serve as a source of persistent elastolytic activity, which might explain the progressive nature of the pulmonary lesion.

INTRODUCTION

Evidence has accumulated that demonstrates the importance of elastolytic mechanisms in the genesis of experimental enzyme-induced emphysema (1). Investigators (2) have reported the progression of the lesion in hamsters for many months after a single instillation of elastase. Data obtained to date support the concept of long-term retention of elastase after its instillation into the lungs (3). 144 d after treatment with enzymatically active ^{3}H-methylated porcine pancreatic elastase (^{3}H-Me-PE),[1] significant levels of radioactivity remained in the lungs, whereas virtually no radioactivity was found in the lungs of hamsters 144 d after treatment with inactivated elastase. However, there is no direct evidence that this residual radioactivity represents active enzyme. In the studies reported in the present communication, we employed ^{3}H-Me-PE to measure the distribution of elastase–antielastase complexes that are present after instillation. The results of these studies suggest an important role for

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[1] *Abbreviations used in this paper*: ^{3}H-Me-CMK-PE, ^{3}H-methylated porcine pancreatic elastase inactivated by *N*-acetyl-(L-alanyl)$_3$-L-alanine chloromethyl ketone; ^{3}H-Me-PE, ^{3}H-methylated porcine pancreatic elastase; α-M, α-macroglobulin; α-1-PI, α-1-protease inhibitor; PiMM and PiZZ, phenotypes MM and ZZ of alpha-1-protease inhibitor, respectively.

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α-macroglobulin-elastase complexes in the pathogenesis of experimental emphysema in the hamster and further suggest that the partitioning of elastase between the complexes formed with α-1-protease inhibitor (α-1-PI) and α-macroglobulin (α-M) is a decisive factor in the emphysema lesion.

METHODS

Experimental emphysema model. Male golden hamsters, *Mesocricetus auratus*, weighing 100–115 g, were used for all animal studies (Engle Laboratory Animals, Inc., Farmersburg, IN). The emphysema-inducing potency of the radiolabeled elastase preparations were compared with unlabeled elastase by transoral intratracheal instillation in hamsters as previously described (3, 4). Other hamsters were instilled with the radiolabeled elastase preparations and later anesthetized and killed by bronchopulmonary lavage at the indicated study time. Bronchopulmonary lavage fluid, plasma, and urine samples were collected as previously described (3).

Tritiated methylated elastase preparations. Porcine pancreatic elastase was purified by the method of Shotton (5) and exhibited the expected amino acid composition (3, 5). The esterolytic and elastolytic activities of elastase preparations were measured as previously described (3, 6). The optical density at 280 nm was used to determine the enzyme concentration ($E^{1\%}_{1\,cm,\,280\,nm}$ = 20.2) (5). Active site titration was carried out with *p*-nitrophenyl-3-(*N*-acetyl-L-alanyl-L-alanyl)-2-methyl carbazate, an azapeptide compound that was kindly provided by Dr. James C. Powers, Georgia Institute of Technology, Atlanta, GA. (7). By this method, the elastase was found to be 95±6% (mean±SE, n = 4) active. Tritiation of porcine pancreatic elastase by reductive methylation was carried out as previously described and did not appear to alter the behavior of the enzyme in vitro or in vivo (3, 8). Hamsters were instilled with a low dose (1.5 or 1.7 μg) of high specific radioactivity ^{3}H-Me-PE (5–9 × 10^5 cpm) or a high dose (160, 180, or 220 μg) of low specific radioactivity ^{3}H-Me-PE (5–9 × 10^5 cpm). Inactivated radiolabeled elastase preparations (^{3}H-methylated porcine pancreatic elastase inactivated by *N*-acetyl-(L-alanyl)$_3$-L-alanine chloromethyl ketone; ^{3}H-Me-CMK-PE) were prepared as previously described and served as controls (3).

Molecular sieve chromatography. Molecular sieve chromatography was performed on aliquots of lavage fluid, plasma, and collected urine at 5°C on columns (1.5 × 100 cm) containing Sephadex G-100, Sephadex G-200, or Sepharose 6B (Pharmacia Fine Chemicals Div. of Pharmacia Inc., NJ) with buffer systems (0.05 M Tris, pH 7.6, 0.14 M NaCl, and 0.05% NaN_3) described previously (9). Each collected fraction was assessed for absorption of light at 280 nm, and aliquots were taken for determination of radioactivity and elastolytic activity as previously described (3). Appropriate standards were used to calibrate the molecular sieve columns.

Antisera. Antisera to porcine pancreatic elastase inactivated with *N*-acetyl-(L-alanyl)$_3$-L-alanine chloromethyl ketone were prepared in rabbits and partially purified by ammonium sulfate precipitation (10). Ouchterlony double-diffusion studies, immunoprecipitation of ^{3}H-Me-CMK-PE and ^{3}H-Me-PE, and studies using antisera to inactivate the elastolytic activity of native porcine pancreatic elastase all indicated a titer of ~150–200 μg of elastase/ml of antiserum.

RESULTS

Elastolytic activity in bronchopulmonary lavage fluid

Elastolytic activity was found in the lavage fluid supernatant up to 4 d after instillation of the high dose

TABLE I
Elastolytic Activity Measured in Bronchopulmonary Lavage Fluid

	Time after instillation of 220 μg of ^{3}H-Me-PE				
	4 h	6 h	1 d	2 d	4 d
A. Lavage fluid supernatant before chromatography					
Tritiated elastase equivalents, μg*	77.9	52.7	33.7	4.6	0.66
Enzymatically active elastase equivalents, μg‡	28.0	17.8	9.4	0.55	0.042
B. Recovered in α-M-elastase complex after molecular sieve chromatography of lavage fluid supernatant					
Percent recovery of radioactivity from column	81	70	67	73	98
Tritiated elastase equivalents, μg§	46.1	26.6	13.7	1.3	0.061
Enzymatically active elastase equivalents, μg‡	56.7	28.1	14.7	1.2	0.042
Percent enzymatically active elastase in α-M-elastase complex‖	123	106	107	92	69

* Tritiated elastase equivalents were calculated by dividing the total radioactivity lavaged from one hamster by the specific radioactivity of the instilled ^{3}H-Me-PE (4.1 × 10^6 cpm/mg).

‡ Determined by the [^{3}H]elastin-SDS solubilization assay. Values are expressed as microgram equivalents of standard porcine pancreatic elastase preparations.

§ Tritiated elastase equivalents in the α-M-elastase complex of lavage fluid from one hamster were calculated by multiplying the tritiated elastase equivalents recovered from the column in the α-M-elastase fractions by the ratio of the entire lavage fluid volume to the volume of lavage fluid applied to the column.

‖ (Micrograms of catalytically active elastase per microgram of tritiated elastase equivalents) × 100.

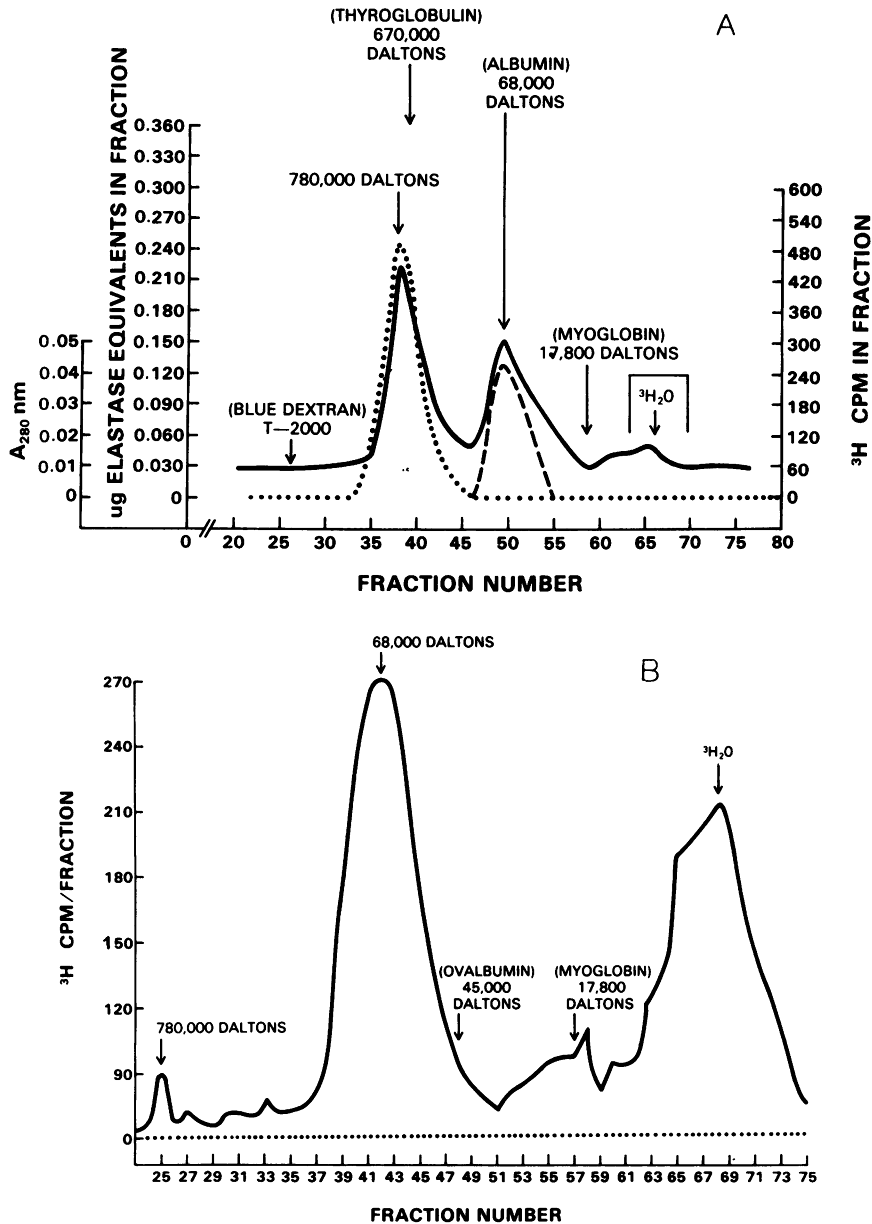

FIGURE 1 The molecular weight distribution of elastase complexes in lavage fluid from instilled hamsters. Lavage fluid supernatant was obtained from hamsters 6 h after instillation with 220 μg of ^{3}H-Me-PE (A), 1.5 μg of ^{3}H-Me-PE (B), 145 μg of ^{3}H-Me-CMK-PE (C), or 1.3 μg of ^{3}H-Me-CMK-PE (D). An aliquot of this lavage fluid was placed on a Sepharose 6B column (A) (1.5 × 100 cm) with a flow rate of 6 ml/h or on a Sephadex G-200 (*B* and *C*) or G-100 (D) molecular sieve column (1.5 × 100 cm) with a flow rate of 12 ml/h. In all cases, 3-ml fractions were collected. The void volume radioactivity recovered from the G-200 column (B) was identified as a 780,000-dalton complex by chromatography of a 2-ml aliquot of the original lavage fluid on a Sepharose 6B column (presented in Table II *A*). The volume and radioactivity of the lavage fluid samples placed on the columns were 1 ml and 16,000 cpm, 1.5 ml and 7,600 cpm, 1 ml and 8,000 cpm, and 3 ml and 4,300 cpm, respectively, for the samples in *A–D*. Each fraction was assessed for absorbance at 280 nm (– – –), tritium radioactivity (——), and elastolytic activity (· · ·). The elution positions of molecular weight standards are indicated.

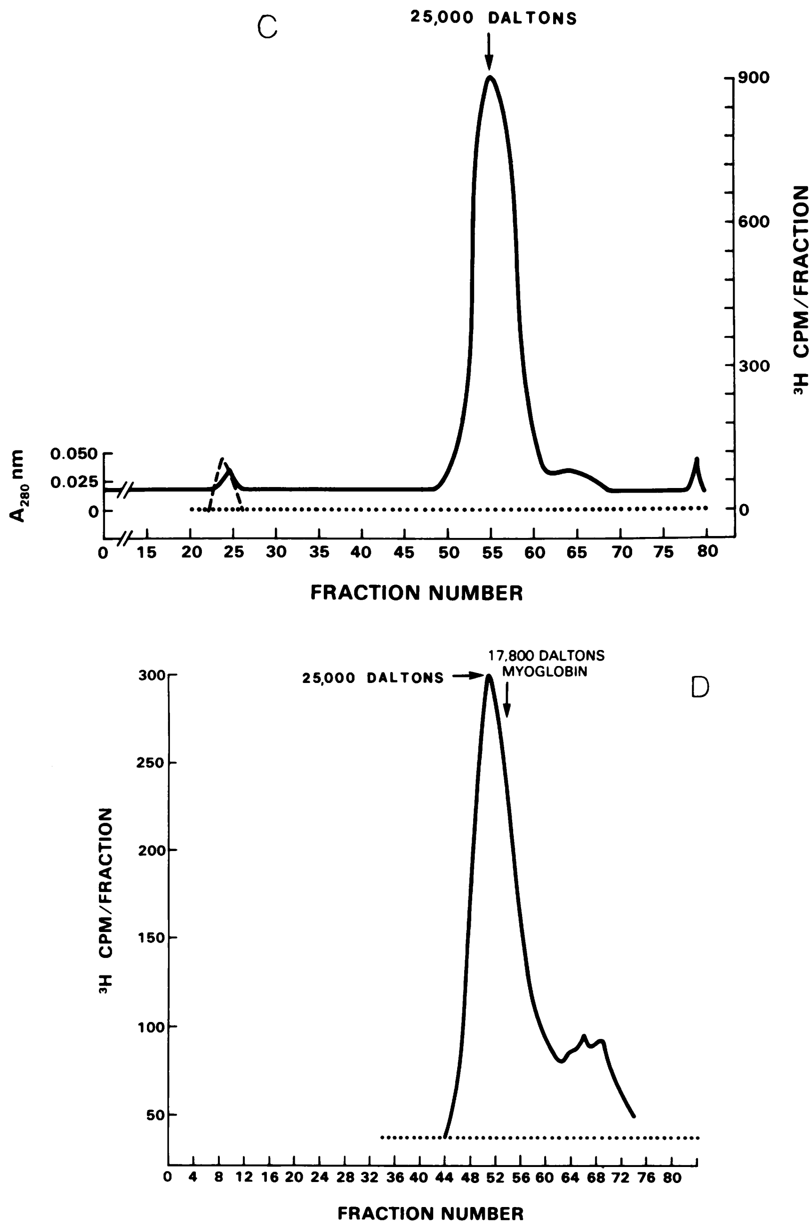

FIGURE 1 (*Continued*)

of ^{3}H-Me-PE (Table I *A*); decreasing amounts were measured as the time interval between instillation and lavage increased. This enzymatic activity was inhibited by the addition to the lavage fluid of *N*-acetyl-(L-alanyl)$_3$-L-alanine chloromethyl ketone. No elastolytic activity could be measured in lung lavage fluid supernatant from animals instilled with saline, ^{3}H-Me-CMK-PE or the low dose of ^{3}H-Me-PE. The homogenized cell pellet from lung lavage fluid of saline-treated hamsters exhibited no elastolytic activity but did inhibit exogenous elastase. The cell pellet from hamsters treated with the high dose of elastase was not tested because of the presence of large numbers of erythrocytes (3).

Molecular sieve chromatography of lung lavage fluid

High dose. Molecular sieve chromatography of lavage fluids from hamsters instilled with the high dose of ^{3}H-Me-PE revealed four radioactive components (Fig. 1 *A* and Table II *A*): 780,000- and 68,000-dalton

TABLE II

Molecular Weight Distribution of Radioactivity after Instillation of Hamsters with Radiolabeled Porcine Pancreatic Elastase

Dose instilled / Study time	No. of samples analyzed	Percent of initial dose recovered‡	Percent recovery from column	Distribution of recovered material: 780,000 daltons (α-M-elastase)	200,000 daltons	68,000 daltons (α-1-PI-elastase)	25,000 daltons (free)	<2,000 daltons (degraded)
						%		
A. Bronchopulmonary lavage fluid supernatant[*]								
^{3}H-Me-PE high dose								
1 h	3	61±12	94±2	16±3		12±3	65±5	8±1
4 h	3	43±3	90±2	61±5		27±0.3	6±4	6±1
6 h	3	37±9	85±10	63±9		23±2	9±8	6±2
1 d	3	11±2	85±10	57±2		28±3	0	15±5
2 d	3	1.8±0.1	84±10	18±11		24±5	0	58±15
4 d	1	0.4	98	10		14	0	76
^{3}H-Me-PE low dose								
1 h	2	58±1	86±1	4±0.5		71±8	7±2	18±5
4 h	2	37±1	89±11	3±0.3		72±1	4±1	21±1
6 h	3	26±7	79±3	3±1		64±8	4±2	29±11
1 d	3	3±1	72±9	9±8		28±8	0	62±7
^{3}H-Me-CMK-PE high dose								
6 h	3	37±7	86±10	1±0.3		0	96±2	3±2
1 d	3	3±1	87±10	1±0.8		0	83±3	16±4
^{3}H-Me-CMK-PE low dose								
6 h	1	7	90	0		0	74	26
1 d	1	0.7	55	0		0	53	47
B. Plasma[§]								
^{3}H-Me-PE high dose								
4 h	2	4±1	102±17	5±1	0	44±2	0	51±1
6 h	3	2±0.3	103±18	7±2	2±1	34±4	0	55±6
1 d	3	4±0.3	77±11	7±1	2±1	29±3	0	62±2
2 d	2	4±0.2	102±9	5±0.5	0	15±2	0	80±2
^{3}H-Me-PE low dose								
4 h	2	3±1	81±0.5	0	0	11±0.5	0	89±0.5
6 h	2	3±0.5	91±10	3±2	0	15±0.5	0	82±2
1 d	3	5±1	85±12	2±1	1±0.8	8±1	0	90±3
^{3}H-Me-CMK-PE high dose								
6 h	3	2±0.5	83±15	2±1	0	0	49±3	49±4
1 d	3	2±0.3	62±16	0	0	0	2±1	98±2
^{3}H-Me-CMK-PE low dose								
6 h	1	3	117	6	0	0	0	94
C. Urine[‖]								
^{3}H-Me-PE high dose								
0–6 h	3	5±3	92±7	0.4±0.2		0.03	0.2±0.1	99±0.3
^{3}H-Me-PE low dose								
0–6 h	2	6±5	90±18	0.5±0.1		0	2±1	97±1

TABLE II (*Continued*)

Dose instilled / Study time	No. of samples analyzed	Percent of initial dose recovered‡	Percent recovery from column	Distribution of recovered material: 780,000 daltons (α-M-elastase)	200,000 Daltons	68,000 Daltons (α-1-PI-elastase)	25,000 Daltons (free)	<2,000 Daltons (degraded)
						%		
^{3}H-Me-CMK-PE high dose								
0–6 h	2	9±3	97±10	0.5±0.4		0	73±1	26±1
^{3}H-Me-CMK-PE low dose								
0–6 h	1	11	92	0.3		0	5	95

* Sephadex G-100, Sephadex G-200, and Sepharose 6B chromatography were used to identify the molecular weights of the tritium-containing fractions of lavage fluid. The absorption of light at 280 nm and the elastolytic activity against tritiated insoluble ligament elastin were determined for each fraction (see Fig. 1 and Table I). The values presented are the mean±1 SE except where $n = 2$, in which case the range is given.
‡ The radioactivity recovered in the sample was expressed as the percent of the initially instilled dose. An aliquot of this sample was then applied to the column.
§ The plasma from 1 ml of heparinized blood obtained from hamsters at the study time indicated was centrifuged (1,000 *g*) and chromatographed as indicated in footnote *.
‖ Urine was collected between the indicated time points. Chromatography was performed on collected urine as indicated in footnote *.

complexes, which are most likely elastase complexes with α-M and α-1-PI, respectively; free elastase, 25,000 daltons; and degraded elastase, <2,000 daltons. Lavage fluid harvested 1 h after instillation was the only sample that contained significant amounts of free ^{3}H-Me-PE; this comprised 65% of the radioactivity recovered and 40% of the ^{3}H-Me-PE initially instilled. In all later samples, elastolytic activity was only associated with the α-M-elastase complex, although traces of free elastase were found in the 4- and 6-h samples (Fig. 1 *A*, Table II *A*). Bronchopulmonary lavage fluid removed from three hamsters 15 d after instillation of 160 μg of ^{3}H-Me-PE was found, after pooling and concentration (UM-10 filter, Amicon Corp., Lexington, MA), to contain 0.009 μg of elastase complexed with α-M. No α-1-PI-elastase was found. Freezing and thawing of lavage fluid samples appeared to dissociate the α-M-elastase complex. Such dissociation in the presence of α-1-PI may have lowered the level of elastolytic activity measured in the prechromatographed lavage fluid as compared with the amount of elastase associated with the α-M-elastase after chromatography (Table I). Paradoxically, the elastase associated with this α-M-elastase complex generally exhibited greater catalytic activity against elastin-sodium dodecyl sulfate (SDS) than a comparable amount of elastase alone. Chloromethyl ketone inhibited the elastolytic activity and did not disaggregate the ^{3}H-Me-PE from α-M-elastase as determined by rechromatography. Lavage fluid from treated hamsters was centrifuged at 25,000 *g* to separate any membrane fragments that might have bound elastase; this procedure had no effect on the chromatographic or elastolytic pattern. In early experiments we examined the possibility that the 780,000-dalton complex was produced in a nonspecific manner: ^{3}H-Me-PE was mixed with bovine serum albumin and then chromatographed. The radioactivity and enzyme activity were both found in the 25,000-dalton fractions. When ^{3}H-Me-PE was incubated with unlabeled ligament elastin and the supernatant from the partially digested elastin pellet was chromatographed on Sephadex G-100, the radioactivity and enzyme activity were found in the 25,000-dalton fractions; however, 3% of the radioactivity and 2% of the elastolytic activity were found in fractions associated with the void volume and may have represented aggregates of elastase with solubilized elastin.

In contrast, most of the radioactivity recovered in lung lavage fluid from hamsters instilled with ^{3}H-Me-CMK-PE was found in the 25,000-dalton fraction rather than in higher molecular weight complexes (Fig. 1 *C* and Table II *A*). No radioactivity was associated with α-1-PI, although trace amounts of radioactivity were associated with α-M.

Low dose. The lung lavage fluid from these hamsters contained only traces of α-M-elastase (Fig. 1 *B* and Table II *A*). Instead, 1, 4, or 6 h after instillation of the low dose, about two-thirds of the radioactivity

in the lavage fluid was associated with α-1-PI, and most of the remainder represented low molecular weight material. Most of the radioactivity recovered in lung lavage fluid from hamsters treated with the low dose (1.3 μg) of ^{3}H-Me-CMK-PE was found in the 25,000-dalton fraction.

Bronchopulmonary lavage fluid from hamsters not treated with enzyme was concentrated (Amicon UM-10 filter) and incubated in vitro with 1.3 μg of ^{3}H-Me-PE. Molecular sieve chromatography revealed that 0.19 μg of the recovered ^{3}H-Me-PE was associated with α-1-PI and only 0.07 μg with α-M (not shown), demonstrating the low concentration of functional α-M in nonhemorrhagic lung. The remaining ^{3}H-Me-PE (0.82 μg) recovered consisted primarily of uncomplexed (25,000-dalton) elastase.

Radioactive complexes in plasma from instilled hamsters

Plasma, which occupied about 60% of the total blood volume, contained 80–95% of the radioactivity in the blood. Molecular sieve chromatography of plasma 6 h after instillation with the high dose of ^{3}H-Me-PE indicated that >50% of the radioactivity was associated with low molecular weight components (Table II *B*). 34% of the plasma radioactivity was associated with α-PI and only 7% was associated with α-M. Because of the small amount of the elastase in α-M-elastase, no significant elastolytic activity was recovered. We found a small amount of radioactive 200,000-dalton material, which may represent a breakdown product of α-M-elastase. During the 1st d the high dose of ^{3}H-Me-CMK-PE cleared rapidly through the plasma, primarily as an intact molecule; little of the low dose of ^{3}H-Me-CMK-PE was found intact in the plasma at study times of 6 h or later.

Radioactive complexes in urine

The major radioactive component in urine collected during the first 6 h after instillation of the high dose of ^{3}H-Me-CMK-PE consisted of 25,000-dalton material, suggesting that the ^{3}H-Me-CMK-PE was excreted as an intact molecule (Table II *C*). On the other hand, little of the low dose of ^{3}H-Me-CMK-PE was found intact in the urine. 99% of the radioactivity in the urine of ^{3}H-Me-PE-treated hamsters consisted of low molecular weight fragments, and the remainder was found in the 780,000-, the 68,000-, and the 25,000-dalton fractions. The urine collected for the first 6 h after instillation of the high dose of ^{3}H-Me-PE contained elastolytic activity equivalent to 0.5 μg of ^{3}H-Me-PE; the elastolytic activity was found only in the 25,000-dalton fraction and was inhibited by addition of the chloromethyl ketone inhibitor. The explanation for the presence of this elastolytic activity is unclear.

Measurement of elastase-binding molecules in plasma

To further elucidate the binding of radiolabeled elastase to antiprotease molecules, we incubated 2.8 μg of ^{3}H-Me-PE with 1.0 ml of plasma from untreated hamsters for 10 min at room temperature (Table III). Molecular sieve chromatography showed that most of the radiolabeled enzyme was associated with α-M. An interesting comparison with human plasma can be made. Addition of more ^{3}H-Me-PE to hamster plasma resulted in significantly larger amounts of α-M-elastase and relatively little additional formation of α-1-PI-elastase as indicated by the ratio of the slopes for α-M-elastase and α-1-PI-elastase in Fig. 2 (20.3 for hamster plasma, 25.6 for human PiZZ plasma and 2.7 for human PiMM plasma). The α-1-PI-elastase values for hamster and human PiZZ plasma, as depicted in Fig. 2, were not much greater than the variability of the measurement. This variability resulted in inaccurate α-1-PI-elastase linear regression lines. Finally, after addition of saturating amounts of elastase, free elastase can be recovered, and the ratio of α-M-elastase to α-1-PI-elastase is lower (Table IV). Although the antielastase titer of hamster plasma is nearly equal to that of normal human plasma, α-M plays a predominant elastase binding role in the hamster and α-1-PI in the normal human (Table IV).

As seen in Table III, [^{3}H]elastin-SDS solubilizing activity was also found in the prechromatographed mixture of elastase and hamster plasma, even though the antielastase level of the plasma had not been exceeded. Of the chromatographic fractions, elastolytic activity was associated with only the α-M-elastase complex so long as the antielastase titer of the plasma was not exceeded (Fig. 3). No elastolytic activity was found when chloromethyl ketone compound was added to the assay mixture.

The following observations suggested that elastase complexed with α-M may not actively digest elastin until the elastase has been set free from the complex. During incubation of hamster-α-M-^{3}H-Me-PE there was a diminution of the amount of elastase that remained complexed with α-M (91±4% initially, 65±1% after 1 d, 41±1% after 2 d, and 31±9% after 3 d; *n* = 2), as determined by chromatographic separation of complexed and uncomplexed elastase. We found a corresponding increase in the amount of free and degraded elastase (data not reported). Addition of *N*-acetyl-(L-alanyl)$_{3}$-L-alanine chloromethyl ketone, an elastase inhibitor, to α-M-elastase increased the stability of the complex. During the incubation, the elastolytic activity increased over 3 d (a mean±SE of 25±2% after 1 d relative to uncomplexed elastase, 51±4% after 2 d, and 85±10% after 3 d), but no activity was observed in the presence of α-1-PI when the

TABLE III
*Recovery of Elastolytic Activity from Hamster Plasma**

	1.0 ml plasma	1.0 ml plasma
Amount of radiolabeled elastase added	2.8 μg (10,800 cpm)	18.6 μg (76,428 cpm)
Elastolytic activity in elastase-plasma mixture	0.01 μg	14.6 μg
Percent tritium recovered from column	64%	89%
Tritium recovered in complex with		
α-1-PI	97 cpm	1056 cpm
α-M	4,684 cpm	46,547 cpm
Ratio: α-M-elastase/α-1-PI-elastase	48	44
Tritiated elastase equivalents in α-M-elastase complex	1.14 μg	11.4 μg
Enzymatic elastase equivalents in α-M-elastase complex	1.05 μg	14.8 μg
Percent enzymatically active elastase in α-M-elastase complex	90%	130%

* The interaction of ^{3}H-Me-PE with protease inhibitors in hamster plasma was determined by assessing the radioactivity and the residual enzyme activity of the elastase-antiprotease complexes. The indicated amount of tritiated elastase in a small volume of water was added to hamster plasma, and the mixture was incubated for 15 min at 23°C. Aliquots of the mixture were assessed for radioactivity, and elastolytic activity was measured in another aliquot by using SDS-pretreated tritiated elastin substrate. The remainder was placed on a Sepharose 6B column. The collected fractions (3 ml) were analyzed for absorption of light at 280 nm, radioactivity, and elastolytic activity as before. The analysis of tritiated elastase equivalents and enzymatic equivalents was carried out as indicated in the footnotes to Table I. Approximately 20–40% of the radioactivity was recovered in fractions associated with low molecular weight material and probably represents autolyzed ^{3}H-Me-PE (5). The entire molecular sieve profile for the mixture containing 18.6 μg ^{3}H-Me-PE and 1 ml of plasma is depicted in Fig. 3. Serum and heparinized plasma were also used occasionally and yielded results with no significant differences.

[^{3}H]elastin substrate had not been pretreated with SDS. Yet the elastolytic activity associated with the α-M-elastase complex had not been inhibited by prolonged contact with the α-1-PI in the plasma mixture from which it had been chromatographically removed for the experiment.

When hamster plasma was in great excess to the added ^{3}H-Me-PE, the specific enzyme activity of the ^{3}H-Me-PE associated with α-M-elastase was <100% of the amount of elastase present, as calculated from the radioactivity and elastolytic activity. No radioactivity or elastolytic activity was recovered in the 25,000-dalton fraction. When ^{3}H-Me-PE was in excess to the antielastase titer of the plasma, as evidenced by the recovery of uncomplexed elastase (radioactivity and enzyme activity in the 25,000-dalton fraction), we found that the specific enzyme activity of ^{3}H-Me-PE in α-M-elastase increased to 147% of the expected catalytic activity against ^{3}H-elastin-SDS and reached 174% when the elastase titer greatly exceeded the antielastase titer of the plasma (the latter data not reported). A similar trend for the specific enzyme activity in lavage fluid from elastase-instilled hamsters was noted above.

Characterization of α-M-elastase and α-1-PI-elastase

Plasma from untreated hamsters was fractionated by Sepharose 6B molecular sieve chromatography and then ^{3}H-Me-PE was added to a fraction containing 750,000-dalton material. After rechromatography of this mixture, all of the enzyme activity and 80% of the radioactivity were found in a 780,000-dalton complex; the remainder of the radioactivity was associated with low molecular weight material. The formation of α-1-PI-elastase was studied as follows: ^{3}H-Me-PE was

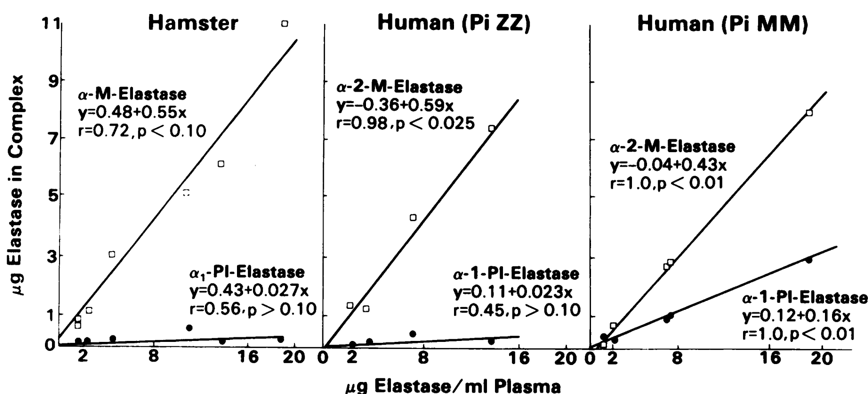

FIGURE 2 Comparison of the functional antielastase profile of hamster plasma with normal human plasma (PiMM) and deficient human plasma (PiZZ). Plasma samples were incubated with ^{3}H-Me-PE and chromatographed on Sepharose 6B columns as described in Fig. 3. The quantity of elastase complexed with α-M and α-1-PI was calculated using the specific radioactivity of the ^{3}H-Me-PE as described in the footnotes to Table I. The equations of the corresponding linear regression lines and their r values are indicated. Two sets of data points for the hamster plot have been presented in greater detail in Table III.

added to the fraction of chromatographed hamster plasma, which contained 53,000-dalton material. After chromatography of this mixture, no enzyme activity was detected in any of the resulting fractions. 55% of the recovered radioactivity was found in 68,000-dalton material and the remainder was found in low molecular weight fractions.

In other studies, 70±4% (mean±SE; $n = 3$) of the hamster α-1-PI-elastase recovered from lavage and 86±5% (mean±SE; $n = 4$) of the complex from plasma was precipitated by rabbit antiserum to porcine pancreatic elastase. Little of the α-M-elastase recovered from hamster plasma or lavage was precipitated by the antiserum, presumably because the elastase is surrounded by the large macroglobulin molecule (11, 12).

DISCUSSION

What is the fate and mode of transport of elastase instilled into hamster's lungs? 1 h after instillation of an emphysema-inducing dose of ^{3}H-Me-PE, 40% remained intact and was not complexed with antiprotease molecules. Subsequently, much of the radioactivity in lung lavage was associated with α-M-^{3}H-Me-PE, while levels of this complex in the blood were quite low as compared with α-1-PI-elastase or low molecular weight material. Levels of lavageable α-M-^{3}H-Me-PE decreased rapidly, although traces could still be detected in lavage fluid 15 d after instillation. Possible explanations for the relatively low level in the blood include a short half-life for the α-M-elastase complex in the blood, which has been noted for human

TABLE IV

*Functional Antielastase Titer of Plasma from Hamsters and Normal and α-1-PI-deficient (PiZZ) Humans**

Source of plasma	No. of samples	^{3}H-Me-PE recovered as complex of‡ α-M	α-1-PI	Percent recovery§	α-M-elastase/ α-1-PI-elastase	Antielastase titer/ml‖
		μg				μg
Normal humans (PiMM)	4	84±18	269±41	76±3	0.3±0.1	353±59
Deficient humans (PiZZ)	5	42±6	36±7	81±6	1.2±0.4	78±13
Hamsters	5	226±33	84±12	95±11	2.7±0.8	310±45

* Plasma and saturating amounts of ^{3}H-Me-PE were incubated and then loaded on molecular sieve columns as described in the text. ^{3}H-Me-PE was recovered complexed with α-M and α-1-PI, quantified by scintillation spectrometry, and assessed for enzyme activity. Free enzyme (25,000 daltons) with full enzymatic activity was recovered, indicating overtitration of the antielastase present.

‡ Mean±SE.

§ The recovery of tritium radioactivity in the eluted volume was compared with the level of radioactivity loaded on the column.

‖ The sum of the amounts of elastase complexed with α-1-PI and α-M per milliliter of plasma.

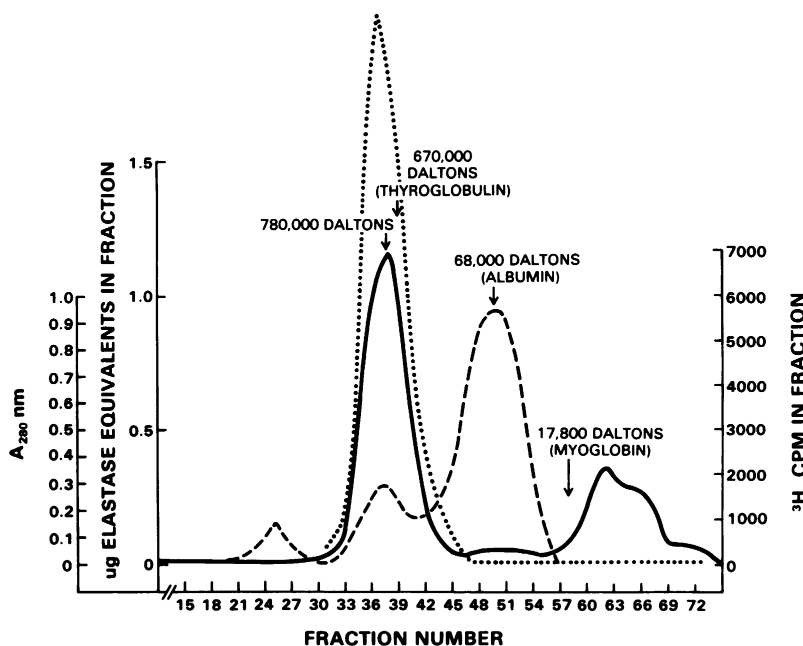

FIGURE 3 The molecular weight distribution of complexes between ^{3}H-Me-PE and hamster plasma. 1 ml of hamster plasma was incubated with 0.2 ml of water containing 18.6 μg of ^{3}H-Me-PE and placed on a Sepharose 6B column. The fractions (3 ml) were assessed for absorbance at 280 nm (- - -), radioactivity (——), and elastolytic activity (• • •) (see the footnotes to Table III). Aliquots of those fractions containing material with a molecular weight of 25,000 daltons or less were rechromatographed on Sephadex G-100 columns to resolve 25,000-dalton material from low molecular weight material. Similar results were obtained whether the plasma was collected in heparin or acid citrate dextrose or whether serum was used. The elution positions of molecular weight standards are indicated.

α-2-macroglobulin-^{131}I-leukocyte elastase (13), and the sequestering of the large α-M-^{3}H-Me-PE complex by the lung matrix with little clearance into the blood (3). Ohlsson and Laurell (13) found that substantial amounts of radioactivity were quickly localized in the liver after administration of ^{131}I-leukocyte elastase. We found substantial amounts of radioactivity in the livers of hamsters after instillation of ^{3}H-Me-PE and much lower levels after instillation of ^{3}H-Me-CMK-PE (3).

The α-M-^{3}H-Me-PE complexes, whether obtained from elastase-hamster plasma mixtures or the lung lavage fluid, exhibited elastolytic activity against bovine ligament, lung, and aorta elastin. Blood was apparently the source of the α-M found in the lung lavage material, because incubation of ^{3}H-Me-PE in vitro with concentrated nonhemorrhagic lung lavage fluid from hamsters that had not been treated with elastase produced relatively small amounts of α-M-^{3}H-Me-PE as compared with α-1-PI-^{3}H-Me-PE. A low dose of elastase instilled into the hamster's lungs produces no apparent hemorrhage or emphysema and, we believe, is inactivated by the endogenous lung antiprotease system, which contains little or no α-M-like inhibitor. Insignificant amounts of α-M-^{3}H-Me-PE and free ^{3}H-Me-PE were found in their lung lavage fluid, and no elastolytic activity could be detected in the fluid. The only complex found resembled α-1-PI-^{3}H-Me-PE. The oversaturation of this endogenous antiprotease system by a large dose of elastase facilitated proteolytic degradation of capillary walls and hemorrhage (3). The formation of α-M-elastase complexes in the lung follows.

Inactivation of ^{3}H-Me-PE with *N*-acetyl-(L-alanyl)$_3$-L-alanine chloromethyl ketone resulted in a dramatic alteration of the rate and manner of clearance (3). Much of the ^{3}H-Me-CMK-PE was excreted as an intact molecule of 25,000 daltons. As expected, little ^{3}H-Me-CMK-PE (1–2%) complexed with antiprotease molecules. Sandhaus and Janoff (14) found that ^{125}I-elastase was bound to α-1-PI, while chloromethyl ketone–inhibited ^{125}I-elastase was uncomplexed. Inasmuch as antiprotease molecules played little or no role in the clearance of ^{3}H-Me-CMK-PE and significant amounts of free ^{3}H-Me-PE were not found after 1 h in lavage fluid or plasma, the slower rate of clearance of ^{3}H-Me-PE from the lungs as compared with ^{3}H-Me-CMK-PE (3) suggests that the formation of α-1-PI-^{3}H-Me-PE and α-M-^{3}H-Me-PE retarded the clearance of elastase from the lung matrix. 15 d after instillation of ^{3}H-Me-PE we were still able to recover small amounts of α-

M-^{3}H-Me-PE from hamsters by bronchopulmonary lavage.

The finding of a low but significant amount of radioactivity (1,200 cpm) in hamster lungs 144 d after instillation of either dose of ^{3}H-Me-PE but virtually none (100 cpm) after ^{3}H-Me-CMK-PE suggested the presence of ^{3}H-Me-PE in some form (3). If some of the radioactivity in the lung matrix represented an α-M-elastase complex, the potential elastolytic activity may be protected from α-1-PI inhibition, and the subsequent release of elastase from the complexes may lead to degradation of newly formed or older lung elastin (3, 15, 16). We (17) have shown that as little as 0.006 μg of elastase binds to elastin and under optimal conditions in vitro continues to degrade significant amounts of the elastin for at least 4 wk. We did not find morphological evidence for pulmonary reaction to inactivated porcine elastase, suggesting that the small amount of foreign protein was not injurious.

Since the discovery of α-1-PI deficiency and its association with pulmonary emphysema, workers have focused on the importance of absolute levels of antiprotease activity. For example, on the basis of the work of Ihrig and co-workers (18), Lieberman (19) has suggested that the ease with which emphysema is induced in hamsters may be related to their low antiprotease level. It would appear from our work that the partitioning of elastase between the antiproteases α-M and α-1-PI may play a key role in the pathogenesis of this enzyme-induced emphysema model. We have used radiolabeled elastase to determine the functional antielastase profile and the titer of plasma and serum samples. Because we find no free inactivated elastase molecules in these mixtures using gel filtration, we believe that the sum of the elastase complexed with α-1-PI and α-M accurately measures the functional inhibitory capacity of the sample. Although the hamster appears to have an adequate antielastase titer, which greatly exceeds that of an α-1-PI-deficient (PiZZ) human, the major elastase inhibitor in the hamster is α-M. α-1-PI apparently plays a significant role only after most of the α-M is saturated with elastase (Table IV). The α-2-M plays a more important role in binding small amounts of elastase added to plasma from α-1-PI-deficient (PiZZ) humans than normal (PiMM) humans. Human leukocytic elastase has been found to produce substantial pulmonary hemorrhage but little emphysema in hamsters (20). Inasmuch as Ohlsson and Olsson (21) have reported that the α-1-PI in human serum binds 92% of the added human leukocyte elastase and that α-2-M binds only 8% in vitro, most of the leukocyte elastase introduced into the hamster's lungs may similarly be inactivated by α-1-PI.

Finally, we wish to speculate on how imbalances of the antielastase system might give rise to emphysema. Elastase, gaining access to the alveolar space, perhaps secreted by an alveolar macrophage or released by a neutrophil, will complex with α-1-PI and be rendered inactive. If the amount of elastase elaborated exceeds the inhibitory capacity of α-1-PI, the type I cell may be injured, and the elastase will gain access to the interstitium. During the course of entry of the elastase into the interstitium, capillary endothelium may also be injured and hemorrhage may occur. As a result of the hemorrhage, circulating α-2-M and α-1-PI enter the air spaces and combine with any free elastase. Damage to elastin fibers may continue as a result of the elastolytic potential of the α-2-M-elastase complexes, which are very large and may remain sequestered in the lungs. It seems reasonable to think that this complex might serve to preserve the elastolytic potential of the enzyme molecule for a long period of time. Deficiency of α-1-PI will result in the formation of higher levels of α-2-M-elastase complex. One implication of the role of the α-2-M-elastase complex in the pathogenesis of emphysema is the prediction that compounds that can inhibit elastase bound to α-2-M will be of greater benefit in inhibiting or preventing the progression of emphysema than treatment with α-1-PI, which cannot inactivate elastase that has been bound to α-2-M.

Galdston et al. (15) have suggested that complexes of α-2-M and human neutrophil elastase may play a role in the pathogenesis of emphysema. Morse (22) has hypothesized that α-2-M-elastase complex in the alveoli of humans might be ingested by macrophages and concentrated centrilobularly. Alveolar macrophages have been shown to concentrate elastase instilled into dogs (23) and hamsters (3). Human alveolar macrophages concentrate human neutrophil elastase (24), much of which retains its enzymatic activity for at least 2 d (25). Alveolar macrophages may secrete relatively large amounts of an elastase when stimulated by such insults as cigarette smoke (26). Recent reports (27, 28) indicate that cigarette smoke inactivates α-1-PI molecules in the lungs of rats and humans. Johnson and Travis (29) have shown that myeloperoxidase from polymorphonuclear leukocytes readily inactivates α-1-PI; and we (30) have demonstrated that this α-1-PI will not bind to and inactivate elastase. Such a combination of events might lead to the overwhelming of endogenous pulmonary antiprotease defenses in smokers and facilitate the formation of α-2-M-elastase complexes, which themselves retain proteolytic activity and may also degrade local α-1-PI. The enzymatic lifetime of the elastase molecules appears to be greatly extended by formation of α-2-M-elastase complexes, which stabilizes the elastase against autolysis. Using an immunohistochemical technique, Cassiman and co-workers (31) have reported that human α-2-M is found associated with connective tissues of organs such as the kidney and lungs. We suggest that the susceptibility

of some patients to emphysema may relate not only to the absolute concentrations of α-1-PI and α-2-M in the blood and lungs but also to their relative concentrations and their individual affinities for elastase.

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REFERENCES

1. Karlinsky, J. B., and G. L. Snider. 1978. Animal models of emphysema. *Am. Rev. Respir. Dis.* **117:** 1109–1133.
2. Snider, G. L., C. B. Sherter, K. W. Koo, J. B. Karlinsky, J. A. Hayes, and C. Franzblau. 1977. Respiratory mechanics in hamsters following treatment with endotracheal elastase or collagenase. *J. Appl. Physiol.* **42:** 206–215.
3. Stone, P. J., J. D. Calore, G. L. Snider, and C. Franzblau. 1979. The dose-dependent fate of enzymatically active and inactivated tritiated methylated pancreatic elastase administered intratracheally in the hamster. *Am. Rev. Respir. Dis.* **120:** 577–587.
4. Snider, G. L., J. A. Hayes, C. Franzblau, H. M. Kagan, P. J. Stone, and A. L. Korthy. 1974. Relationship between elastolytic activity and experimental emphysema-inducing properties of papain preparations. *Am. Rev. Respir. Dis.* **110:** 254–262.
5. Shotton, D. M.. 1970. Elastase. *Methods Enzymol.* **19:** 113–140.
6. Stone. P. J., C. Franzblau, and H. M. Kagan. Proteolysis of insoluble elastin. *Methods Enzymol.* **82a:** In press.
7. Powers, J. C., and D. L. Carroll. 1975. Reaction of acyl carbazates with proteolytic enzymes. *Biochem. Biophys. Res. Commun.* **67:** 639–644.
8. Rice, R. H., and G. E. Means. 1971. Radioactive labelling of proteins *in vitro*. *J. Biol. Chem.* **246:** 831–832.
9. Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* **91:** 222–233.
10. Garvey, J. S., N. E. Cremer, and D. H. Sussdorf. 1977. Methods in Immunology. 3rd edition. W. A. Benjamin, Inc., Reading, MA. 220–222.
11. Baumstark, J. S. 1967. Studies on the elastase-serum protein interaction. I. Molecular identity of the inhibitors in human serum and direct demonstration of inhibitor-elastase complexes by zone and immunoelectrophoresis. *Arch. Biochem. Biophys.* **118:** 619–630.
12. Barrett, A. J., P. M. Starkey, and E. A. Munn. 1974. The unique nature of the interaction of α_2-macroglobulin with proteinases. *In* Bayer Symposium 5: Proteinase Inhibitors. H. Fritz, H. Tschesche, L. J. Greene, and E. Truscheit, editors. Springer Verlag, Berlin. 72–77.
13. Ohlsson, K., and C.-B. Laurell. 1976. The disappearance of enzyme-inhibitor complexes from the circulation of man. *Clin. Sci. Mol. Med.* **51:** 87–92.
14. Sandhaus, R. A., and A. Janoff. 1976. Retention and localization of exogeneous elastases in rat lung in an animal model of emphysema. *Am. Rev. Respir. Dis.* **113:** 214 (Abstr.)
15. Galdston, M., V. Levytska, I. E. Liener, and D. Y. Twumasi. 1979. Degradation of tropoelastin and elastin substrates by human neutrophil elastase, free and bound to alpha$_2$-macroglobulin in serum of the M and Z (Pi) phenotypes for alpha$_1$-antitrypsin. *Am. Rev. Respir. Dis.* **119:** 435–441.
16. Christner, P., G. Weinbaum, B. Sloan, and J. Rosenbloom. 1978. Degradation of tropoelastin by proteases. *Anal. Biochem.* **88:** 682–688.
17. Stone. P. J., G. Crombie, and C. Franzblau. 1977. The use of tritiated elastin for the determination of subnanogram amounts of elastase. *Anal. Biochem.* **80:** 572–577.
18. Ihrig, J., J. Kleinerman, and D. J. Rynbrandt. 1971. Serum antitrypsins in animals. Studies of species variations, components, and the influence of certain irritants. *Am. Rev. Respir. Dis.* **103:** 377–389.
19. Lieberman, J. 1976. Elastase, collagenase, emphysema, and alpha$_1$-antitrypsin deficiency. *Chest.* **70:** 62–67.
20. Senior, R. M., H. Tegner, C. Kuhn, K. Ohlsson, B. C. Starcher, and J. A. Pierce. 1977. The induction of pulmonary emphysema with human leukocyte elastase. *Am. Rev. Respir. Dis.* **116:** 469–475.
21. Ohlsson, K., and I. Olsson. 1974. Neutral proteases of human granulocytes. III. Interaction between human granulocyte elastase and plasma protease inhibitors. *Scand. J. Clin. Lab. Invest.* **34:** 349–355.
22. Morse, J. O. 1978. Alpha$_1$-antitrypsin deficiency. *N. Engl. J. Med.* **299:** 1099–1105.
23. Janoff, A., B. Sloan, G. Weinbaum, V. Damiano, R. A. Sandhaus, J. Elias, and P. Kimbel. 1977. Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. *Am. Rev. Respir. Dis.* **115:** 461–478.
24. Campbell, E. J., R. R. White, R. M. Senior, R. J. Rodriquez, and C. Kuhn. 1979. Receptor-mediated binding and internalization of leukocyte elastase by alveolar macrophages in vitro. *J. Clin. Invest.* **64:** 824–833.
25. McGowan, S. E., P. J. Stone, and G. L. Snider. 1981. The fate of human leukocyte elastase incorporated into alveolar macrophages. *Am. Rev. Respir. Dis.* **123**(Pt. 2): 226. (Abstr.)
26. Rodriquez, R. J., R. R. White, R. M. Senior, and E. A. Levine. 1977. Elastase release from human alveolar macrophages: Comparison between smokers and non-smokers. *Science (Wash. D. C.).* **198:** 313–314.
27. Janoff, A., H. Carp, D. K. Lee, and R. T. Drew. 1979. Cigarette smoke inhalation decreases α_1-antitrypsin activity in rat lung. *Science (Wash. D. C.).* **206:** 1313–1314.
28. Gadek, J. E., G. A. Fells, and R. G. Crystal. 1979. Cigarette smoking induces functional antiprotease deficiency in the lower respiratory tract of humans. *Science (Wash. D. C.).* **206:** 1315–1316.
29. Johnson, D., and J. Travis. 1979. The oxidative inactivation of human α-1-proteinase inhibitor. Further evidence for methionine at the reactive center. *J. Biol. Chem.* **254:** 4022–4026.
30. Clark, R. A., P. J. Stone, A. El Hag, J. D. Calore, and C. Franzblau. 1981. Myeloperoxidase-catalyzed inactivation of α-1-protease inhibitor by human neutrophils. *J. Biol. Chem. 256:* 3348–3353.
31. Cassiman, J. J., F. van Leuven, B. van der Schueren, and H. van den Berghe. 1980. Immunohistochemical localization of human α_2-macroglobulin in connective tissue. *Cell Tissue Res.* **213:**301–310.