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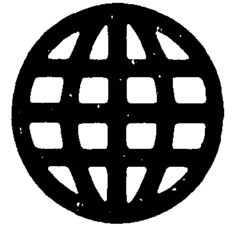

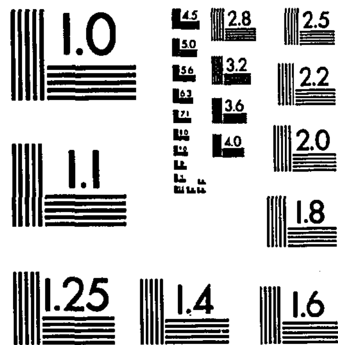

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PROPERTIES OF MACROPHAGE ELASTASE

by

MITCHELL HOROWITZ

A dissertation submitted to the
Graduate Faculty in Biomedical Sciences
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy,
The City University of New York.

1985

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Abstract

PROPERTIES OF MACROPHAGE ELASTASE

by

Mitchell Barry Horowitz

Advisor: Jerome Kleinerman, M.D.

Horseradish peroxidase-coupled elastin was used to confirm the elastolytic potential of alveolar macrophage (AM) conditioned medium (CM). AM were collected via bronchoalveolar lavage from DBA/2 mice. After 60 h in culture, the CM of 1.5 x 10^6 AM contained _in vitro_ elastolytic activity equivalent to 125 ng of porcine pancreatic elastase (PPE). This represented a distinct AM-derived elastase, rather than contaminating neutrophil elastase, since 1) it had the characteristics of a metalloproteinase; 2) it was resistant to inactivation by serine protease inhibitors; 3) it was continuously discharged during culture; 4) its pH profile differed from that reported for neutrophil elastase, and 5) its secretion was dependent upon AM protein synthesis. Elastolytic activity was also detected in the CM of the $P388D_1$ macrophage cell line, from which a homogeneous 15k MW elastase was isolated by affinity chromatography.

CM from AM cultured in the presence of 2 μM colchicine contained 360% the elastase activity of controls. A similar effect was obtained with nocodazole, an unrelated microtubule-disrupting agent, but not with taxol, which promotes microtubule assembly. Theophylline alone more than doubled elastase secretion, and combined with colchicine produced a 5-fold increase. Cycloheximide eliminated basal elastase activity but failed to suppress 12% of colchicine-induced elastase secretion, suggesting the presence of a small, intracellular pool of AM elastase. Although alpha$_2$-macroglobulin (α_2-M) inhibited AM elastase, endogenous α_2-M did not appear to be masking elastase activity since methylamine, which inactivates α_2-M, did not increase free elastase. Methylamine did, however, produce a 6-fold increase in β-glucuronidase secretion, which suggests AM elastase is not similarly lysosomally stored.

To assess the in vivo elastolytic potential of AM elastase, AM CM containing in vitro activity equivalent to 500 ng PPE was instilled intratracheally into 5 DBA/2 mice. After 2 weeks, although mean linear intercept (Lm), an index of airway diameter, was not significantly increased, histologic lung sections from 3 mice revealed areas suggestive of emphysema. However, as these subjective impressions were not corroborated by the Lm data, a definitive role for AM elastase in the pathogenesis of emphysema awaits future studies using higher elastase challenges.

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INTRODUCTION

Emphysema is a lung disease characterized anatomically by an enlargement of distal air spaces. According to the protease-antiprotease theory of emphysema, elastase is the specific protease responsible for the degradation of elastin, a major structural protein of the lung. In experimentally induced emphysema, elastin is initially digested then subsequently resynthesized in a morphologically defective manner (52). Normally, the respiratory tract is lined with antiproteases capable of inactivating elastase, thereby providing homeostatic control against unrestrained elastolysis. When there is an imbalance, however, due either to protease excess or antiprotease deficiency, the lung may be predisposed to the elastic fiber injury which can lead to emphysema.

The protease-antiprotease theory evolved from two studies: Gross et al (28) in 1965 injected rats intratracheally with papain, an exogenous elastin-degrading protease, to produce the first animal model of experimentally-induced emphysema after Laurell and Eriksson (56) had noted a high incidence of early-onset emphysema in individuals genetically deficient in the major serum antiprotease, α_1-antitrypsin (now referred as α_1-proteinase inhibitor: α_1-PI). However, less than five percent of the

cases of emphysema are patients with a genetic deficiency of α_1-PI (84). Therefore, while these studies implicated protease-antiprotease imbalance in the pathogenesis of emphysema, they neither explained the usual mechanism of the disease nor demonstrated the existence of an endogenous lung elastase.

Subsequent research has identified several forms of elastase, including proteases derived from the neutrophil (44) and the alveolar macrophage (93, 105). Unlike the alveolar macrophage, neutrophil cell lysates reveal significant amounts of elastase (44), and, consonant with current concepts of the importance of α_1-PI in preventing lung protease injury, neutrophil elastase is inhibited by α_1-PI (107).

The pathologic potential of human neutrophil elastase was demonstrated by Janoff and Scherer (43) in 1968 when they produced experimental emphysema by instilling the purified enzyme into dogs. Cohen and Rossi (16) have extrapolated animal data to calculate that the yearly load of elastase released from human neutrophils in the lung would, if left uninhibited, have the capability to produce emphysema in man.

Although these findings support the idea of a pathogenetic neutrophil function in the development of emphysema, other studies lead to a questioning of the importance of

this role: 1) elastase activity has been reported in other cells which have access to the lung environment, including alveolar macrophages (105), monocytes (81), and mast cells (62), and in platelets (39); 2) using urinary desmosine as an index of lung catabolism, Janoff (41) found that depressed levels of circulating neutrophils do not lead to reduced elastin catabolism; 3) depletion of neutrophils and neutrophil elastase in hamsters, using antineutrophil antibody, caused no alteration in the extent of emphysema produced in response to a cadmium chloride challenge (36); and 4) Senior et al (83) reported that at an *in vitro* dosage equivalent to that of pancreatic elastase which produced severe emphysema but no mortality in hamsters, purified neutrophil elastase produced extensive lung hemorrhage, high mortality, and only mild emphysema.

Involvement of alveolar macrophage elastase in the development of emphysema was first implied in the last decade from three observations: 1) macrophages accumulate in young cigarette smokers at the sites of early lung injury (66); 2) conditioned medium from cultured macrophages expresses elastolytic activity (102); and 3) macrophage homogenates (60) and macrophage lysosomal contents (40) have induced emphysema in experimental animals. However, since macrophage lysates demonstrate low elastolytic activity relative to neutrophil lysates (81), neutrophil elastase has generally been considered to be the prime

agent of emphysema. Furthermore, since neutrophils are phagocytosed by macrophages, the lung injury observed using crude macrophage extracts may be attributable to internalized neutrophil elastase (34). Likewise, elastase activity in conditioned medium might be neutrophil-derived, via either culture contamination with neutrophils or _in vitro_ release of neutrophil elastase internalized through phagocytosis or receptor-mediated endocytosis (11).

More recent studies, however, provide more compelling evidence for the existence and pathologic importance of a distinct macrophage elastase. Banda and Werb (5) and White et al (107) have independently purified an elastase from mouse peritoneal macrophages and characterized it as a metalloproteinase, in contradistinction to neutrophil elastase, which is a serine protease. Likewise, Valentine and Fisher have recently identified a calcium-dependent bovine alveolar macrophage elastase (93). _In vivo_ demonstration of macrophage elastase was made by Padmanabham et al who found elastolytic activity with the characteristics of macrophage elastase in pulmonary lavage fluid from rats exposed to cadmium aerosolization (70).

The inability of several investigators to detect macrophage elastase may be due to limitations in assay sensitivity. Elastase activity in macrophage cultures is very low, and the initial objective of this project was there-

fore to evaluate various elastase assays, to determine which procedure provided the maximum sensitivity and specificity requisite for enzyme detection, purification, and characterization. Elastolytic activity may be low due to 1) enzyme secretion in an inactive form; 2) partial enzyme inactivation by endogenous intracellular or secreted antiproteases; 3) low-level enzyme secretion; or 4) weak enzyme reactivity. Macrophage elastase cleaves elastin on the amino side of leucyl residues, in contrast with neutrophil elastase which cleaves on the carboxyl side of valyl and alanyl residues (99). Since leucyl residues are less common in elastin than alanyl and valyl sidechains, macrophage elastase is probably not as reactive as neutrophil elastase. Unlike neutrophil elastase however, macrophage elastase retains its activity toward elastin substrates in the presence of α_1-PI (5, 99). The potential significance of this observation is that, if left unrestrained, even low levels of macrophage elastase could produce lung lesions. In fact, Chapman and Stone have recently reported (14) that in serum-free medium, when cells are grown in contact with an elastin substrate, neutrophils degrade eight times as much elastin per cell as macrophages, whereas when the cells are cultured in the presence either of human serum or alveolar fluid, it is the macrophage which degrades 5-fold more substrate than the neutrophil. Presumably, serum antiproteases may be relatively ineffective against macro-

phage elastolysis either due to innate resistance of macrophage elastase to inactivation, or because degradation proceeds as an intimate pericellular event, isolated from significant antiprotease interaction. In addition, macrophage elastase has been shown to actually inactivate α_1-PI (3), further disturbing the lung's protease-antiprotease balance.

Along with α_1-PI, α_2-macroglobulin (α_2-M) a highly conserved 725k MW serum glycoprotein, constitutes one of the two major mammalian antiproteases (7). Whereas the 52k MW α_1-PI attains 70% of its serum concentration in the bronchoalveolar fluid of humans, the high MW α_2-M is relatively excluded from alveoli (24). There is however, local production of α_2-M by alveolar macrophages (104) which may contribute to intra-alveolar antiprotease levels. Gadek et al (24) have calculated that, with respect to neutrophil elastase, α_1-PI levels in the lower respiratory tract provide over 200 times the antiprotease activity of α_2-M. For such proteases as macrophage elastase, however, which resist inactivation by α_1-PI (5), α_2-M may serve as the primary antiprotease in the lung, although it has not been conclusively established whether even α_2-M can inhibit macrophage elastase. Regardless, the pericellular locus of elastin degradation by macrophages may preclude enzyme inactivation by potential inhibitors.

To definitively address the issue of whether alveolar macrophage elastase plays a role in the pathogenesis of emphysema requires first purifying the enzyme and then evaluating whether it is capable of causing elastin degradation in vivo. Macrophage elastase has been previously purified from the conditioned medium of peritoneal macrophages (5, 99) since these cells can be harvested in large numbers from thioglycollate-injected mice. In addition to facilitating purification of elastase by increasing the number of peritoneal macrophages, the macrophage population elicited by intraperitoneal thioglycollate secretes more elastase per cell (105). Peritoneal macrophages become activated by such stimuli as lymphokines, bacterial products, allergens, and phagocytosable particles (54). Both resident and thioglycollate-elicited peritoneal macrophages are activated to secrete elevated levels of elastase when presented with a phagocytic challenge (105). Purification of alveolar macrophage elastase might therefore be aided by cell activation. However, although basal alveolar macrophage elastase secretion is equivalent to that of peritoneal exudative macrophages receiving a phagocytic load, phagocytosis does not promote elastase release from resident alveolar macrophages (105). This suggests that alveolar macrophage elastase secretion might be refractory to activating agents. However, White et al (106) showed that cytoskeleton-disrupting drugs increase elastase secretion

from alveolar macrophages two to three fold. Gordon and Werb (27) had first demonstrated this effect of colchicine, vinblastine and cytochalasin B on elastase release by peritoneal macrophages, and Banda and Werb (5) used colchicine treatment in their elastase purification protocol.

The mechanism, however, by which colchicine induces increased elastase activity is not known. In addition to its effect on microtubules, there are cell surface receptors for colchicine (8), and colchicine has been shown to elevate cAMP levels (35) and increase DNA synthesis (15). Alternatively, the increase in elastase activity seen with colchicine may be secondary to a decreased secretion of an endogenous elastase inhibitor, thereby leading to the greater expression of free elastase. The results of Banda and Werb (5), who recovered 800% of their initial elastolytic activity after purification, suggest that an endogenous inhibitor may be reversibly masking potential enzyme activity. Valentine et al (94) have subsequently isolated an elastase inhibitor from bovine alveolar macrophages. Purification of alveolar macrophage elastase may therefore be aided by employing conditions which promote dissociation of enzyme from endogenous inhibitors. Banda and Werb's findings can be alternatively explained as an activation during purification of a macrophage proelastase. Although a macrophage zymogen has not been identified, there is recent evidence for physiological activation of latent

macrophage elastolytic activity by plasmin (13).

To determine whether alveolar macrophage elastase is characteristically distinct from polymorphonuclear leukocyte elastase, it was first necessary to verify the enzyme is derived from the alveolar macrophage and produced via active protein synthesis. Secondly, we had to provide evidence that the alveolar macrophage elastase has characteristic properties which distinquish it from neutrophil elastase. To facilitate these studies, a murine-derived macrophage cell line was used. On the basis of morphologic appearance, phagocytic capability, adherence properties, presence of Fc and C_3 receptors, and the absence of surface immunoglobulin, Koren et al (51) have characterized these $P388D_1$ cells as "macrophage like." The cells are known to secrete various neutral proteases, including a proteolytic enzyme which resembles macrophage elastase (100). Conditioned medium from cultured $P388D_1$ cells was used to try to develop an elastase purification scheme to be used subsequently for the purification of mouse alveolar macrophage elastase. As a proliferating cell line the system has the advantage of providing a homogeneous macrophage population with the potential to produce a relatively large quantity of elastolytic material for study and possible purification. In addition, the effects of intratracheally instilled purified $P388D_1$ elastase can be determined and compared histologically and morphometrically to the lung

injury induced by similarly injected mouse alveolar macrophage elastase.

Further studies using mouse alveolar macrophages will be performed to investigate the effects of potential modulators of elastase secretion, identify stimulating factors, and help elucidate the mechanism of elastase release. Microtubule stabilizing agents were evaluated together with agents such as colchicine, which depolymerizes microtubules and elevates intracellular cAMP. Other drugs which also affect cAMP were studied to determine their effect on elastase secretion.

The objectives of this investigation were therefore 1) to compare various elastase assays; 2) to purify and characterize mouse alveolar macrophage elastase; 3) to identify factors _in vitro_ which can regulate the synthesis, secretion, and expression of macrophage elastolytic activity toward native elastin; and 4) to determine whether macrophage elastase has the capability to degrade lung elastin _in vivo_. The significance of the study is a clarification of 1) the potential contribution of macrophage elastase to the development of emphysema, and 2) the physiological role of endogenous antiproteases such as α_2-M and α_1-PI in regulating macrophage elastase activity.

METHODS

I. ELASTASE ASSAYS

Six methods were evaluated for use in detecting elastase activity in macrophage conditioned medium and cell lysates, and in monitoring elastolytic activity during macrophage elastase purification.

A) HORSERADISH PEROXIDASE (HRP)-COUPLED ELASTIN

Substrate was prepared according to the procedure of Saunders et al (78). Aldehyde groups were introduced into the carbohydrate portion of Type VI HRP (Sigma) via periodate oxidation (65). The HRP-aldehyde was reacted with 5 mg of elastin (Calbiochem) previously sieved to 200-325 mesh. The resulting Schiff bases were stabilized through the addition of 8 mg sodium borohydride. The conjugate was then incubated 18 h at 37°C with 5.5 mg each of trypsin and chymotrypsin so that the substrate remaining would be resistant to further cleavage by nonelastase proteases. After repeatedly washing the substrate with saline it was suspended in 50 ml saline and frozen.

The assay quantifies elastase indirectly by using cleaved HRP as an indicator enzyme in a second reaction

with an HRP substrate, 2,2' -azino -di(3 -ethyl benzthiazoline -6 -sulfonate) (ABTS; Sigma). To assay, a 1% (vol/vol) suspension of the conjugate is dispersed by sonication and stirring, and 1-ml aliquots are added to centrifuge tubes. After 3 washes at 1000 x g, each with 2 ml of 0.01 M phosphate-buffered saline (PBS), pH 8.0, containing 0.05% Tween 80, the substrate is equilibrated for 30 min at 37°C in reaction buffer (0.01 M Tris, pH 9.0, containing 2mM $CaCl_2$). The conjugate is then centrifuged at 1000 x g and the buffer is aspirated off and discarded. One ml of buffer is then dispensed into each assay tube, followed by 0.2 ml of sample. After 3-15 h incubation at 37°C the tubes are centrifuged, and 0.8 ml of supernatant from each tube is recentrifuged. HRP substrate is prepared by adding 120 µl 3% H_2O_2 to 10 ml of 4 mM ABTS in 0.1 M citrate buffer, pH 3.9. Equal volumes (175 µl) of supernatant and substrate solution were added to wells of a 96-well flat-bottomed plastic microplate (Costar) and the OD_{414} read after 10-30 min. Alternatively, 350 µl each of supernatant and ABTS solution were added to 1-cm semi-micro cuvettes. For certain experiments the ABTS reation was terminated by the addition of 0.7 ml of stop solution to an equal volume of supernatant plus ABTS. Stop solution was prepared by mixing 1 ml of hydrogen fluoride pyridine (70% HF; Aldrich), 4 ml of 1 M NaOH, and 400 ml of 1 mM EDTA.

ABTS is susceptible to cleavage from peroxidases in

biological samples. HRP-elastin was incubated with and without alveolar macrophage conditioned medium to determine whether ABTS digestion is due to endogenous macrophage peroxidase, rather than HRP cleaved from the HRP-elastin. After 15 h, conditioned medium was added to the tube lacking sample, and the contents of both tubes were assayed for apparent elastase activity.

The resistance of HRP-elastin to cleavage by a non-elastase serine protease was evaluated by comparing the elastolytic activity of 100 ng of trypsin to that of 100 ng of PPE, assayed in triplicate.

B) RADIOIODINATED ELASTIN COUPLED TO SEPHAROSE

Ten mg of solubilized elastin, prepared from bovine ligamentum nuchae (47) was iodinated with 5 mCi of Na[^{125}I] using the Enzymobead lactose peroxidase reagent (Bio-Rad). The radiolabeled elastin was then coupled to Sepharose through a modification of the procedure of Rifkin and Crowe (75). Radioactive elastin was mixed with 1 ml CNBr-activated Sepharose 4B (Pharmacia), and rotated overnight at 4°C in 4 ml 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl. Residual active groups on the gel were blocked by incubating the derivatized Sepharose with 1 M ethanolamine, pH 8, for 2 h. Excess uncoupled ligand and blocking agent were removed by alternate washes with coupling buffer and 0.1 M

acetate buffer, pH 4, containing 0.5 M NaCl. The radioiodinated elastin-Sepharose was stored at 4°C in 0.02 M Hepes buffer, pH 8.1, containing 5mM $CaCl_2$ and 0.1 mM NaN_3.

To assay elastase, the derivatized Sepharose was washed by centrifugation at 2000 x g in 0.02 M Hepes buffer until less than 500 cpm of radioactivity appeared in the supernatant. The substrate was then resuspended in the same buffer containing 500 µg/ml bovine serum albumin (BSA: RIA grade; Sigma) at 80,000-100,000 cpm/ml. One ml of swirled substrate suspension was dispensed into 12 x 75 ml polypropylene centrifuge tubes and the radioactivity was measured in a Beckman Gamma 5500 counter. One hundred µl of each sample to be assayed for elastase activity was added, and the suspension was vortexed. After a 5-15 h incubation at 37°C the tubes were vortexed, the Sepharose was pelleted at 2000 x g for 5 min, and the radioactivity in 110 µl of supernatant was measured. Results are calculated as percent release of total initial radioactivity. A standard curve was generated at each assay from the results of duplicate or triplicate determinations of known concentrations of porcine pancreatic elastase. The elastolytic activity of samples was expressed as ng equivalents of porcine pancreatic elastase via linear regression analysis.

C) N-SUCCINYL-(L-ALANINE)$_3$-p-NITROANILIDE (SLAPN)

A stock solution of substrate was prepared by adding 73 mg of N-succinyl-(L-alanine)$_3$-p-nitroanilide (SLAPN; Calbiochem) to one ml of N-methyl-pyrrolidinone (J. T. Baker) as described by Bieth (9). The substrate was dissolved by heating at 60°C for 10 min. To assure accuracy of concentration, the stock solution was standardized so that at a 1:5000 dilution with 0.2 M Tris-HCl, pH 8.0, containing 5 mM $CaCl_2$, the OD_{315} was 0.365.

To assay esterolytic activity, 2.5 ml of Tris assay buffer were poured into a 1-cm cuvette, 10 µl of sample were added, and the solution mixed by inversion. After 5 minutes the reaction was started by adding 20 µl of stock substrate solution to the cuvette and inverting it twice; immediately the Δ OD_{410}/min was recorded over a 3 min interval at room temperature.

With SLAPN, 1 U of elastase activity corresponds to the hydrolysis of 1 micromole of substrate per minute. Units are calculated by multiplying the Δ OD/min by 0.284 (9).

D) ELASTIN-AGAR

Plastic Petri dishes containing agar gel uniformly inpregnated with elastin-fluorescein of particle size less than 37 µm, in 0.02 M Tris-HCl, pH 8.8, as described by

Senior et al (82), are commercially available (Elastin Products). Fluorescein-conjugated elastin is used because it is easier to disperse in agar than unconjugated elastin. Wells, 4-mm in diameter, were punched in the agar and 10 μl of sample were added to each well. The diffusion plates were then incubated at 37°C in a sealed polyethylene bag containing 3 ml of water to prevent dehydration. Elastolysis produces a zone free of elastin particles which presents as a clear area with a defined border. After 24 hours the diameters of the zones of solubilization were measured and the areas calculated.

E) [^{3}H]-ELASTIN

Tritiated elastin substrate was prepared according to Banda et al (4). Elastin from bovine ligamentum nuchae (Sigma) was finely sieved (230-325 mesh); 2.5 g was suspended in 50 ml H_2O in a 500-ml Erlenmeyer flask on a magnetic stirplate and the pH adjusted to 9.2 with 0.1 M NaOH. Twenty five mCi of [^{3}H]-$NaBH_4$ (ICN; specific activity 350 mCi/mole) in 200 μl of 3mM NaOH were added, followed 10 min later by 250 mg of nonradioactive $NaBH_4$ in 3 mM NaOH. After 2 h, 3 drops of Pakowet (Pako Corp) antifoam agent were added; the suspension was adjusted to pH 3 with glacial acetic acid and mixed for an additional 30 min. The elastin was washed 6 times with cold H_2O by centrifuging at

10,000 x g for 30 min. After stirring overnight in water, the substrate was resuspended in H_2O at 16 mg/ml, and 5-ml aliquots were dispensed into 50-ml screwcap centrifuge tubes.

To assay for elastase, a 5-ml aliquot of substrate was resuspended in 40 ml of 0.3 M Tris-HCl, pH 8.2, containing 15 mM $CaCl_2$ amd 0.02% NaN_3. One hundred μl of substrate suspension were added to 200 μl of sample, and incubated for 16 h at 37°C. The suspensions were then centrifuged for 3 min at 10,000 x g and the radioactivity in 100 μl of supernatant was counted in 15 ml of Aquasol-2 (New England Nuclear), using a Beckman liquid scintillation counter which provided automatic quench compensation by the external standard ratio method. Photolumimnescence and chemiluminescence were minimized by counting samples after a 24-h period of dark adaptation. A series of porcine pancreatic elastase standards were included in every assay.

F) ELASTIN-RHODAMINE

When elastin-labeled rhodamine is digested by elastase, rhodamine is released and can be measured by spectrophotometry (38). Its concentration is proportional to the elastolytic activity of the enzyme.

A 20 mg/ml suspension of 200-400 mesh elastin-

rhodamine (Elastin Products) was added at room temperature, with gentle stirring, to 0.2 M Tris-HCl, pH 8.8, containing 0.01% Triton X-100. The substrate was then washed until colorless on coarse, ashless filter paper using the same buffer but without Triton X-100. The washed elastin-rhodamine was then resuspended in buffer (without Triton X-100) to a concentration of 20 mg/ml. Five hundred µl of Pakosol (Pako Corp) were added per 100 ml of suspension to suppress foaming and inhibit bacteral growth.

Before elastolytic activity could be evaluated, the optical density per mg of substrate had to be determined. From 0 to 1 ml of substrate in enough buffer to provide a total volume of 2.90 ml was added to five 10-ml conical flasks. After adding 0.1 ml of an elastase solution containing 20 units of activity, the reaction flasks were stoppered, placed in a Dubnoff incubator, and incubated at 37°C at 60 excursions per minute, until the substrate in all the flasks was totally solubilized. Incubates were brought to a final 10-ml volume with buffer, and the OD per mg of substrate was determined from the slope of optical density at 550 nm plotted versus elastin concentration in mg.

Elastase standards were prepared in triplicate in 2 ml of buffer in conical flasks. One ml of substrate was added to each flask and incubated as above. After 20 min, 7.0 ml

of buffer were added to each flask, the contents filtered through coarse, ashless filter paper, and the OD_{590} read. Elastase activity was calculated using the OD/mg previously determined for the substrate. One unit of activity is defined as the amount of elastase which will solubilize 1 mg of elastin in 20 min at pH 9.8 at 37°C. This procedure was used to verify the specific activity of commercially obtained porcine pancreatic elastase; no biological samples were assayed.

II. MACROPHAGE COLLECTION

A) PERITONEAL MACROPHAGES

Thioglycollate treatment

The mouse peritoneal cavity is a convenient site for the collection of macrophages (63). Inflammatory peritoneal macrophages may be induced by sterile irritants. Dehydrated Brewer thioglycollate medium (Difco) was rehydrated to provide a solution containing 0.5% sodium thioglycollate, and autoclaved. Male DBA/2 mice, 10-12 weeks of age, were injected i.p, with 2 ml of thioglycollate, and peritoneal cells were harvested 2,3, and 4 days later to determine the time course of optimal macrophage yields. Strain variation in responsiveness to inflammatory stimuli was determined for DBA/2, BDF_1, and C57BL/6 mice.

Collection of peritoneal macrophages

Peritoneal macrophages were collected by the method of Meltzer (63). Mice were exsanguinated by guillotine decapitation to avoid contamination of peritoneal cells with blood cells. The skin over the abdomen was wetted with 70% ethanol and lifted up with forceps away from the abdominal wall. A small subdermal incision was made in the mid-abdomen, taking care not to penetrate the peritoneum.

Using the thumb and forefinger of each hand the skin at the site of the incision was completely retracted cranially and caudally. With a 10-cc syringe and 20-gauge needle, 8 ml of Dulbecco's modified Eagle's medium (GIBCO) containing 10 U/ml preservative-free sodium heparin (Abbott Laboratories) was forcefully injected into the peritoneal cavity, entering just below the level of the xiphoid process. Without removing the needle, the abdomen was lightly tapped with a finger, and, positioning the needle so that the tip would not be occluded, the peritoneal fluid was slowly withdrawn into the syringe, then transferred to 50-ml polypropylene centrifuge tubes and placed in ice. Typically, 6 ml of fluid was recovered. A small slit was then made in the wall, and an additional 2 ml of medium was added to the cavity, using a 2-ml serological pipette. The remaining peritoneal fluid was then withdrawn using the pipette. Cells from syngeneic mice were pooled, and the peritoneal lavage fluid was centrifuged at 200 x g for 10 min at 4°C.

B) ALVEOLAR MACROPHAGES

Mouse alveolar macrophage collection

Male DBA/2 mice (Jackson) were sacrificed by aortic exsanguination following light pentobarbital anesthesia. The lungs were collapsed by slitting the diaphragm and the trachea was surgically exposed using sterile technique. An

18-gauge Teflon catheter was inserted into the trachea through a sublaryngeal incision, and 0.8-1.0 ml of 37°C sterile saline was introduced into the lungs using a 1-ml syringe. After 1 min the lavage fluid was drained by gravity into iced centrifuge tubes and the lavage procedure repeated a total of 6 to 18 times. The pooled lavage fluid was centrifuged for 10 min at 200 x g at 4°C to pellet the cells.

BCG injection

Infiltration of macrophages into the lungs and spleen of mice can be induced by intravenous vaccination with emulsified mycobacterial cell walls (6). A preparation of heat-killed Bacillus Calmette-Guerin (BCG) of the strain *M. bovis* was supplied by Dr. Quentin Myrvik (Bowman Gray School of Medicine, Winston-Salem, NC). The emulsion was prepared according to the method of Barclay et-al (6). Light mineral oil was added to lyophilized cell walls (0.12 ml oil/25 mg BCG) in a mortar, and ground to a smooth paste with a pestle. A 0.2% solution of Tween-80 in normal saline was gradually added, with grinding, until a 1.5 mg/ml oil-in-saline emulsion of BCG was obtained. The emulsion was then heated for 30 min at 65°C.

Eight- to ten-week-old male C57Bl/KsJ and C57Bl/6 mice were injected via the tail vein with 0.2 ml of the emulsi-

fied BCG. Control mice were injected with 0.2 ml of 0.2% Tween-80 in saline. After 2-5 weeks, animals were killed, their lungs lavaged, cell counts performed, and the macrophages cultured. At three weeks, the cell yield obtained from lung minces (48) was compared to the cell recovery from lung lavage. Whole lung were cut into approximately 0.1-mm^2 pieces using two passes, 90° apart, of a McIlwain tissue chopper (Mickle Lab Eng Co). Cells were recovered by suspending the minced lungs in 10 ml of saline in a 50-cm centrifuge tube, vigorously shaking by hand for 2 min, followed by filtration through 6-ply sterile gauze, and centrifugation at 200 x g.

Hamster alveolar macrophage collection

To lavage alveolar macrophages from male Syrian Golden hamsters, tracheas were cannulated with a 5-French infant feeding tube (Argyle), cut to a length of 10 cm; lavage volumes were 3.5 ml. Otherwise, the procedure was as described for mice.

III. MACROPHAGE CULTURE

A) CULTURE OF THE P388D$_1$ CELL LINE

Flask cultures of P388D$_1$ macrophages were obtained from the American Type Culture Collection. Cells were grown in a 37°C incubator in room air in 75-cm^2 screw-cap tissue culture flasks (Corning) in 20 ml RPMI medium 1640 (GIBCO) containing phenol red and L-glutamine, supplemented with 10% heat-inactivated, acid-treated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 20 µg/ml gentamycin. Acid treatment involves lowering the pH of the serum to 3.2 with 2 M HCl, allowing it to remain at room temperature for 2 h, then restoring neutrality with 2 M NaOH. The purpose of acidification is to inactivate serum α_2-M (92). When the cells achieved confluence they were either subcultured in growth medium or maintained in serum-free medium.

Subcultures were prepared at a ratio of 1:5 by pouring off half of the old medium, adding fresh medium to a volume of 100 ml, dislodging the cells by smartly slapping the flask against the thigh, and dispensing the suspension into new flasks. Serum-free maintenance medium was prepared by supplementing RPMI 1640 medium with 0.2% lactalbumin hydrolysate and antibiotics. Confluent flasks were rinsed twice with Hank's balanced salt solution (HBSS; GIBCO) containing

antibiotics, and 25 ml of maintenance medium were added. Conditioned maintenance medium was collected every 48 h and replaced with fresh medium. Pooled conditioned medium was centrifuged at 200 x g for 10 min at 4°C to pellet any cellular material; the supernatant was stored at -20°C.

B) CULTURE OF ALVEOLAR MACROPHAGES

Cell pellets from lavage fluid were resuspended in RPMI Medium 1640 without phenol red (Biofluids Inc) supplemented with 15% heat-inactivated, acid-treated fetal calf serum (FCS; GIBCO), 2.0 mM L-glutamine, and antibiobics (100 U/ml penicillin; 100 μg/ml streptomycin; 20 μg/ml gentamycin) (RPMI-FCS). Cell viability was assessed by the method of Trypan Blue exclusion using an aliquot of cell suspension. A differential cell count was performed on a Wright-Giemsa stained slide preparation, and a total cell count taken using a hemacytometer. The cell suspension was adjusted to a final density of 1×10^6 cells/ml and plated onto 16-mm wells of plastic tissue culture dishes (Costar) in a volume of 1 ml/well. The macrophages were allowed to adhere for 4-6 h in a humidified CO_2 incubator (5% CO_2 in air), and the wells were then rinsed twice with HBSS to remove contaminating cells. After an additional 24 h in RPMI-FCS (0.5 ml/well), wells of macrophage monolayers were again rinsed twice with HBSS, then maintained in 0.5 ml

serum-free RPMI containing 0.2% lactalbumin hyrolysate, 2 μM colchicine, 2 mM L-glutamine, and antibiotics (RPMI-LH-colchicine). Conditioned medium was collected after 48 h in RPMI-LH-colchicine, centrifuged for 10 min at 1000 x g at 4°C, and the supernatant stored at -70°C. Fresh RPMI-LH-colchicine was added to the cells, and medium again harvested, centrifuged, and frozen, every 48 h for a total of 8 days in serum-free medium.

C) CULTURE OF PERITONEAL MACROPHAGES

The procedure for the culture of peritoneal macrophages was the same as for alveolar macrophages, except the cells were cultured in 25 cm^2 plastic tissue culture flasks (Corning), and Dulbecco's modified Eagle's medium (DMEM; GIBCO) was used rather than RPMI 1640. Total and differential cell counts were performed, and cell viability was determined by Trypan Blue exclusion. The cells were cultured at a density of approximately 20 x 10^6 cells/flask, in 10 ml of medium.

IV. PURIFICATION PROCEDURES

A) PURIFICATION OF $P388D_1$ ELASTASE

Five liters of conditioned medium from $P388D_1$ macrophages were concentrated 1000-fold by ultrafiltration across a 10,000 molecular weight cut-off membrane (Amicon YM-10:) at 60 lb/in^2 of nitrogen. Concentrated medium was then dialyzed at 4°C against two changes of 50 mM Tris-maleate buffer, pH 7.6, containing 200 mM NaCl and 5 mM $CaCl_2$ (107). As an alternative procedure, five liters of conditioned medium were concentrated to 50 ml, then treated with solid ammonium sulfate to obtain seven fractions: 0-35% saturation, 35-45%, 45-55%, 55-65%, 65-75%, and 85-100%. Ammonium sulfate was added, followed by 30 min of stirring at room temperature and 10 min of centrifugation at 10,000 x g. Each pellet was reconstituted in 3 ml of 0.2 M Tris-HCl, pH 8.0, then dialyzed against two changes of 0.02 M Hepes buffer, pH 8.0, containing 2 mM $CaCl_2$. The supernatant remaining with 100% saturated ammonium sulfate was also dialyzed, then concentrated to 3 ml.

A 1.6 x 22 cm column of oxalic acid-solubilized elastin covalently linked to agarose beads was prepared as described by White et al (107). Sodium dodecyl sulfate (SDS) was added to a slurry of α-elastin-agarose (Elastin Products) in 0.02 M sodium acetate buffer, pH 5, at an

SDS:elastin ratio of 1:16 (w/w). The slurry was rotated at room temperature for 24 h prior to loading the column. After equilibrating the column at 4°C with 50 mM Tris-maleate buffer, pH 7.6, containing 200 mM NaCl and 5 mM $CaCl_2$, 1 ml of concentrated, dialyzed sample was applied, and 1-ml fractions were collected at a flow rate of 15 ml/h.

A 1.3 x 90 cm column and a 2.2 x 100 cm column were packed with Fractogel TSK HW 55 (S) (MCB Reagents), and calibrated using ribonuclease A, chymotrypsinogen, and ovalbumin molecular weight standards (Polysciences Inc) and albumin. Select fractions from affinity chromatography which exhibited elastase activity, and the 45-55% split from ammonium sulfate precipitation were separately pooled, concentrated by ultrafiltration (Amicon YM-10), and fractionated by high performance liquid chromatography (HPLC) using one of the Fractogel columns, collecting 5-ml fractions at a 0.5 ml/min flow rate. An inline variable wavelength UV detector (Beckman) monitored absorbance at 254 and 280 nm.

An alternative purification attempt followed the procedure of Banda and Werb (5). Crude, concentrated conditioned medium was dialyzed against two changes of 10 mM ammonium bicarbonate, followed by two changes of 15 mM Tris-HCl, pH 7.6. After equilibration, the conditioned,

dialyzed medium was concentrated to 2 ml across a YM-10 membrane. One ml of this concentrate was applied to a 1.0 x 23.5 cm column of DEAE-Sephadex A-25 equilibrated with 20 mM Tris-HCl, pH 7.6. The sample was eluted with the Tris buffer at a flow rate of 5.4 ml/h, and 2-ml fractions were collected. After elution of 100 ml, a linear NaCl gradient (0-0.7 M; 70 ml total volume) in buffer was begun, followed by final elution with 0.7 M NaCl in starting buffer.

A 1.6 x 93 cm column of Ultrogel AcA54 gel filtration medium (LKB) was packed at a flow rate of 50 ml/h, and equilibrated at 12 ml/h in 50 mM Tris-HCl, pH 7.6, containing 150 mM NaCl and 50 mM $CaCl_2$. The column was calibrated with molecular weight standards. Peaks of elastase activity from the DEAE-Sephadex A-25 column were separately pooled and concentrated; 1-ml samples were loaded on the AcA54 column and eluted at 6 ml/h, while collecting 5-ml fractions.

Elastolytic activity was most frequently assayed using radioiodinated elastin coupled to Sepharose (75); HRP-coupled elastin (78) and tritiated elastin (4) substrates were otherwise used. Purification was followed by discontinuous SDS polyacrylamide gel electrophoresis (53). Both tube and slab gels were usually formed of a 4% T, 2.7% C stacking gel and a 10% T, 2.7% C separating gel; gradient gels contained from 20 to 5% T. Tube gels were run at a

constant current of 3 mA/tube; two slab gels were run simultaneously at a current of 6 mA. Pharmacia low molecular weight standards (14.4-94 kDaltons) were always included.

B) PURIFICATION OF PERITONEAL MACROPHAGE ELASTASE

Elicited macrophages were collected from 20 male DBA/2 mice, 12 to 16 wk of age, 3 d after i.p. injection of thioglycollate. The cells were cultured, and once 600 ml of pooled conditioned medium were accumulated, purification by sequential anion exchange chromatography, gel filtration chromatography, and cation exchange chromatography was attempted. Concentration, dialysis, and application to DEAE-Sephadex A-25 and to Ultrogel AcA54 proceeded as described for $P388D_1$ macrophages. Peak elastase-containing fractions which eluted between 102 and 128 ml from the AcA54 column were pooled, dialyzed against 3 changes of 0.1 M borate buffer, pH 7.6, and concentrated across a 5000 molecular weight cut-off membrane (Amicon YM-5). Two ml of concentrated sample were loaded on a 1 x 30 cm SP-Sephadex C-25 column, and 1.85-ml fractions were collected at a flow rate of 7.4 ml/h. After 33 ml were eluted with 0.1 M borate buffer, pH 7.6, gradient elution was begun: 0-1.0 M NaCl in borate buffer; 75 ml total volume.

C) PURIFICATION OF ALVEOLAR MACROPHAGE ELASTASE

Resident alveolar macrophages from thirty-five male DBA/2 mice, 10-12 weeks of age, were collected by bronchoalveolar lavage, and cultured. Seventy-five ml of conditioned medium were concentrated 50-fold by ultrafiltration, dialyzed, and applied to a 1 x 27 cm elastin-Sepharose column (107) as described for the $P388D_1$ elastase purification, except 1-ml fractions were collected at a flow rate of 3.6 ml/h. Elastase-containing fractions were concentrated, dialyzed and fractionated on a DEAE-Sephadex A-25 column (5), again as described above for the $P388D_1$ cells, except that fractions were collected at an elution rate of 3.6 ml/h, and a 0-0.7 M NaCl gradient was started after 35 ml had been collected.

D) PURIFICATION OF ALPHA$_2$-MACROGLOBULIN

Hamster alpha$_2$-macroglobulin (α_2-M) was purified from the pooled sera collected by aortic exsanguination 48 h after subcutaneous injection in the posterior flank of technical grade turpentine (0.25 ml/100 g body weight). Okubo et al (67) report that turpentine injury raises α_2-.M levels over 1000-fold in rats. The purification scheme was adapted from Hayashida et al (32). Serum was stored at -70°C and therefore incubated for 1 h in a 37°C water bath prior to beginning the purification, in order to promote

reassociation of α_2-M subunits (85). To pellet and remove low density lipoproteins, 12 ml of hamster serum were mixed with 0.24 ml of 10% dextran sulfate and 1.2 ml of 1 M $CaCl_2$, and centrifuged at 4°C and 2500 x g. The supernatant was dialyzed overnight against 0.03 M acetate buffer, pH 5.1, then applied to a 2.6 x 27 cm DEAE-cellulose (Whatman DE-52) anion exchange column. A two-step elution was performed, beginning with 0.03 M acetate buffer, pH 5.1 at a flow rate of 34 ml/h and monitoring the OD_{280}. After one hundred 7.5-ml fractions were collected, the buffer was changed to 0.07 M acetate, pH 5.1, and an additional 35 fractions were collected.

Alpha$_2$-M activity was monitored by the method of Cullmann and Dick (18), described on page 41. Fractions 112-114 contained α_2-M activity and were pooled, dialyzed against 0.03 M acetate buffer, pH 5.1, and concentrated to 20 ml by ultrafiltration (Amicon YM-10 membrane). After re-equilibrating the DE-52 column with 0.03 M buffer, the concentrated crude α_2-M was reapplied, and eluted with a linear gradient created with 560 ml each of 0.03 M and 0.12 M acetate buffer, pH 5.1;6-ml fractions were collected at a flow rate of 15 ml/h. The eluate from 307-390 ml displayed α_2-M activity and was pooled, concentrated to 2 ml, and dialyzed against 0.04 M Tris-HCl, pH 7.6, containing 0.03 M NaCl, in preparation for gel filtration chromatography. This sample was then applied to a 1.6 x 85 cm Sephacryl

S-300 Superfine (Pharmacia) column, and fractionated using the 0.04 M Tris buffer into 5-ml aliquots at a flow rate of 8 ml/h. Fractions 13-15 under the first peak off the S-300 column were pooled and concentrated to 1 ml. Agarose electrophoresis and immunoelectrophoresis were performed as described on page 44.

E) PURIFICATION OF ALPHA$_2$-MACROGLOBULIN ANTISERUM

Specific rabbit antiserum to hamster α_2-M was provided by Drs. Kleinerman and Ip. To remove endogenous rabbit α_2-M from the antiserum, the IgG fraction, which contained the anti-α_2-M IgG, was isolated by anion exchange chromatography. Antiserum was dialyzed overnight against 0.0175 M phosphate buffer, pH 6.8, then applied to a 1.6 x 19 cm DEAE-Sephacel (Pharmacia) column. Two-ml fractions were collected at a flow rate of 20 ml/h with inline detection of OD_{280}. Fractions from the single protein-containing peak which eluted with 0.0175 M buffer were pooled, and concentrated across a YM-10 membrane (Amicon).

Further purification of anti-α_2-M IgG was attempted by the affinity chromatography procedure of Guzman et al (29). Dr. Guzman (Mt. Sinai School of Medicine, New York) generously provided a 0.7 x 14 cm column prepared with the collagen from <u>Ascaris</u> cuticle. Stepwise elution (4.5 ml/h; 1.5-ml fractions) of DEAE-Sephacel-purified anti-α_2-M IgG

was conducted in 0.01 M Tris HCl, pH 7.3, containing, successively, no salt, 0.1 M NaCl, and 1 M NaCl. Column fractions were screened for anti-α_2-M activity, OD_{280} was measured, and the peak fractions eluting with each buffer were separately pooled.

Column fractions containing anti-α_2-M IgG were identified by double radial immunodiffusion (69). A 1% (w/v) agarose (SeaKem ME; Marine Colloids) gel was prepared in normal saline. Wells (4-mm) were punched in the agarose in a repeating design of 6 wells radially arranged around a central well. Whole hamster serum (10 μl) was added to the center well, and 10 μl from select column fractions was added to the surrounding wells. Activity of peak column fractions toward goat anti-(rabbit IgG) (Cappell) was also tested. After overnight incubation the gels were stained and evaluated for evidence of immunoprecipitation.

The ability of rabbit anti-hamster IgG to inactivate hamster α_2-M in vitro was assessed by adding 25 μl of partially purified IgG to 10 μl of hamster serum (diluted 1:20 with normal saline). Functional α_2-M remaining in hamster serum was assayed (18) after a 1-min incubation.

V. MODULATION OF MACROPHAGE ELASTASE

A) *IN VIVO* STUDIES

Four control DBA/2 mice were instilled intratracheally with 50 μl of PBS, pH 7.4; four mice received PBS containing 200 μg of lipopolysaccharide (*E. coli* serotype 055:B5; Sigma); and four had 125 μg of polysinosinic:polycytidylic acid (poly I:C; Sigma) instilled in PBS. After 48 h the lungs were lavaged and the macrophages cultured as above, except that colchicine was omitted from the maintenance medium. Elastase activity in the conditioned medium from cells cultured 48 h in maintenance medium was assayed using HRP-elastin substrate (78).

Twelve animals were housed in specially designed Plexiglass chambers (50) and exposed 22 h daily for 30 d to room air containing 30 ± 5 ppm nitrogen dioxide, monitored by a nitrogen dioxide analyzer (Columbia Scientific Industries). Elastase activity from cultured alveolar macrophages of experimental animals was compared with that of controls.

B) *IN VITRO* STUDIES

DBA/2 alveolar macrophages

The effect of the following putative macrophage activators on elastase secretion, glucose uptake, and β-glucuronidase secretion by cultured murine alveolar macrophages was determined: 2 μM colchicine (Sigma); 2 mM theophylline (Sigma); 3 μM methyl [(5-2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate (nocodazole; Sigma), 10 μM taxol (courtesy of Dr. Matthew Suffness, National Cancer Institute), 200 nM phorbol myristate acetate (PMA; Sigma), 1 μM calcium ionophore A23187 (Sigma); 100 μg/ml polyI:C (Sigma); and 50 μg/ml lipopolysaccharide (E. coli serotype 055:B5; Sigma). To assess whether enzyme inactivation by endogenous α_2-M was reducing expression of elastase activity, cells were cultured in the presence of 50 mM methylamine (Sigma) (7). Cells were additionally cultured in the presence of cycloheximide (0.25 mg/ml) alone and cycloheximide plus colchicine, to determine whether elastase secretion requires de novo synthesis.

All the above reagents were prepared in RPMI 1640 without phenol red (Biofluids Inc) supplemented with 0.2 % lactalbumin hydrolysate, 2 mM L-glutamine and antibiotics (RPMI-LH). Four of the drugs required preliminary solubilization in dimethyl sulfoxide (DMSO), in which case appropriate blanks were used to determine the effect of DMSO alone: taxol, nocodazole, and A23187 were dissolved in a final concentration of 0.1 % DMSO; PMA in 0.01% DMSO.

Alveolar macrophages lavaged from seventy-five 12-13 week old male DBA/2 mice were plated over a 5-d period at a density of 1.5×10^6 macrophages per 16-mm tissue culture well on RPMI-FCS. To ensure uniform macrophage composition in each well, each day's pooled cells were equally divided among the wells. When the desired cell density was achieved, the cells were rinsed with HBSS, and 0.7 ml of RPMI-LH containing the various agents was added to each well. Three wells were used for each treatment group. After 60 h in the maintenance medium, the conditioned medium was collected and centrifuged at 200 x g. The supernatants were tested for levels of elastase activity using HRP-elastin, and for LDH, β-glucuronidase and glucose.

$P388D_1$ CELL LINE

To determine whether $P388D_1$ cells secrete a proelastase which requires enzymatic cleavage to express full activity, the effect of 100 μg of trypsin and of 200 ng of porcine pancreatic elastase on the elastase activity of concentrated $P388D_1$ conditioned medium were evaluated.

VI. OTHER ASSAYS AND BIOCHEMICAL PROCEDURES

A) GLUCOSE UTILIZATION

As an independent measure of macrophage activation, glucose utilization by cultured macrophages was assayed by the method of Ryan et al (77). The assay is based upon the phosphorylation of glucose in the presence of ATP, and hexokinase. The resulting glucose-6-phosphate reacts with NADP, catalyzed by glucose-6-phophate dehydrogenase (G6PD), to yield NADPH, which can be monitored spectrophotometrically. All reagents were obtained from Sigma, and solutions were prepared in 0.1 M Tris-HCl, pH 7.5, containing 64 mM NaCl, 3.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$. For each assay, 0.7 ml of buffer, 0.1 ml of 20 mM ATP, 0.1 ml of 5 mM NADP, and 5 μl of a commercial mixture of hexokinase (200 U/ml) and G6PD, were dispensed into a cuvette. After ten μl of sample were added to the cuvette, and mixed, the OD_{340} was read after allowing 2 min for completion of the reactions. Assays were performed in triplicate, and glucose utilization was calculated from the ratio [(initial OD of sample) - (final OD of sample)] / [(initial OD of control) - (final OD of control)], where initial OD represents the glucose concentration of the culture medium at the beginning of the culture period (2 mg/ml for RPMI 1640), where final OD measures the glucose concentration in the

medium after incubation, and where culture medium lacking the specific chemical agent being investigated in the sample serves as the control.

B) BETA-GLUCURONIDASE ASSAY

To determine any lysosomotropic effect by any of the agents investigated above, conditioned medium was assayed for β-glucuronidase, a marker lysosomal enzyme, by the method of Hall et al (30).

Beta-glucuronidase hydrolyzes p-nitrophenyl β-glucuronide to glucuronic acid and p-nitrophenol. The p-nitrophenol can be measured spectrophotometrically at 400 nm at alkaline pH. A 0.1 M substrate solution of p-nitrophenyl β-glucuronide was prepared in 0.1 M sodium acetate buffer, pH 5.0. To assay, 0.2 ml of substrate was added to 0.3 ml of buffer and 0.1 of sample. After a 2 h incubation at 37°C, the reaction was stopped with the addition of 1 ml of 50 mM NaOH, and the absorbance was read. Enzyme activity was expressed as nanomoles of substrate cleaved per hour: the complete hydrolysis of 1 micromole of substrate yields an OD_{400} of 12.1.

C) LDH ASSAY

Lactate dehydrogenase (LDH) is a cytosolic enzyme. As

a general indication of cell death or injury, LDH was assayed in the conditioned medium of cultured macrophages after the method of Wroblewski and LaDue (108).

In the presence of reduced nicotinamide adenine dinucleotide (NADH), LDH catalyzes the conversion of pyruvate to lactate, and oxidizes a molar equivalent of NADH. The rate of change in absorbance at 340 nm due to the depletion of NADH is proportional to LDH activity.

LDH was assayed using LDH-P Reagent (Calbiochem-Behring), which, when rehydrated, contains 0.23 mM NADH and 0.62 mM pyruvate in 0.05 M phosphate buffer, pH 7.5. To assay, 75 μl of sample were added to 1 ml of assay reagent at 30°C, and the delta OD_{340}/min was recorded for 3 min using a Beckman DU-8 spectrophotometer. From the molar absorptivity of NADH at 340 nm (6.22×10^3 L $mole^{-1}cm^{-1}$), units of LDH activity are defined as (Δ OD/min $\times 10^6$)/(6.22×10^3).

D) DNA ASSAY

To determine possible toxicity of various agents, and to permit standardized comparisons of elastase activity under conditions of variable cell number, cellular DNA content was measured (17) as a means of quantifying cell number. Wells were washed in PBS, 0.5 ml PBS was added to each

well, and the plate was then frozen. Lysates were thawed over ice, and 0.5 ml of 4% Triton-X 100 was added to each well. After a 30 min incubation, lysates were transferred to iced test tubes containing 30 μl of 7 M perchloric acid, incubated on ice for 30 min, then centrifuged at 4°C for 15 min at 10,000 x g. The supernatant was discarded and the pelleted DNA was hydrolyzed by incubating for 30 min with 0.5 ml of 1 M perchloric acid at 70°C. Hydrolysates were transferred to clean tubes, and 0.5 ml of freshly prepared colorimetric reagent (10 ml of 3% diphenylamine, 0.2 ml conc H_2SO_4, and 0.1 ml 1.6% acetaldehyde) was added to each tube. Samples were incubated for 16 h at 37°C, centrifuged at 4°C for 10 min at 10,000 x g, and the OD at 600 nm then measured against a water blank. DNA content was calculated by linear regression analysis using similarly treated standards of calf thymus DNA (Type 1; Sigma).

E) ALPHA$_2$-MACROGLOBULIN ASSAYS

Functional assays

Cullmann and Dick (18) have recently reported a highly sensitive spectrophotometric assay for α_2-M which uses the chromogenic trypsin substrate carbobenzoxy-valyl-glycyl-arginine-p-nitroanilide (Chromozym-TRY; Boehringer-Mannheim). Analogous to the classical assay of Ganrot (25), α_2-M activity is determined from the residual amidolytic

activity of α_2-M-bound trypsin, after adding trypsin in excess, then inactivating the free trypsin with aprotinin.

The assay was performed in 0.05 M Tris-HCl, pH 8.0, containing 0.15 M NaCl and 2.6 mg/L trypsin (Type III; 10,000 U/mg; Sigma). In a semimicrocuvette 10 µl of sample were added to 0.5 ml of 37°C-buffer, and mixed by inversion. After a 1 min incubation, 10 µl of a 15 U/ml aqueous solution of aprotinin (Boehringer-Mannheim) were added, and the solution again incubated for 1 min, followed by the addition of 25 µl of 5 mM substrate solution. The Δ OD_{405}/min was followed for 3 min at 37 °C. Plasma samples were diluted 1:25 with normal saline to bring them within the linear range of the assay. Units of α_2-M activity using Chromozym-TRY are equal to (Δ OD/min)/10.4.

Alpha$_2$-M activity was also measured by a direct adaptation of the method of Ganrot (25). Fifty µl of sample were added to 2 ml of 0.1 M Tris-HCl, pH 8.2, containing 10 µg/ml of trypsin. Free trypsin was inactivated with 100 µl of a 0.02% solution of aprotinin, and 1 ml of the trypsin substrate, α-N-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA; Sigma) was then added. After a 10 min incubation, the reaction was terminated with 0.5 ml of 30% acetic acid, and the OD_{410} was measured in a Beckman DU-8 spectrophotometer.

Immunologic assay

Rocket immunoassay of α_2-M followed Laurell's original procedure (55). Rabbit antiserum to α_2-M was incorporated at a concentration of 2% (v/v) into a 1% (w/v) agarose (SeaKem ME; Marine Colloids) gel, in pH 8.3 Tris-borate buffer (90 mM Tris; 80 mM boric acid), containing 3 mM disodium EDTA (7). The gel was cast vertically on GelBond film (Marine Colloids) between two glass plates spaced 1.5 mm apart. When the gel had set, a row of 3-mm holes were punched, and 7 μl of sample were added to each well. Electrophoresis was run overnight at 7 V/cm, with α_2-M migrating anodally.

After electrophoresis, proteins were fixed for 10 min in a picric acid bath prepared by adding 14 g picric acid to 1 L of distilled water, heating to 60°C, filtering, then adding 200 ml of glacial acetic acid. Excess picric acid was removed by a 10 min wash in 95% ethanol, and the gel was then pressed into a thin film, under filter paper and a weight. After three 15-min washes (twice in 0.1 M NaCl and finally in distilled water) the gel was pressed dry, and stained for 10 min in Coomassie Brilliant Blue R-250 (50 g stain + 4.5 L 95% ethanol + 1 L glacial acetic acid + 4.5 L distilled water). Excess stain was removed in destaining solution (4.5 L 95% ethanol + 1 L glacial acetic acid + 4.5 L distilled water) and the gels were allowed to dry. The

height of each "rocket" is directly proportional to its concentration; samples were quantified using a standard curve obtained from rocket immunoassay of serial dilutions of pooled normal serum.

F) PURITY ASSESSMENT OF α_2-MACROGLOBULIN

Electrophoresis

Homogeneity of purified α_2-M was preliminarily evaluated by electrophoretic comparison with whole hamster serum using the Paragon serum protein agarose electrophoresis kit (Beckman). Electrophoresis proceeded for 60 min at 100 V in a Bio-Rad Model 1405 cell maintained at 5°C. Densitometric scans of stained gels were performed at 600 nm with a Beckman DU-8 spectrophotometer.

Immunolectrophoresis

Immunolectrophoresis was performed using 5-in x 7-in buffered agarose gels with a precut pattern of wells and trenches (Bioware Products), following the manufacturer's recommended procedure. Briefly, 3 μl of sample was added to a well and electrophoresed at 160 V for 30 min. After electrophoresis, the gel surface was gently blotted and 100 μl of goat anti-hamster serum (Cappel Laboratories) was added to the adjacent trough. After an overnight incubation, gels

were deproteinized by washing in saline, dried, and stained with Imido Black.

G) pH PROFILES

The pH profiles of porcine pancreatic elastase (PPE) and murine alveolar macrophage elastase were determined using peroxidase-coupled elastin substrate. The assay was conducted in 0.01 M Tris-HCl, pH 6.8-9.0, containing 2 mM $CaCl_2$. Actual pH was measured after the addition of sample to buffer. The samples were 1) 10 ng of PPE (Elastin Products; 114 U/mg), and 2) dialyzed conditioned medium from strain DBA/2 murine alveolar macrophages. Elastase activity was expressed as ng PPE equivalents, assuming the pH at which the highest activity of PPE was observed represented 10 ng of PPE.

H) INHIBITOR PROFILE

The effect of several protease inhibitors on $P388D_1$, alveolar macrophage, peritoneal macrophage, and porcine pancreatic elastase was determined. The specific inhibitors evaluated included: 10 mM ethylene diamine tetra-acetic acid (EDTA); 50 μg/ml hamster α_1-PI; 70 μg/ml hamster α_2-M; 5 mM acetyl-alanyl-alanyl-prolyl-alanyl-chloromethyl ketone (AAPACK) or acetyl-alanyl-alanyl-prolyl-valyl-

chloromethyl ketone (AAPVCK); and 10 mM phenylmethane sulphonyl fluoride (PMSF; Sigma). Both AAPACK and AAPVCK were a gift from Dr. J. Powers (Georgia Institute of Technology; Atlanta, Georgia). A stock solution of PMSF was prepared in 2-propanol; experiments with PMSF included appropriate controls.

Inhibitors were preincubated with 20-100 ng of PPE or a macrophage elastase solution of equivalent activity for 10-60 min at 37°C. The final pH was adjusted as necessary with NaOH. Enzyme activity was determined using HRP-elastin, [^{125}I]-elastin, or [^{3}H]-elastin.

Specific metal dependency of murine alveolar macrophage elastase was determined by dialyzing the crude enzyme against 10 mM EDTA, then removing the EDTA by dialysis against distilled water (68), and then attempting reactivation with 50 mM $CaCl_2$ or 10 mM $ZnSO_4$. For this experiment, $CaCl_2$ was omitted from the assay buffer.

I) PROTEIN DETERMINATION

Protein concentrations were determined by the method of Lowry et al (58). Protein in individual column fractions was estimated by measuring the absorbance at 280 nm and assuming an OD of 1.0 corresponded to a 1 mg/ml protein solution.

VII. INDUCTION AND EVALUATION OF EXPERIMENTAL EMPHYSEMA

A) ELASTASE INSTILLATION INTO HAMSTERS

The *in vivo* elastolytic and hemorrhagic potential in hamster lungs of two different porcine pancreatic elastase preparations were compared: Type III Elastase (75 U/mg) from Sigma and high purity elastase (112.7 U/mg) from Elastin Products. Ten Units of high purity elastase in 100 µl of PBS, pH 7.4, and an equivalent dosage, as determined from its esterolytic activity toward SLAPN, of the Sigma elastase were instilled into hamsters.

Thirteen male Syrian Golden hamsters (Charles River), 35-38 d of age, weighing 68-86 g, were separated into three weight-matched treatment groups. One at a time, the animals were anesthetized by i.p. injection of 0.15 ml of a 1:1 (v/v) solution of pentobarbital (64.8 mg/cc) and normal saline. After swabbing the epiglottis with lidocaine, a polyethylene catheter was introduced endotracheally under direct vision. Immediately, 0.1 ml of PBS alone or containing the Sigma or the high purity elastase, was slowly instilled by a syringe connected to a hand-operated mechanical infuser. Instillation was coordinated with the inspiratory phase of the animal's breathing. At 48 h post-instillation, the animals were sacrificed by aortic exsanguination following pentobarbital anesthesia. The lungs, with the trachea, were removed from the thorax and

intratracheally inflation-fixed by Bouin's solution at a pressure of 20 cm H_2O for 15 min. Midcoronal lung slices were processed, embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin.

B) INSTILLATION INTO MICE

Pancreatic elastase instillation

Male DBA/2 mice were anesthetized by intraperitoneal injection of 50 μl of a 1:1 solution of pentobarbital (64.8 mg/ml) and normal saline, and taped by their limbs to a dissection board. The skin overlying the trachea was swabbed with alcohol, and a 5-mm incision was made inferior to the level of the cricoid cartilage. A 30-gauge needle was connected via 10 cm of polyethylene tubing (PE-10; 0.28 mm i.d.; Clay Adams) to a 1-cc syringe containing Dulbecco's PBS, either alone, as a control, or supplemented with 5 U or 10 U per 50 μl of porcine pancreatic elastase (112.3 U/mg; Elastin Products). The board was inclined to an angle 30°C from the horizontal, the trachea was exposed, and 50 μl of solution was slowly instilled endotracheally over a 1-min period, using a Harvard infusion pump. During the infusion the animal was rotated to a left lateral dependent then a right lateral dependent position. The animal then was returned to the horizontal and the incision was closed with 4-0 chromic gut (Ethicon).

After 48 h the animals were anesthetized and killed by severing the aorta. The diaphragm and sternum were cut, and PE-90 polyethylene tubing (1.27 mm o.d.; Clay Adams) was tied into the trachea. The ribcage was freed, and the lungs were inflation-fixed in situ at 25 cm H_2O pressure with Ito and Karnovsky fixative. The entire thorax was then removed and immersed in fixative, still maintaining inflation pressure. For some experiments the lungs and trachea were dissected out and inflation-fixed ex situ. After 24 h, the fixed lungs were removed from the thorax if necessary, processed, and embedded in paraffin. Five-μm thick mid-coronal lung sections were stained with hematoxylin and eosin.

Instillation of macrophage conditioned medium

Conditioned RPMI-LH medium, harvested from macrophages from DBA/2 mice, was ultrafiltered using an Amicon YM-10 membrane to a concentration equivalent in activity to 10 μg/ml of porcine pancreatic elastase. Fifty μl of the conditioned medium, containing the elastolytic equivalent of 500 ng of PPE, was injected into each of five 12- to 14-week old male DBA/2 mice. Five control mice received 50 μl of RPMI-LH which had not been exposed to cells. Two weeks later, the animals were sacrificed and their inflation-fixed lungs were processed for histologic exami-

nation.

C) MORPHOMETRY

Evaluation of emphysema

To measure mean linear intercept (Lm), an index of airway diameter (90), a film overlay grid was used to divide lung section slides into uniform fields. At a standard locus within each field, the number of points of interception of tissue elements with a microscope crosshair were counted separately along horizontal and vertical axes at a magnification of 430X. Observed Lm was calculated by dividing the total horizontal and vertical length of the cross by the mean total number of intercepts per field. Due to tissue shrinkage and distortion incurred during histologic processing, observed Lm at a right angle to the microtome blade is approximately 73% of actual Lm (90). To adjust for such dimensional alterations, actual Lm was calculated using a correction factor of 1.39, previously determined by Dr. Kleinerman for this laboratory.

Evaluation of hemorrhage

The extent of hemorrhage was quantified using the same grid and lung sections as for the Lm measurements. The number of fields in which there was visual microscopic evi-

dence of hemorrhage was expressed as a percentage of the total number of lung fields.

VIII. STATISTICAL ANALYSES

The null hypothesis that the difference of two population means equals zero was tested at the α = .05 level of significance, using the two-tailed Student t-test. In experiments involving three or more groups, one-way analysis of variance (ANOVA) was used to determine whether the samples were derived from the same population. Multiple pair-wise comparisons were performed only upon rejection of the null hypothesis that all sample means were equal.

Linear regression analysis was calculated by the method of least squares; data was not force-fitted through the origin. The strength of the relationship between X and Y was provided by the coefficient of correlation, r, calculated by dividing the XY-product sum by the square root of the product of the sum of X^2 and the sum of Y^2 (105).

RESULTS

I. ELASTASE ASSAYS

Table 1 (p 78) summarizes, with respect to the detection of porcine pancreatic elastase activity, the useful ranges of several elastase assays. Subnanogram sensitivity was achieved both with [^{125}I] -elastin-Sepharose and with HRP-elastin substrates. These were the only assays tested capable of consistently demonstrating elastolytic activity in the crude conditioned medium of cultured macrophages. Within the range of elastase values typically encountered with our experimental protocols, 0.5 to 20 ng, both assays were linear, with coefficients of correlation greater than 0.98 (Table 1, p 78; Fig. 1, p 107; Fig. 2, p 108).

To generate standard curves suitable for the detection of subnanogram levels of PPE with HRP-elastin, samples were generally incubated overnight, and the secondary reaction with ABTS was allowed to proceed up to 30 min prior to reading the absorbance (Fig. 3; p 109). For extended range standard curves, a 3-h incubation was sufficient, and the absorbance was read within 10 min after reaction with ABTS (Fig. 4; p 110).

Likewise, sensitivity of the radioiodinated-elastin

assay was optimized with a 15-h incubation (Fig. 5; p 111), whereas shorter reaction times avoided nonlinearity due to substrate depletion, and expanded the useful range of the assay (Fig. 6; p 112). The daily coefficient of variation was generally less than 10% when 1 to 10 ng was assayed after five hours of incubation (Fig. 7; p 113).

At the lowest limit of the two assays, standards differing by 0.25 ng could be discriminated with HRP-elastin, and differences at least as small as 0.5 ng were significant using [^{125}I]-elastin-Sepharose (Table 2; p 79). As a measure of precision, 50 ng of porcine pancreatic elastase was assayed by four methods: the [^{125}I]-elastin-Sepharose, elastin agar, and HRP-elastin substrates all provided data which deviated less than 5% in triplicate determinations, and the reproducibility using [^{3}H]-elastin was within 8% (Table 1; p 78).

The lower limit of dependable elastase detection using tritiated elastin was, at 5 ng (Fig. 8; p 114), an order of magnitude less sensitive than the HRP-elastin or [^{125}I]-elastin assays. However, the assay was useful up to 500 ng and quite linear (Fig. 9; p 115). These three substrates were relatively resistant to cleavage by trypsin. The elastolytic activity of 100 ng of trypsin was near the lower limit of detection when assayed with HRP-elastin or radioiodinated elastin, and the amount of tritiated elastin

degraded by 100 μg of trypsin was only half that degraded by 0.1 μg of PPE (Table 3; p 80).

For higher levels of elastase activity, either the elastin-agar assay or elastin-rhodamine substrate were suitable. From 30 ng to 10 μg of porcine pancreatic elastase were detectable using the elastin-agar plate (Fig. 10, p 116; Fig. 11, p 117). Standard curves using elastin-rhodamine (Fig. 12; p 118) were perfectly linear up to 50 μg (Fig. 13; p 119).

When biological material is tested for elastase activity using HRP-elastin, it is necessary to correct for endogenous peroxidase activity. However, macrophage conditioned medium expressed activity against ABTS only after incubation with HRP-elastin (Table 4; p 81), indicating that the reaction was due to the HRP uncoupled from elastin, rather than from a macrophage-derived peroxidase.

II. EVIDENCE FOR MACROPHAGE ELASTASE SECRETION

The crude conditioned medium from 48 h cultures of hamster alveolar macrophages contained less than 0.5 ng PPE equivalent activity per 200 μl (Table 5; p 82), which was at the lower limit of detection of the HRP-elastin assay. When concentrated 16-fold, however, elastase was easily detectable, and 80-fold concentration produced the anticipated 5-fold additional increase in activity.

Elastase from murine alveolar macrophages was evident without concentration: 2.5 ng per 200 μl of conditioned medium in this experiment (Table 5; p 82). Crude conditioned medium from P388D_1 macrophages contained 0.9 ng/200 μl. Dialysis did not increase the elastase activity either of concentrated P388D_1 conditioned medium, or of crude DBA/2 alveolar macrophage conditioned medium (Table 5; p 82).

After the preliminary incubation of murine alveolar macrophages in growth medium, elastase activity was continuously secreted over a six-day period, both from control and colchicine-treated cells (Fig. 14; p 120).

From its pH profile, optimal elastase activity of crude DBA/2 alveolar macrophage conditioned medium was expressed at pH 7.6 (Fig. 15; p 121). This contrasts with

the pH 8.2 optimum recorded for porcine pancreatic elastase under identical conditions (Fig. 15; p 121) and reported for neutrophil elastase (105).

Pancreatic elastase is completely inactivated by AAPVCK and by PMSF, but retains half its activity in the presence of EDTA (Table 6; p 83). In contrast, the elastase asociated with the crude conditioned medium of alveolar macrophage is completely inhibitable by EDTA and resists inactivation by AAPVCK and PMSF. After treatment with EDTA, partial restoration of alveolar macrophage elastase activity was achieved by dialysis and subsequent addition of either calcium or zinc.

Alpha$_2$-M inhibited both pancreatic and macrophage elastase. However, α_1PI, which is known to inhibit both pancreatic and neutrophil elastase, was without effect on alveolar macrophage elastase (Table 6; p 83).

Two observations suggest a protease inhibitor is secreted by the P388D$_1$ cell line: 1) 40-fold concentration of the P388D$_1$ conditioned medium by ultrafiltration yielded less than a 4-fold increase in elastase activity (Table 5; p 82); and 2) the concentrated conditioned medium substantially inhibited the activity of both PPE and trypsin (Table 7; p 84).

III. MODULATION OF ELASTASE SECRETION

A) *IN VIVO* STUDIES

Neither lipopolysaccharide nor polyinosinic:polycytidylic acid activated alveolar macrophages to produce more elastase *ex vivo* (Table 8; p 85). Macrophages from the lipopolysaccharide-treated mice were less viable than either control or poly I:C-instilled animals, as indicated by an approximate 40% reduction in DNA after a 2-day culture period (Table 8; p 85). Elastase levels, however, were less than 1/3 of controls, and therefore would remain reduced even if expressed in relation to DNA.

One-month exposure to nitrogen dioxide did not activate the alveolar macrophages to secrete more elastase per cell (Table 8; p 85), but the mean number of macrophages recovered per animal from 6 lavages was 1.4×10^6 versus 0.4×10^6 for controls. NO_2 may therefore increase the potential elastase burden by recruiting additional macrophages to the lung.

B) *IN VITRO* STUDIES

After 60 h in culture, the conditioned medium of 1.5×10^6 murine alveolar macrophages contained the elastolytic equivalent of 125.4 ng of PPE (Table 9, p 86; calculated

from the regression equation plotted in Fig. 1b, p 107). Consistent with reports by Gordon and Werb (27) and White et al (106), the microtubule disaggregator colchicine produced over a 3.5-fold increase in elastase activity of macrophage conditioned medium. Nocodazole, a compound chemically unrelated to colchicine, but which also disrupts microtubules, likewise produced almost a 3-fold increase in elastase activity. On the contrary, taxol, which promotes microtubule assembly, and is potentially antagonistic in action to colchicine, did not produce a significant increase in elastase secretion.

Theophylline, when added to a final 2 mM concentration, produced over a 2-fold increase in the elastolytic level detected. In the presence of both colchicine and theophylline, the elastolytic activity was 5 times the control value.

Elastase activity was not significantly affected by DMSO, methylamine, or PMA, but was completely suppressed both by cycloheximide and A23187. When cells were maintained in medium containing both cycloheximide and colchicine, only 12% of the elastase activity found in the conditioned medium of macrophages cultured in colchicine alone was detected, indicating that protein synthesis is a required process for elastase production in culture at an elastolytic level of 450 ng/1.5 x 10^6 cells/60 h.

Approximately 90% of the glucose in the control medium was depleted after 60 h. This may have limited the effect of those agents which increased glucose utilization above controls. Despite this limitation, a significant increase was still observed with colchicine and with PMA (Table 10; p 87). The effect of PMA was probably unrelated to the presence of DMSO, which alone produced no significant alteration in glucose utilization. Both theophylline and cycloheximide substantially reduced glucose uptake; smaller reductions were noted when cells were maintained in medium containing colchicine plus either theophylline or cycloheximide.

The macrophages maintained in control medium released 53.8 U of the lysosomal enzyme, β-glucuronidase. Colchicine, nocodazole, and theophylline, all of which promoted elastase secretion, increased the release of β-glucuronidase to a lesser extent (Table 11; p 88). Unlike the additive increase in elastase activity of colchicine and theophylline, β-glucuronidase secretion in the presence of both agents was less than with either alone.

The greatest increase in β-glucuronidase release resulted from exposure to methylamine, which had no effect on elastase secretion. Both cycloheximide and A23187 reduced β-glucuronidase release, and the effect of cylohex-imide persisted in the presence of colchicine. Taxol and

DMSO did not have any effect on β-glucuronidase secretion. PMA produced a small increase, but it was not statistically significant. Regression analysis of PPE and β-glucuronidase indicated that the above parameters do not correlate ($r= 0.095$; Fig. 16; p 122).

The highest LDH levels were seen in the conditioned medium from colchicine-treated and from theophylline-treated macrophages (Table 12; p 89). The 5-fold increase in elastase activity produced by colchicine plus theophylline did not correlate with the 65% increase in LDH, which was less than that from either agent alone. The correlation coefficient of LDH secretion and elastase activity in response to the various agents is 0.687 (Fig. 17; p 123).

DMSO treatment resulted in a significant decrease in LDH in conditioned medium. Therefore, although nocodazole appeared not to increase LDH beyond control values, it produced a 64% increase relative to its appropriate DMSO blank. In contrast, the large reductions observed with taxol and A23187 appear largely attributation to DMSO. Cycloheximide had no effect on LDH levels, and when added with colchicine, prevented the increase seen with colchicine alone. Methylamine almost completely prevented the appearance of LDH in conditioned medium.

The only agent studied that produced a statistically significant increase in functional α_2-M was methylamine

(Table 13; p 90). Of the non-combination treatments, only theophylline reduced α_2-M levels. Whereas colchicine or cycloheximide alone lacked any significant effect on α_2-M secretion, a large reduction was observed in combination.

IV. MACROPHAGE COLLECTION

A) BCG TIME COURSE

The injection of emulsified BCG resulted in an intense granulomatous reaction in the lungs (Fig. 18; p 124) and spleens of both C57BL/6 and C57BL/KsJ mice. Whether animals were sacrificed after 2 wk, 4 wk, or 5 wk, there was no significant difference either in the number of macrophages recovered by lavage, or in spleen weight (Table 14; p 91), when compared one against another. However, when considered as a single experimental group, BCG-injected animals had twice as many lavageable macrophages as saline-injected controls, and their spleens were four times the weight of control spleens (Table 15; p 92). The BCG emulsion recruited lymphocytes and neutrophils into the lungs as well as macrophages. Therefore, when expressed relative to the total cell lavage population, the percentage of macrophages recovered from experimental mice was 1/3 that of control mice. Macrophage viability was the same in both groups (>96%).

B) CELL RECOVERY FROM LUNG MINCES

The lungs of three BCG-injected C57BL/6 mice were minced after performing six serial lavages of each. The

lavage procedure yielded an average of 0.84 x 10^6 macrophages per animal; an additional 3.6 x 10^6 macrophages were recoverable through mincing (Table 16; p 93). However, the viability of the macrophages obtained from lung minces was significantly compromised. Compared with the percentage of macrophages in the lavage fluid, twice as large a percentage of the total cells recovered from mincing were macrophages (Table 16; p 93).

C) COMPARISON OF 6 vs 18 SERIAL LUNG LAVAGES

Approximately 500,000 macrophages were collected from six 1-ml lavages of the lungs of DBA/2 mice (Table 17; p 94). An additional 400,000 macrophages were obtained when a total of 18 serial lavages were performed.

D) THIOGLYCOLLATE TIME COURSE IN DBA/2 MICE

Approximately 2 million resident cells were lavaged from the peritoneal cavity of control DBA/2 mice, 3/5 of which were macrophages (Table 18; p 95). Two days after i.p. administration of thioglycollate, the total cell count increased to 6 million, and the macrophage fraction rose to 3/4. At day 3, the cell count peaked at 10 million, with macrophages still comprising 75% of the total cell population. Between days 3 and 4 there was a rapid resolution of

the cellular influx as the lavage fluid composition returned to baseline values by day 4.

E) STRAIN COMPARISON OF RESPONSE TO THIOGLYCOLLATE

Three days after i.p. thioglycollate, macrophage elicitation in BDF_1 mice was similar to that in DBA/2 mice (Table 19; p 96). Strain C57BL/6 mice were, however, much more responsive: over 25 million macrophages were recoverable, and they comprised over 80% of the total cell population lavaged.

V. PURIFICATION PROCEDURES

A) ALPHA$_2$-MACROGLOBULIN PURIFICATION

Alpha$_2$-M was purified from the serum of turpentine-injected hamsters. Unlike the large increase in serum α_2-M levels achieved by injecting turpentine into rats (67), this procedure did not elevate hamster α_2-M. Pre-turpentine serum levels were not determined, but α_2-M activity 48 h post-injection was 11.3 U/ml (i.e. 136 U ÷ 12 ml; Table 20, p 97) versus 16.7 U/ml (Table 21a; p 98) in pooled serum from normal hamsters.

During purification, α_2-M activity eluted from the first DEAE-cellulose column with 0.07 M acetate buffer (Fig. 19; p 125). After reapplication of this partially purified material to the DEAE column and elution with a linear gradient, all the α_2-M activity was found in the first peak (Fig. 20; p 126). Ultimate purification was achieved by gel filtration by collecting the first peak from a Sephacryl S-300 column (Fig. 21; p 127). Apparent protein homogeneity was established by noting a single peak on agarose electrophoresis (Fig. 22; p 128), and a single precipitin arc upon immunoelectrophoresis (Fig. 23; p 129). During the purification procedure, 33% of the starting α_2-M activity was recovered, and the α_2-M was enriched over 20-fold (Table 20; p 97).

B) ALPHA$_2$-MACROGLOBULIN ANTISERUM

Specific antiserum to hamster α_2-M, available from Drs. Kleinerman and Ip, was used 1) to develop an α_2-M immunoelectrophoretic assay, and 2) to evaluate the potential of the antiserum to inactivate the antiprotease function of α_2-M. If the antiserum effectively reduced functional α_2-M levels it could be used in macrophage cultures to prevent the interaction of elastase with α_2-M secreted by the macrophages, and possibly lead to greater elastolytic expression of the elastase in the conditioned medium.

The antiserum produced a single arc in reaction with whole hamster serum (Fig. 24; p 130). The results of rocket immunoelectrophoresis of serum standards, using whole antiserum, is shown in Fig. 25 (p 131). A plot of the standard curve (Fig. 26; p 132) had a correlation coefficient of 0.995 (Fig. 27; p 133).

Whole rabbit antiserum contains α_2-M which had to be removed, so as not to introduce exogenous α_2-M while attempting to reduce functional levels of endogenous α_2-M secreted by cultured hamster macrophages. When the rabbit antiserum was applied to a DEAE-Sephacel column, the pooled, concentrated material which eluted with 0.0175 M phosphate buffer (Fig. 28; p 134) contained negligible α_2-M activity (Table 21a; p 98).

Approximately 25% of the functional serum α_2-M resisted inactivation by the IgG fraction (Table 21b; p 98). Affinity chromatography of the partially purified IgG resulted in one major peak being eluted with each buffer change (Fig. 29; p 135). Rabbit IgG was present in all fractions preceding 1 M salt, when tested immunologically against goat anti-(rabbit IgG) (Fig. 24; p 130). Anti-(hamster α_2-M) IgG, assessed by precipitation with whole hamster serum, was demonstrated only in fractions 7, 10, and 19 (Fig. 24; p 130). The poor yield prevented further evaluation of the α_2-M inactivating potential of these fractions.

C) PURIFICATION OF P388D$_1$ MACROPHAGE ELASTASE

The concentrated, dialyzed conditioned medium of P388D$_1$ macrophages was fractionated by affinity chromatography, using SDS-treated α-elastin-Sepharose (Fig. 30a; p 136). Elastase activity was measured in selected fractions (Fig. 30b; p 136) and the entire effluent was combined into 6 pools.

Fractions 1-20, 21-30, and 31-60 each contained over 20% of the total elastase activity (Fig. 31; p 137), but were contaminated with the bulk of protein. Pool 61-70 appeared almost homogeneous on electrophoresis, with a molecular weight of 15k (Fig. 32; p 138), but contained

only 13% of the elastase activity. An additional 16% of the elastase was found in pool 71-83, and a residual 7% in the final pool. The same two major bands were visible in each of the last two pools (Fig. 33; p 139): one, corresponding to 15k was the predominant band of pool 71-83, but the secondary band of pool 84-156; the other band corresponded to a molecular weight of approximately 50k.

Pool 61-70 was further fractionated by gel filtration HPLC into 3 elastase-containing peaks: a minor peak with an elution volume, based on molecular weight standards, indicative of 40k MW; a second peak corresponding to an apparent MW of 10-15k; and a third peak with an apparent MW below 10k (Fig. 34; p 140). When pool 31-60 was applied to the column, only 2 peaks contained elastase: the predominant peak eluted as a 40k MW protein and the small peak at approximately 10k (Fig. 35; p 141).

Pools 71-83 and 84-156 were combined and likewise applied to the Fractogel column, which succeeded in resolving the two major proteins into two peaks (Fig. 36; p 142): one at 15-25k MW, and the other at approximately 10k. Over 80% of the elastase activity was in the second peak; its specific elastase activity was 60-fold greater than the first peak (Table 22a; p 99). Passage through the Fractogel column resulted in an absolute increase in the total elastase activity recovered from each pool (Table 22b; p

99).

Ammonium sulfate fractionation of the crude conditioned medium was attempted as a preliminary purification step. Seventy percent of the total elastase activity was contained between 35 and 55% saturation (Table 23; p 100). The 45-55% fraction was applied directly to the Fractogel column, without preliminary separation by affinity chromatography. Again, activity was seen at 10-15k, but the 40k peak was very small, and there were at least two additional higher MW peaks, including a very prominent peak corresponding to a MW of 50-55k (Fig. 37; p 143).

Anion exchange chromatography of the $P388D_1$ conditioned medium isolated the elastase activity into two main peaks: one at the void volume and the other at the beginning of the salt gradient (Fig. 38; p 144).

D) PURIFICATION OF DBA/2 PERITONEAL MACROPHAGE ELASTASE

Anion exchange chromatography separated DBA/2 peritoneal macrophage conditioned medium into two peaks of elastase activity: a sharp peak eluted at the void volume, and a smaller, broader peak appeared at the beginning of the salt gradient (Fig. 39; p 145). The fractions under each elastase peak were separately pooled and concentrated

and further fractionated by gel filtration chromatography.

The void volume sample eluted from the AcA54 column with one main peak expressing elastase activity at an apparent molecular weight--with respect to calibration standards--of 25k (Fig. 40; p 146). Two earlier protein peaks contained significantly less elastase activity (Fig. 40, p 146; Table 24a, p 101). Electrophoresis of the descending portion of the main elastase peak (peak 3) revealed a major band at 23k and a fainter band at 21k (Fig. 41; p 147). Molecular weights were assigned by linear regression analysis of migration distance of molecular weight standards aginst the log of molecular weight. The regression plot (Fig. 42; p 148) had a correlation coefficient of -.997. These two bands were also visible in the electrophoresis gel of fractions from the ascending portion of the peak (Fig. 40; p 146), along with several higher molecular weight proteins. Most of the protein and elastase activity in AcA54 fractions 51-64 subsequently eluted at the void volume from a cation exchange column (Fig. 43; p 149). However, erratic elastase activity was seen in post-void fractions. Electrophoresis of pooled fractions 18-47 from the SP-Sephadex column demonstrated a doublet at 55-60k MW (Fig. 44; p 150).

The material which was slightly retained on the DEAE column (fractions 64-81) separated into two sharp peaks and

one broad peak on the AcA54 column (Fig. 45; p 151). All contained comparable elastase activity (Table 24a; p 101), and eluted at the same volumes as the three peaks from the void sample elution. The first peak appeared purer than the second on electrophoresis (Fig. 46; p 152), with a doublet at 55-60k apparently equivalent to that seen from the SP-Sephadex column. The upper band from both samples was the predominant protein. The tube gel of the second peak (Fig. 46; p 152), in addition to the major band at 57-60k, contained protein corresponding to 27k and 35k MW.

The elastase activity under each of the three peaks from the AcA54 column was completely inhibitable by EDTA; at least 80% activity was retained by each peak in the presence of the serine protease inhibitor, PMSF (Table 25; p 102).

E) PURIFICATION OF C57BL/6 PERITONEAL MACROPHAGE ELASTASE

C57BL/6 peritoneal macrophage conditioned medium was also subjected to sequential anion exchange and gel filtration chromatography. As with the DBA/2 conditioned medium, a sharp peak of elastase activity occurred at the void volume of the DEAE-Sephadex column (Fig. 47; p 153). However, unlike the single peak from DBA/2 mice, this was followed by a second peak only slightly retarded in its pas-

sage through the column. In addition, whereas over 40% (Table 24a; p 101) of the total elastase activity recovered from the DEAE column from DBA/2 conditioned medium required elution with salt, less than 10% of the C57BL/6 elastase came through the same column with NaCl-containing buffer.

The material under the double peak at the DEAE void volume was further fractionated on the AcA54 column. Three peaks of elastase activity occurred at the exact elution volumes as the three peaks from the DBA/2 samples (Fig. 48; p 154). Total elastase activity, as well as peak specific activity, was greatest in the third AcA54 peak of both DBA/2 and C57BL/6 conditioned medium (Table 24a; p 101). Upon electrophoresis, the doublet at approximately 57k was evident in all peaks, whereas the 23k doublet was only seen in peak 2 (Fig. 49; p 155).

F) PURIFICATION OF DBA/2 ALVEOLAR MACROPHAGE ELASTASE

Elastase from DBA/2 alveolar macrophage conditioned medium, unlike $P388D_1$ elastase, was not partially retarded beyond the major protein peak in its passage through an SDS- α-elastin-Sepharose column (Fig. 50; p 156). Elastase-containing fractions were therefore pooled, concentrated, and fractionated by anion exchange chromatography. Peaks of elastase activity eluted at the void volume and at low salt concentration (Fig. 51; p 157). The

limited enzyme activity recovered from these two peaks precluded any attempt at further purification of alveolar macrophage elastase.

VI. INDUCTION AND EVALUATION OF EXPERIMENTAL EMPHYSEMA

A) ELASTOLYTIC AND HEMORRHAGIC ACTIVITY OF PORCINE PANCREATIC ELASTASE IN HAMSTERS

The esterolytic activity of the Sigma porcine pancreatic elastase (S-PPE) toward SLAPN was found to be 38% that of the Elastin Products elastase (EP-PPE) (Table 26a; p 103). Subsequently, a 10 U/0.1 ml solution of EP-PPE was prepared according to the specific activity data provided by the manufacturer, and a solution of S-PPE was dosage-matched in terms of SLAPN activity (Table 26b; p 103).

Forty-eight hours after intratracheal instillation into hamsters, the Lm of EP-PPE-injected animal (68.3 ± 3.1 μm) was significantly greater than that of saline-injected controls (57.8 ± 4.7 μm) or S-PPE -injected animals (60.8 ± 2.9 μm) (Table 27a; p 104). The difference in Lm between saline-injected and S-PPE-injected hamsters was not statistically significant.

Evidence of pulmonary hemorrhage was relatively rare in control animals (Table 27b; p 104). The EP-PPE was responsible for a 2.5-fold increase in the percentage of hemorrhagic fields, and, with one-fifth of the fields affected, the S-PPE-injected hamsters had the highest incidence of hemorrhage.

B) DISTRIBUTION OF INSTILLATE IN MOUSE LUNGS

All lobes of all three mice injected intratracheally with drawing ink were completely and homogeneously blackened when visually inspected 15-min post-instillation.

C) INSTILLATION OF PANCREATIC ELASTASE INTO MICE

There was 100% mortality among the mice instilled with 10 U of porcine pancreatic elastase. They all survived the instillation but died of apparent respiratory distress within 30 min. At autopsy, the lungs displayed extensive hemorrhage and edema (Fig. 52; p 158).

In contrast, all the mice tolerated 5 U of elastase. Upon gross examination 48-h post-instillation, coarsly textured regions of emphysema were seen on the lung surface. Widespread parenchymal damage was evident on histologic sections (Fig. 53c; p 159). Lesions of both panlobular and centrilobular character were noted. The mean Lm of these experimental animals was twice the value of controls (Table 28; p 105).

D) INSTILLATION OF MACROPHAGE CONDITIONED MEDIUM INTO MICE

Two weeks after the instillation of the concentrated

conditioned medium from cultured murine alveolar macrophages, the lungs of the experimental animals appeared normal in gross examination. Likewise, their Lm, although 2 μm longer, was not statistically different from control Lm (Table 29a; p 106). However, in three of the five experimental mice, focal areas of alveolar damage were seen on lung sections: most notably toward the periphery of the basal lobes of two of these animals (Fig. 53b; p 159). Local airway enlargement in similar regions from control animals appeared generally less extensive (Fig. 53a; p 159).

Almost twice as large a percentage of the lung fields of experimental animals had an Lm greater than one standard deviation above the sample mean than control animals (Table 29a; p 106) but this difference lacked statistical significance.

ASSAY	USEFUL DETECTION RANGE of PPE	COEFFICIENT of CORRELATION	REPRODUCIBILITY 50 ng ± 1 SD
SLAPN	100 ng - 50 µg*	-	-
Elastin-Rhodamine	5 - 50 µg	1.0	-
Elastin-Agar	30 ng - 10 µg*	-	50 ± 2.0
	80 - 500 ng	0.974	
HRP-Elastin	0.25 - 100 ng*	-	50 ± 2.4
	0.5 - 20 ng	0.990	
	5 - 100 ng	0.994	
[^{3}H]-Elastin	5 - 500 ng*	-	50 ± 3.8
	5 - 50 ng	0.992	
[^{125}I]-Elastin	0.5 - 100 ng*	-	50 ± 1.3
	0.5 - 10 ng	0.987	
	5 - 50 ng	0.968	

Table 1. Comparison of useful ranges of six elastase assays.

*This range achieved by combining data obtained under 2 different assay conditions; correlation coefficient therefore not applicable.

a)

PPE (ng)	HRP-Elastin Assay (OD_{414})	T-test Pairs
0	0.345 ± 0.005	
0.25	0.367 ± 0.004	p<.005 (0.25 vs 0 ng)
0.5	0.386 ± 0.003	p<.005 (0.5 vs 0.25 ng)

b)

PPE (ng)	[^{125}I]-Elastin Assay (% Release ^{125}I)	T-test Pairs
0	8.95 ± 0.16	
0.5	10.60 ± 0.43	p<.005 (0.5 vs 0 ng)
1	11.97 ± 0.13	p<.01 (1 vs 0.5 ng)

Table 2. Detection of low level elastase activity using a) HRP-elastin and b) [^{125}I]-elastin.

	HRP-Elastin (OD_{414})	[^{125}I]-Elastin (% Release ^{125}I)	[^{3}H]-Elastin (cpm)
Blank	0.230	10.5	163
PPE, 1 ng	-	14.2	-
PPE, 5 ng	-	22.8	-
PPE, 6 ng	0.299	-	-
PPE, 100 ng	0.596	-	15229
Trypsin, 100 ng	0.247	11.2	-
Trypsin, 100 μg	-	-	6612

Table 3. Susceptibility of three elastin substrates to cleavage by trypsin.

TREATMENT	OD_{414}		
	Initial	30 Min	60 Min
CM added after incubation	0.244	0.248	0.251
CM added before incubation	0.268	0.504	0.755

Table 4. Absence of endogenous peroxidase activity in alveolar macrophage conditioned medium (CM).

CM was added to HRP-elastin in assay buffer either before or after a 15 h incubation period. The supernatant was tested for peroxidase activity at 0, 30, and 60 min after addition of ABTS substrate solution.

SAMPLE	ELASTASE ACTIVITY (ng PPE equiv)		
	Hamster Alveolar Macrophages	Mouse Alveolar Macrophages	$P388D_1$ Macrophages
Crude CM	<0.5	2.5	0.9
Dialyzed CM	-	2.0	-
16X conc CM	5.5	-	-
80X conc CM	25.5	-	-
40X conc CM	-	-	3.1
40X conc, dialyzed CM	-	-	3.1

Table 5. Elastase activity of macrophage conditioned medium.

1.7×10^6 macrophages were cultured for 48 h in 0.5 ml of medium. The elastase activity in 200 microliters of conditioned medium was assayed using HRP-elastin.

	ELASTASE ACTIVITY (% of Control)		
	Mouse AM CM	Hamster AM 16X-CM	PPE
Control	100	100	100
EDTA, 1 mM	75	-	-
EDTA, 10 mM	0	-	49
EDTA, 20 mM	-	0	-
EDTA, 10 mM + $ZnSO_4$, 10 mM	20	-	-
EDTA, 10 mM + $CaCl_2$, 50 mM	43	-	-
AAPVCK, 5 mM	77	-	0
AAPACK, 2.5 mM	-	92	-
Hamster $Alpha_2$-M, 70 μg/ml	14	-	6
Hamster $Alpha_1$-PI, 50 μg/ml	100	-	-
PMSF, 10 mM	80	-	2

Table 6. Inhibitors of elastase activity.

Mouse AM CM = DBA/2 alveolar macrophage crude conditioned medium; Hamster AM 16X CM = 16-fold concentrated conditioned medium from hamster alveolar macrophages; PPE = 20 *ng* porcine pancreatic elastase. Elastase activity was assayed using HRP-elastin.

SAMPLE	CPM
CM	10,400
Trypsin, 100 μg	6,600
PPE, 100 ng	15,200
Trypsin + PPE	23,000
Trypsin + CM	12,600
PPE + CM	11,100

Inhibition of trypsin:

[6,600 - (12,600 - 10,400)] / 6,600 x 100 %

= 67 %

Inhibition of PPE:

[15,200 - (11,100 - 10,400)] / 15,200 x 100 %

= 95 %

Table 7. Inhibition of trypsin and porcine pancreatic elastase by $P388D_1$ conditioned medium.

CM = 40-fold concentrated $P388D_1$ conditioned medium; CPM = counts per minute released from tritiated elastin in 100 microliters of supernatant.

TREATMENT	ELASTASE ACTIVITY	DNA (μg)
Control	1.3	5.4
Poly I:C, 125 μg	0.8	5.3
Control	1.6	3.9
LPS, 200 μg	0.5	2.4
Control	1.3	
NO_2, 30 ppm	0.7	

Table 8. In vivo modulation of alveolar macrophage elastase-secreting capability.

LPS = *E. coli* lipopolysaccharide 055:B5. Poly I:C = polyinosinic:polycytidylic acid.

Poly I:C and LPS were instilled intratracheally. NO_2 was present in environmnent; controls breathed room air. Elastase activity/48h/10^6 cells/200μl of conditioned medium was determined using HRP-elastin. DNA was measured in lysates of cells which remained at end of culture period.

TREATMENT	PPE EQUIV (ng ± 1 SD)	T-TEST	% CONTROL
Colchicine, 2 μM	451.1 ± 70.8	p<.001	360
Theophylline, 2 mM	286.9 ± 3.0	p<.001	229
Colch + Theo	626.2 ± 54.9	p<.001	499
Cycloheximide, 0.25 μg/ml	0	p<.001	0
Colch + Cyclohex	54.0 ± 4.7	p<.001	43
Nocodazole, 3 μM	343.4 ± 47.2	p<.002*	282*
Taxol, 10 μM	168.4 ± 19.2	ns (p=.063)*	138*
PMA, 0.2 μM	143.2 ± 21.5	ns (p=.28)	114
A23187, 1 μM	0	p<.001*	0*
DMSO, 0.1%	121.8 ± 25.2	ns (p>.3)	97
Methylamine, 50 mM	130.1 ± 10.9	ns (p>.3)	104
Control	125.4 ± 12.0		100

*Relative to DMSO

ANOVA: p<.001 (excluding cycloheximide and A23187)

Table 9. The effect of various agents on elastase secretion by cultured murine alveolar macrophages.

The total elastase activity secreted by 1.5 x 10^6 DBA/2 alveolar macrophages during 60 h of culture was assayed using HRP-elastin, and calculated from the regression equation plotted in Fig. 1b.

TREATMENT	GLUCOSE UTILIZATION (% of Control)	T-TEST
Colchicine, 2 μM	106.8 ± 3.0	p<.05
Theophylline, 2 mM	54.3 ± 0.7	p<.001
Colch + Theo	89.9 ± 1.2	p<.01
Cycloheximide, 0.25 μg/ml	48.3 ± 4.2	p<.001
Colch + Cyclohex	70.8 ± 2.5	p<.001
Nocodazole, 3 μM	103.9 ± 3.4*	ns (p=.21)*
Taxol, 10 μM	103.2 ± 1.5*	ns (p=.17)*
PMA, 0.1 μM	106.7 ± 2.8	p<.05
A23187, 1 μM	101.0 ± 1.4*	ns (p>.3)*
DMSO, 0.1 %	98.4 ± 3.4	ns (p>.3)
Methylamine, 50 mM	107.0 ± 4.6	ns (p=.09)
Control	100.0 ± 3.0	

*Relative to DMSO

ANOVA: $p<.001$

Table 10. The effect of various agents on glucose utilization by cultured murine alveolar macrophages.

TREATMENT	BETA-GLUCURONIDASE (nmol substrate cleaved/h)	T-TEST	% CONTROL
Colchicine, 2 μM	97.0 ± 13.8	p<.001	180
Theophylline, 2 mM	74.1 ± 6.7	p<.001	138
Colch + Theo	61.8 ± 4.1	p<.02	115
Cycloheximide, 0.25 μg/ml	27.6 ± 0.5	p<.001	51
Colch + Cyclohex	30.7 ± 2.8	p<.001	57
Nocodazole. 3 μM	104.7 ± 16.1	p<.01*	183*
Taxol, 10 μM	53.1 ± 4.3	ns (p=.27)*	93*
PMA, 0.1 μM	61.2 ± 3.2	ns (p=.26)	114
A23187, 1 μM	34.9 ± 2.3	p<.001*	61*
DMSO, 0.1 %	57.3 ± 3.9	ns (p=.21)	107
Methylamine, 50 mM	314.8 ± 2.2	p<.001	585
Control	53.8 ± 3.4	-	100

*Relative to DMSO

ANOVA: p<.001

Table 11. The effect of various agents on beta-glucuronidase secretion by cultured murine alveolar macrophages.

Total beta-glucuronidase activity in conditioned medium from 1.5×10^6 DBA/2 alveolar macrophages assayed using p-nitrophenyl beta-glucuronide substrate.

TREATMENT	LDH (Units ± 1 SD)	T-TEST	% CONTROL
Colchicine, 2 μM	145.0 ± 9.8	p<.001	221
Theophylline, 2 mM	124.0 ± 17.1	p<.001	189
Colch + Theo	108.1 ± 4.5	p<.001	165
Cycloheximide, 0.25 μg/ml	65.5 ± 3.7	ns (p>.3)	100
Colch + Cyclohex	52.0 ± 4.6	p<.01	79
Nocodazole, 3 μM	61.0 ± 12.2	ns (p>.3)	93 (164*)
Taxol, 10 μM	33.0 ± 8.4	p<.001	50 (89*)
PMA, 0.1 μM	74.6 ± 4.9	p<.05	114
A23187, 1 μM	25.0 ± 4.3	p<.001	38 (67*)
DMSO, 0.1 %	37.1 ± 3.7	p<.001	57
Methylamine, 50 mM	5.0 ± 7.4	p<.001	8
Control	65.5 ± 4.9		100

*Relative to DMSO

ANOVA: p<.001

Table 12. Effect of various agents on LDH levels in conditioned medium of cultured murine alveolar macrophages.

TREATMENT	$ALPHA_2$-MACROGLOBULIN (Microunits ± 1 SD)	T-TEST	% CONTROL
Colchicine, 2 μM	55.0 ± 8.3	ns (p=.11)	134
Theophylline, 2 mM	23.2 ± 7.7	p=.05	57
Colch + Theo	46.3 ± 7.7	ns (p>.3)	113
Cycloheximide, 0.25 μg/ml)	33.8 ± 6.1	ns (p>.3)	83
Colch + Cyclohex	14.7 ± 8.9	p<.02	36
Nocodazole, 3 μM	50.4 ± 5.6	ns (p=.24)*	118*
Taxol, 10 μM	36.1 ± 1.2	ns (p>.3)*	85*
PMA, 0.1 μM	47.4 ± 3.2	ns (p=.26)	116
A23187, 1 μM	41.4 ± 11.6	ns (p>.3)*	97*
DMSO, 0.1 %	42.6 ± 5.4	ns (p>.3)	104
Methylamine, 50 mM	62.0 ± 3.8	p<.05	152
Control	40.9 ± 11.8		100

*Relative to DMSO

ANOVA: p<.001

Table 13. Effect of various agents on $alpha_2$-macroglobulin activity in conditioned medium of cultured murine alveolar macrophages.

TIME POST-BCG	MACROPHAGE COUNT ($\times 10^6$; ± 1 SD)	% MACROPHAGES	SPLEEN WEIGHT (mg)
2 weeks (n=4)	1.60 ± 0.63	45.4 ± 7.7	407 ± 103
4 weeks (n=6)	1.50 ± 0.51	41.3 ± 6.0	253 ± 101
5 weeks (n=5)	1.83 ± 0.53	26.5 ± 6.6	272 ± 111

STATISTICS

	MACROPHAGE COUNT	% MACROPHAGES	SPLEEN WEIGHT
ANOVA	ns (p>.3)	p<.001	ns (p=.1)
T-test (2 wk; 4 wk)	-	ns (p=.25)	-
T-test (2 wk; 5 wk)	-	p<.001	-
T-test (4 wk; 5 wk)	-	p<.001	-

Table 14. Time course inflammatory response to i.v. emulsified BCG in C57 mice.

	SALINE (n=8)	BCG (n=24)	(T-TEST)
MACROPHAGE COUNT (x 10^6)	0.89 ± 0.34	1.79 ± 0.86	$p<.001$
WBC COUNT (x 10^6)	0.068 ± 0.044	3.84 ± 2.24	$p<.001$
SPLEEN WEIGHT (mg)	75 ± 21	290 ± 91	$p<.001$
% MACROPHAGES	90.9 ± 7.9	33.5 ± 8.7	$p<.001$
MACROPHAGE VIABILITY (%)	96.7 ± 4.6	96.1 ± 2.9	ns ($p>.3$)

Table 15. Spleen weight and alveolar lavage cell count 2-5 d after emulsified BCG was administered i.v. to C57 mice.

TREATMENT	MACROPHAGE COUNT ($x 10^6$)	MACROPHAGE VIABILITY (%)	WBC COUNT ($x 10^6$)	% MACROPHAGES
Lavage (n=3)	0.84 ± 0.28	92.9 ± 5.0	3.08 ± 0.28	21.1 ± 4.1
Mince (n=3)	3.60 ± 0.81	68.4 ± 11.4	4.63 ± 0.88	43.7 ± 7.3
(T-test)	($p<.01$)	($p<.05$)	($p<.01$)	($p<.05$)

Table 16. Comparison of cell yield obtained by bronchoalveolar lavage and lung mincing

Six serial lung lavages were performed on BCG-injected C57BL/6 mice, and the lungs were then minced.

NUMBER OF LAVAGES	MACROPHAGES RECOVERED ($\times 10^5$; $\pm$ 1 SD)
6 (n=8)	4.82 $\pm$ 1.59
18 (n=10)	8.95 $\pm$ 1.19
(T-test)	($p<.001$)

Table 17. Comparison of macrophage yield from DBA/2 mice obtained with six and eighteen serial lung lavages.

	MACROPHAGE COUNT (x 10^6 ; ± 1 SD)	WBC COUNT (x 10^6 ; ± 1 SD)	PERCENT MACROPHAGES
CONTROL (n=6)	1.13 ± 0.21	0.81 ± 0.17	58.2 ± 7.6
DAY 2 (n=6) (T-test)	4.33 ± 0.70 (p<.001)	1.45 ± 0.46 (p<.01)	74.8 ± 7.5 (p<.01)
DAY 3 (n=8) (T-test)	7.53 ± 1.77 (p<.001)	2.38 ± 0.63 (p<.001)	75.6 ± 6.6 (p<.001)
DAY 4 (n=6) (T-test)	1.25 ± 0.40 (ns; p>.3)	0.98 ± 0.14 (ns; p=.07)	54.7 ± 6.5 (ns; p>.3)
[ANOVA]	[p<.001]	[p<.001]	[p<.001]

Table 18. Time course of peritoneal inflammatory cell response following i.p. thioglycollate in DBA/2 mice.

STRAIN	MACRAPHAGE COUNT (x 10^6 ± 1 SD)	WBC COUNT (x 10^6 ± 1 SD)	% MACROPHAGES
C57BL/6 (n=7)	26.4 ± 6.6	5.6 ± 1.5	82.5 ± 2.0
BDF_1 (n=4)	8.2 ± 2.1	3.9 ± 2.1	68.1 ± 14.1
DBA/2 (n=8)	7.5 ± 1.8	2.4 ± 0.6	75.6 ± 6.6
(ANOVA)	(p<.001)	(p<.001)	(p<.05)

T-TEST PAIRS	MACROPHAGE COUNT	WBC COUNT	% MACROPHAGES
C57BL/6; BDF_1	p<.001	ns (p=.15)	p<.05
C57BL/6; DBA/2	p<.001	p<.001	p<.05
BDF_1; DBA/2	ns (p>.3)	ns (p=.08)	ns (p=.23)

Table 19. Strain comparison of inflammatory cell yield obtained by peritoneal lavage.

Mice were lavaged 3 days after i.p. injection of 2 ml thioglycollate solution.

STAGE	ALPHA$_2$-M ACTIVITY (Units)	PROTEIN (mg)	SPECIFIC ACTIVITY (U/mg)	ENRICHMENT (fold)
Serum	136	540	0.25	1
DEAE - cellulose (application I)	92	47	2.0	7.8
DEAE - cellulose (application II)	ND	29	-	-
Sephacryl S-300	45	8.8	5.2	21

Table 20. Summary of purification of α_2-M from hamster serum.

Activity of α_2-M assayed using chromozym - TRY

a)

SAMPLE	ALPHA$_2$-MACROGLOBULIN ACTIVITY (U/ml)
Hamster whole serum	16.7
Partially purified rabbit IgG	0.013

b)

SAMPLE	α_2-M ACTIVITY (mUnits)	% INHIBITION OF CONTROL
Control (serum alone)	7.94	-
Serum + 25 μl IgG	2.36	70
Serum + 100 μl IgG	1.83	77

Table 21a. Comparison of α_2-M activity in hamster serum and partially purified rabbit IgG.

Table 21b. Inactivation of hamster α_2-M by partially purified rabbit anti-α_2-M IgG.

a)

ELUTION VOLUME (ml)	ELASTASE ACTIVITY (µg PPE Equiv)	TOTAL PROTEIN (µg)	SPECIFIC ACTIVITY
97-104	0.19	839	0.00023
111-121	2.74	193	0.014

b)

POOL FROM ELASTIN-SEPHAROSE (fractions)	ELASTASE ACTIVITY APPLIED TO FRACTOGEL (µg PPE equiv)	ELASTASE ACTIVITY RECOVERED FROM FRACTOGEL PEAKS (µg PPE equiv)		
		40k	15k	<10k
31-60	0.26	1.76	0.71	-
61-70	0.16	0.80	5.72	8.17
71-156	0.27	-	0.19	2.74

Table 22a. $P388D_1$ elastase activity isolated on Fractogel column from fractions 71-156 from elastin-Sepharose column.

Table 22b. Summary of $P388D_1$ elastase activity recovered from elastin-Sepharose fractions applied to Fractogel column.

AMMONIUM SULFATE CUT (% Saturation)	ELASTASE ACTIVITY	
	(μg PPE equiv)	(% of total)
0-35	2.96	11
35-45	7.53	29
45-55	10.44	41
55-65	4.92	19
65-75	0	0
75-85	0	0
85-100	0	0

Table 23. Ammonium sulfate fractionation of $P388D_1$ conditioned medium Elastase assayed with [^{125}I]-elastin-Sepharose.

a)

ELASTASE ACTIVITY (μg PPE equiv)

	DEAE Void Pk	DEAE Grad Pk	AcA54/Pk1	AcA54/Pk2	AcA54/Pk3
DBA/2	7.14	-	0.08	0.20	1.26
	-	5.48	0.34	0.42	0.34
C57BL/6	13.96	-	0.37	0.65	2.92
	-	1.30	-	-	-

b)

SPECIFIC ACTIVITY (ng activity/μg protein)

	Peak 1	Peak 2	Peak 3
DBA/2	0.10	0.09	0.46
C57BL/6	0.36	0.80	3.14

Table 24a. DBA/2 and C57BL/6 peritoneal macrophage elastase activity recovered from AcA54 column after application of DEAE-Sephadex peak fractions.

24b. Specific DBA/2 and C57BL/6 peritoneal elastase activity of peaks off AcA54 column

Specific activity of DBA/2 AcA54 Peaks 1 and 2 were from fractionation of DEAE gradient peak; calculation for Peak 3 was from DEAE void peak separation. Elastase was assayed using HRP-elastin.

	ELASTASE ACTIVITY (% of CONTROL)		
	EDTA (10 mM)	PMSF (10 mM)	CONTROL
Peak 1	2	81	100
Peak 2	4	94	100
Peak 3	0	80	100

Table 25. Activity of AcA54 peak purified fractions from DBA/2 peritoneal macrophage conditioned medium in the presence of EDTA or PMSF.

Peaks 1 and 2 were from AcA54 fractionation of DEAE gradient peak; Peak 3 was from application of DEAE void peak on AcA54 column. Elastase activity was assayed with tritiated elastin.

a)

ELASTASE	SPECIFIC ACTIVITY (according to label)	SPECIFIC ACTIVITY (assayed with SLAPN)
Elastin Products PPE	112.7	30.7
Sigma PPE	75	11.7

b)

ELASTASE	SLAPN ASSAY (Delta OD_{405}/min)
Elastin Product PPE, 0.887 mg/ml	0.868 ± 0.001
Sigma PPE, 2.328 mg/ml	0.870 ± 0.003

Table 26a) Comparison of activity of Sigma and Elastin Products formulations of porcine pancreatic elastase.

b) Equivalent esterolytic activity of elastase instillates.

a)

TREATMENT GROUP	MEAN LINEAR INTERCEPT (L_m ± 1 SD; micrometers)	T-TEST
Saline (n=5)	57.8 ± 4.7	-
Sigma PPE (n=4)	60.8 ± 2.9	ns (p>.3)
Elastin Products PPE (n=4)	68.3 ± 3.1	p<.01 (p<.05 vs Sigma PPE)
	[ANOVA: p<.01]	

b)

TREATMENT GROUP	EXTENT OF HEMORRHAGE (% of lung fields)	T-TEST
Saline (n=5)	3.4 ± 2.2	-
Sigma PPE (n=4)	20.0 ± 7.5	p<.005
Elastin Products (n=4)	8.4 ± 3.1	p<.05 (p<.05 vs Sigma PPE)
	[ANOVA: p<.001]	

Table 27a) Mean linear intercept, 48 h post-instillation of elastase into hamsters.

b) Extent of hemorrhage, 48 h post-instillation of elastase into hamsters.

TREATMENT GROUP	MEAN LINEAR INTERCEPT (MICROMETERS ± 1 SD)	T-TEST
Control (PBS)	49 ± 4	
PPE, 5 U	89 ± 8	p<.001

Table 28. Effect of 5 units of porcine pancreatic elastase on mean linear intercept of mouse lungs.

Mice were sacrificed 48-h after intratracheal instillation of phosphate-buffered saline, pH 7.4 (PBS), or PBS containing 5 units of porcine pancreatic elastase.

a)

TREATMENT GROUP	MEAN LINEAR INTERCEPT (Micrometers ± 1 SD)	T-TEST
Control (RPMI-LH) (n=5)	45.2 ± 3.8	
Conditioned Medium (n=5)	47.2 ± 3.1	ns (p>.3)

b)

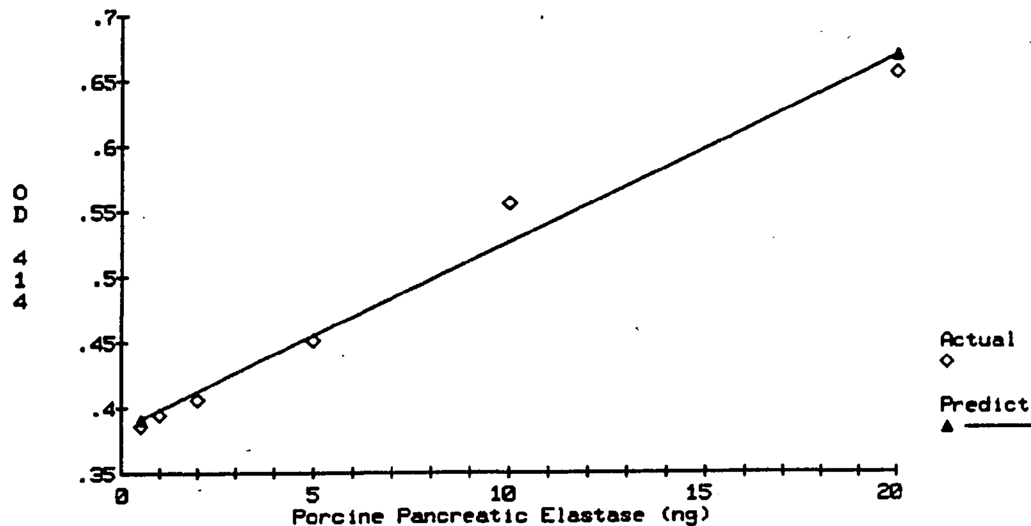
TREATMENT GROUP	% LUNG FIELDS WITH HIGH MEAN LINEAR INTERCEPT	T-TEST
Control (RPMI-LH) (n=5)	10.2 ± 9.7	
Conditioned Medium (n=5)	17.1 ± 8.8	ns (p=.3)

Table 29a) Effect of instilled conditioned medium on mean linear intercept of mouse lungs.

Conditioned medium from macrophages of DBA/2 mice contained elastase activity equivalent to 0.5 micrograms of porcine pancreatic elastase.

29b) Percentage of individual lung fields with high L_m after instillation of conditioned medium.

L_m was considered high when it was greater than 1 standard deviation above the sample mean. All lung fields were counted; there were at least 25 lung fields per animal.



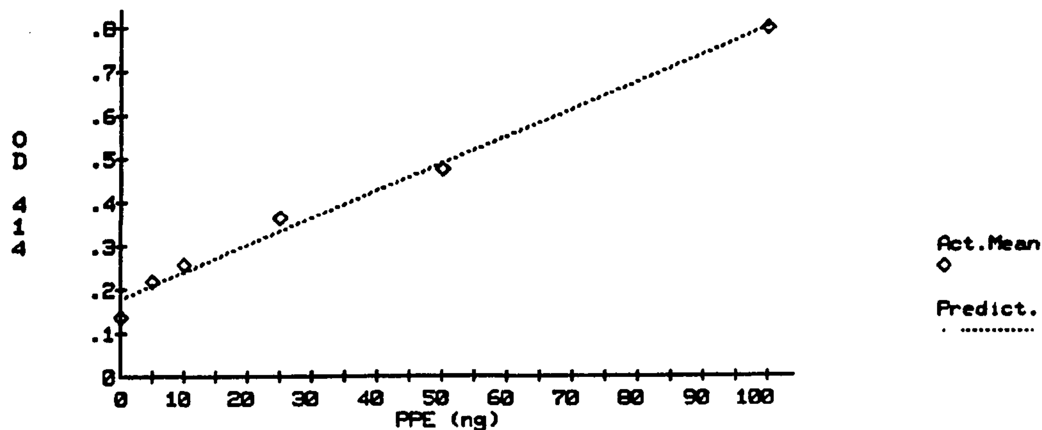


Fig. 1. Linear regression plots of porcine pancreatic elastase standard curves using HRP-elastin.

a) 0.5-20 ng; 3 h incubation; 30 min reaction with ABTS.
b) 5-100 ng; 3 h incubation; 10 min reaction with ABTS.

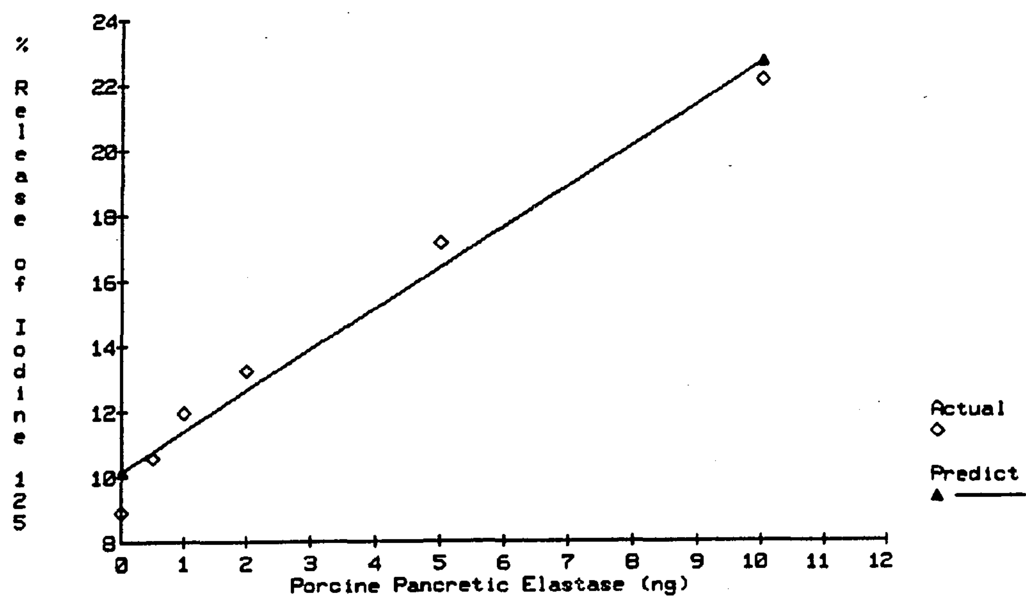


Fig. 2. Linear regression plot of PPE standard curve using [^{125}I]-elastin-Sepharose.

Duplicate determinations; 5 h incubation.

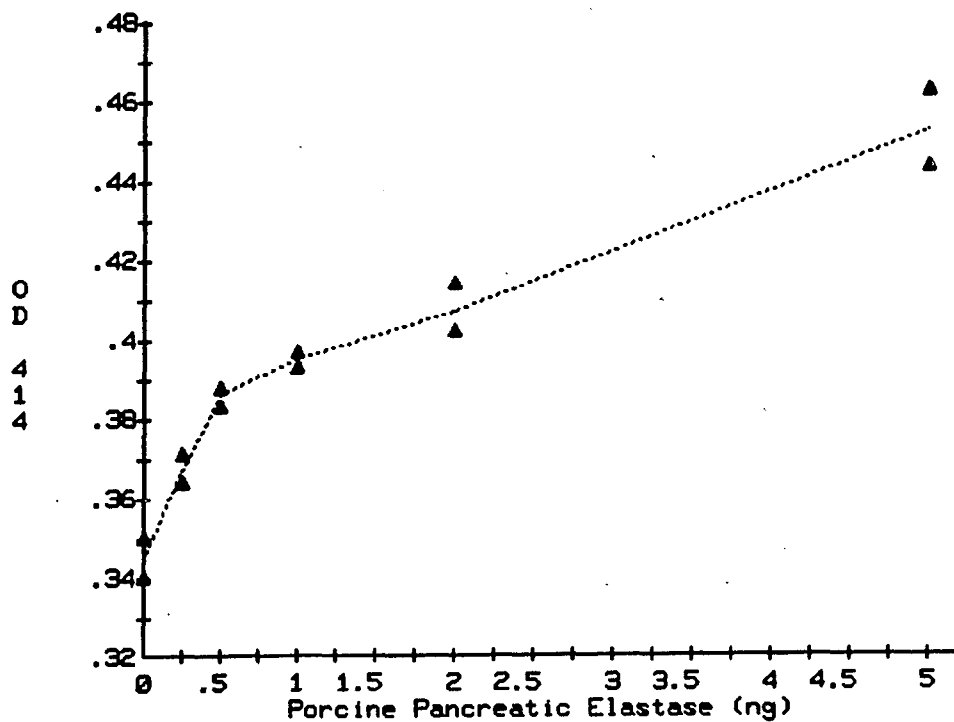


Fig. 3. PPE standard curve (0-5 ng) using HRP-elastin.

12 h incubation; 30 min ABTS reaction.

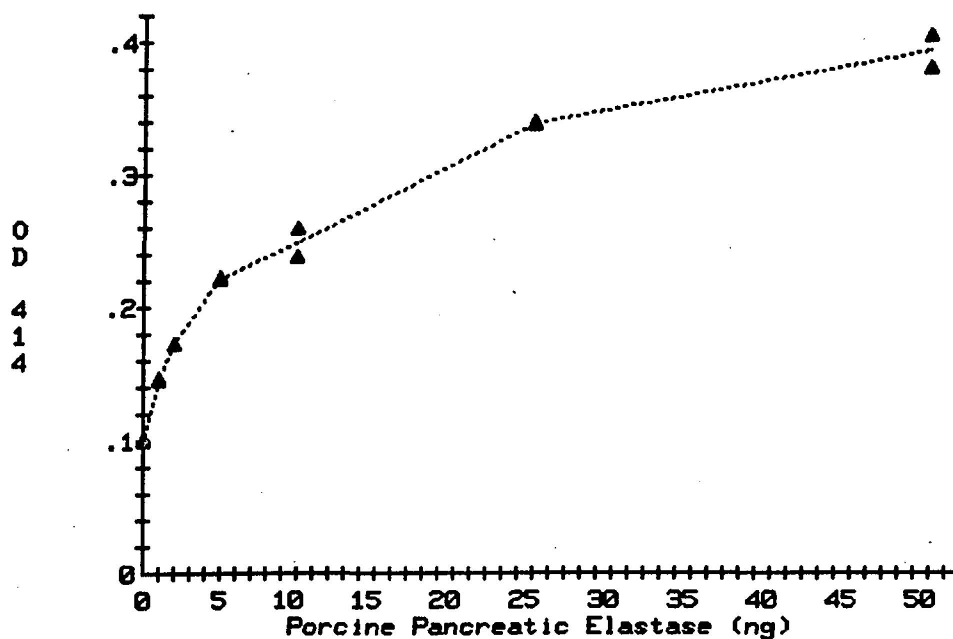


Fig. 4. PPE standard curve (0-50 ng) using HRP-elastin.

3 h incubation; 10 min ABTS reaction.

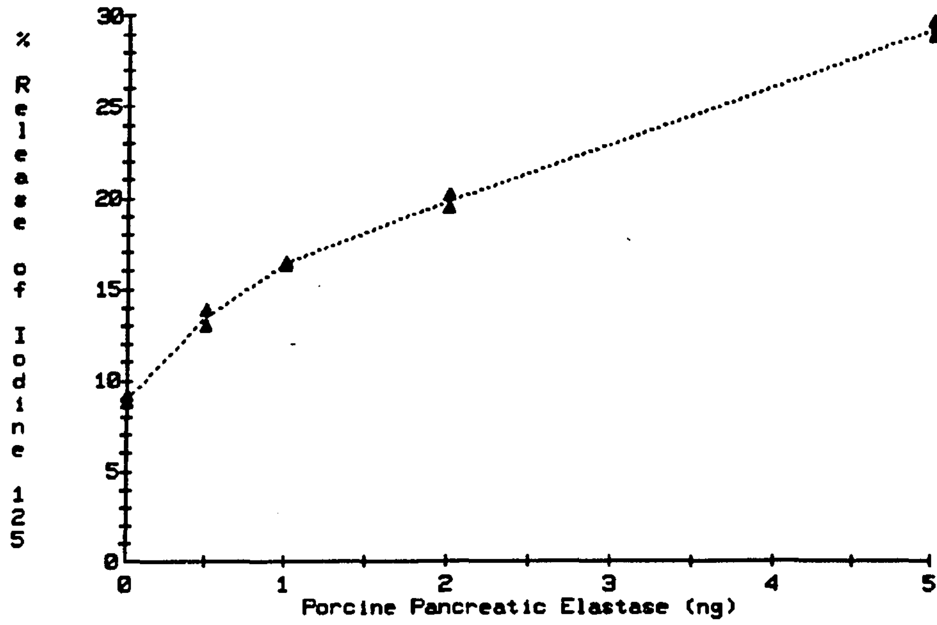
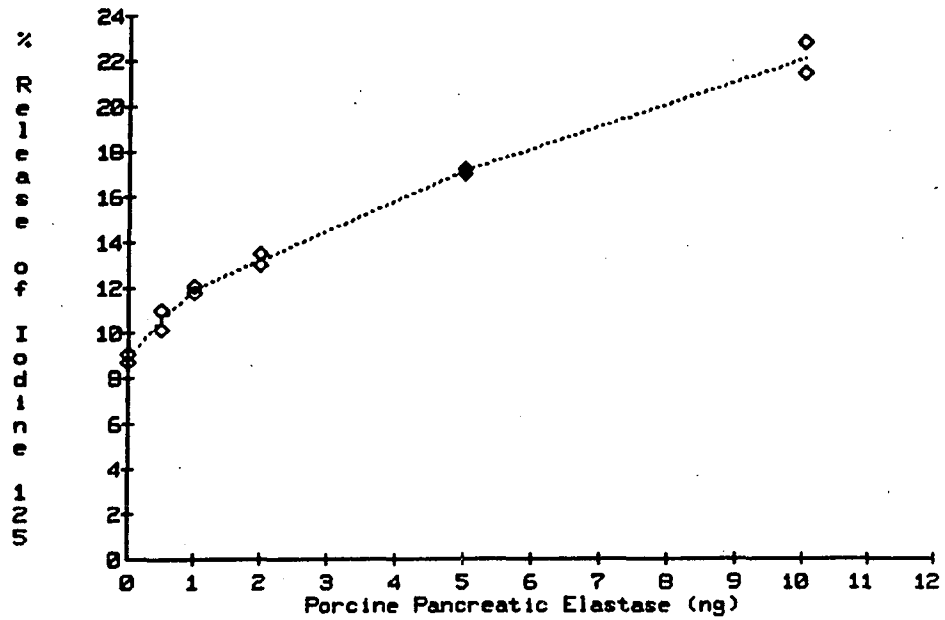


Fig. 5. PPE standard curve (0-5 ng) using [^{125}I]-elastin-Sepharose.

15 h incubation.

a)



b)

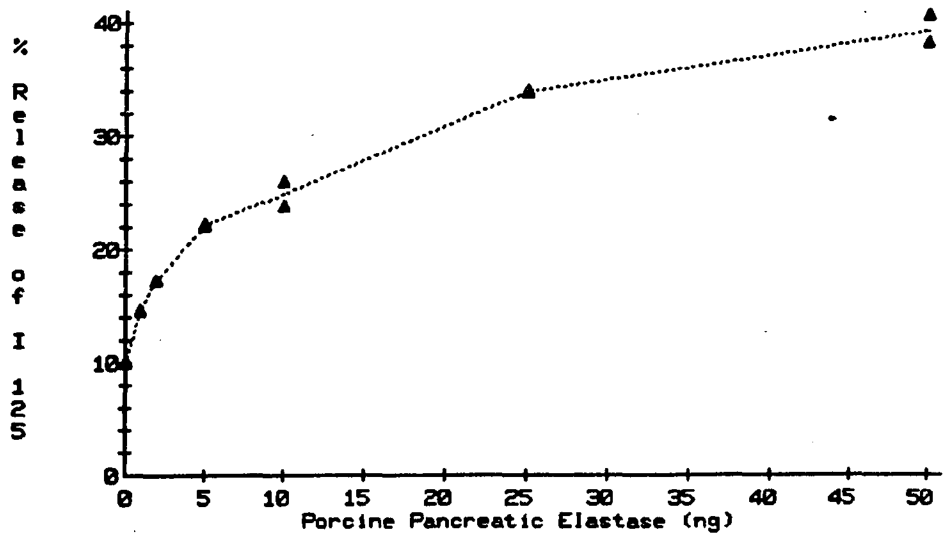


Fig. 6. PPE standard curves using [^{125}I]-elastin-Sepharose. a) 0-10 ng; 5 h incubation; b) 0-50 ng; 5 h incub.

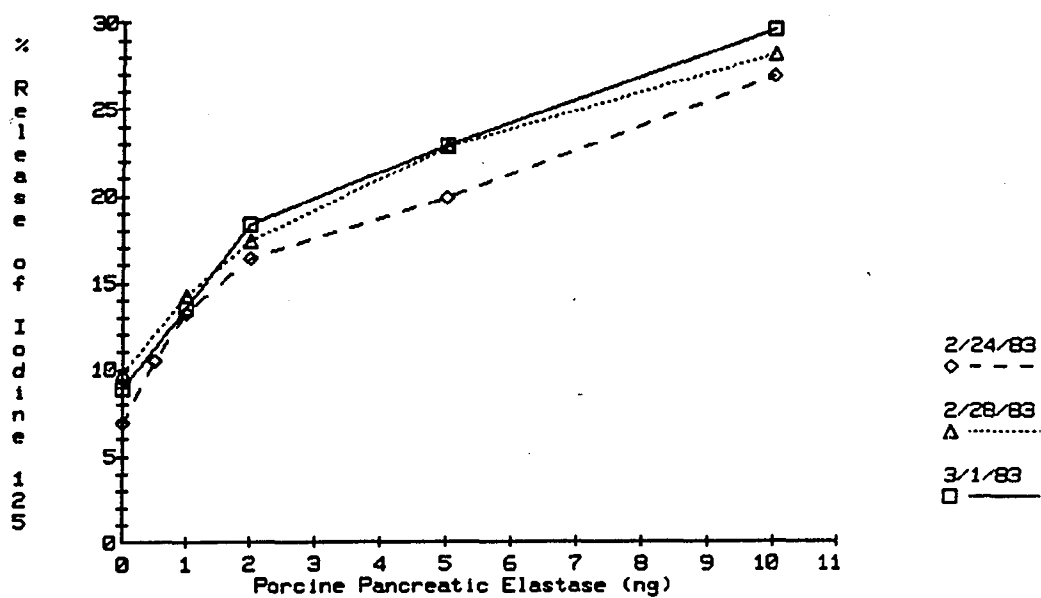


Fig. 7. Day-to-day reproducibility of elastase assay using [^{125}I]-elastin-Sepharose.

5 h incubation.

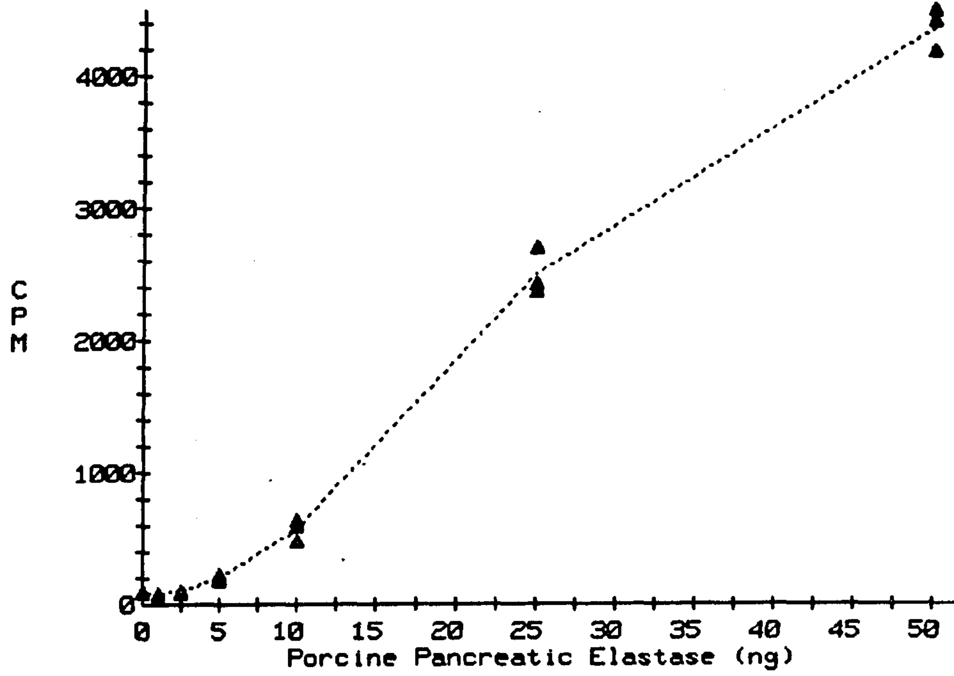


Fig. 8. PPE standard curve using tritiated elastin.

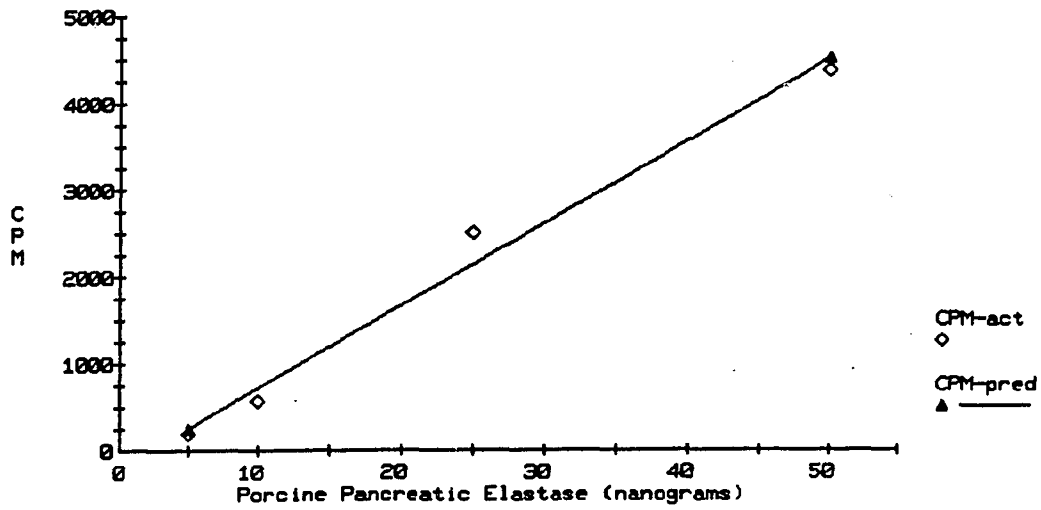


Fig. 9. Linear regression plot of PPE standard curve using tritiated elastin.

Mean of triplicate determinations plotted.

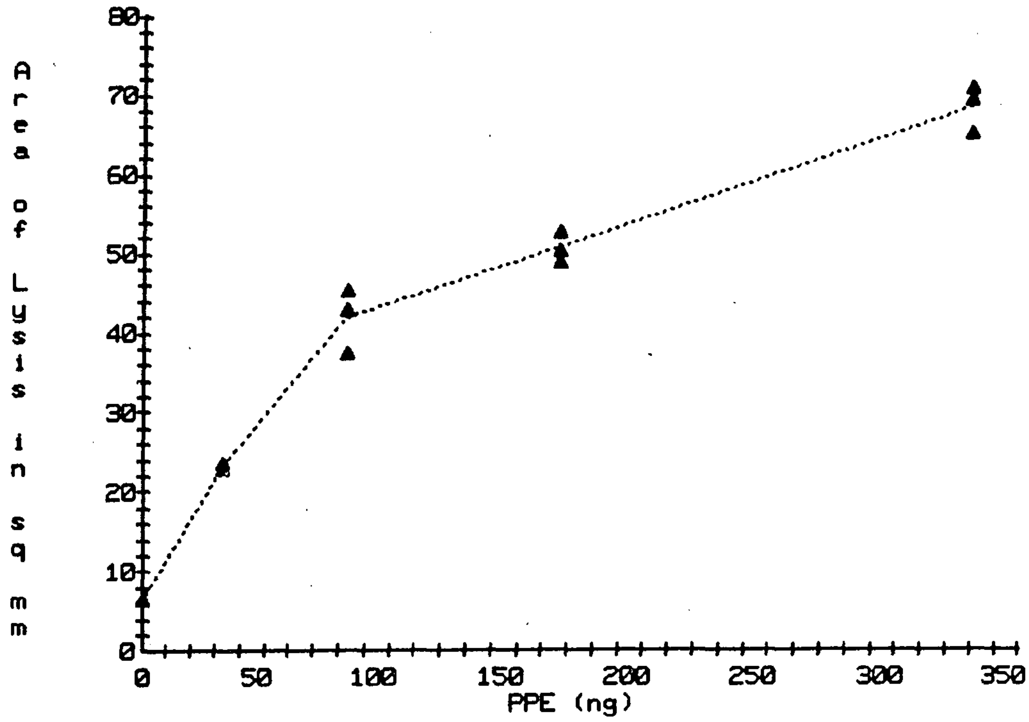


Fig. 10. PPE standard curve (0-350 ng) using elastin-agar plate.

24 h incubation.

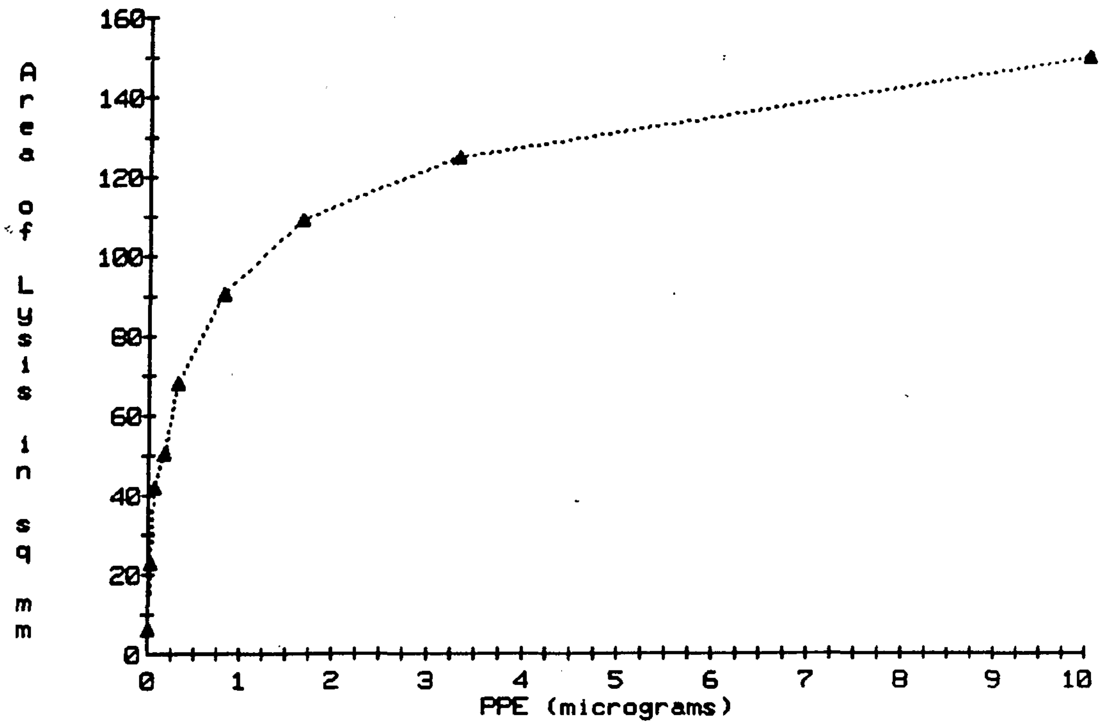


Fig. 11. PPE standard curve (0-10 μg) using elastin-agar plate.

Mean of triplicate determinations; 24 h incubation.

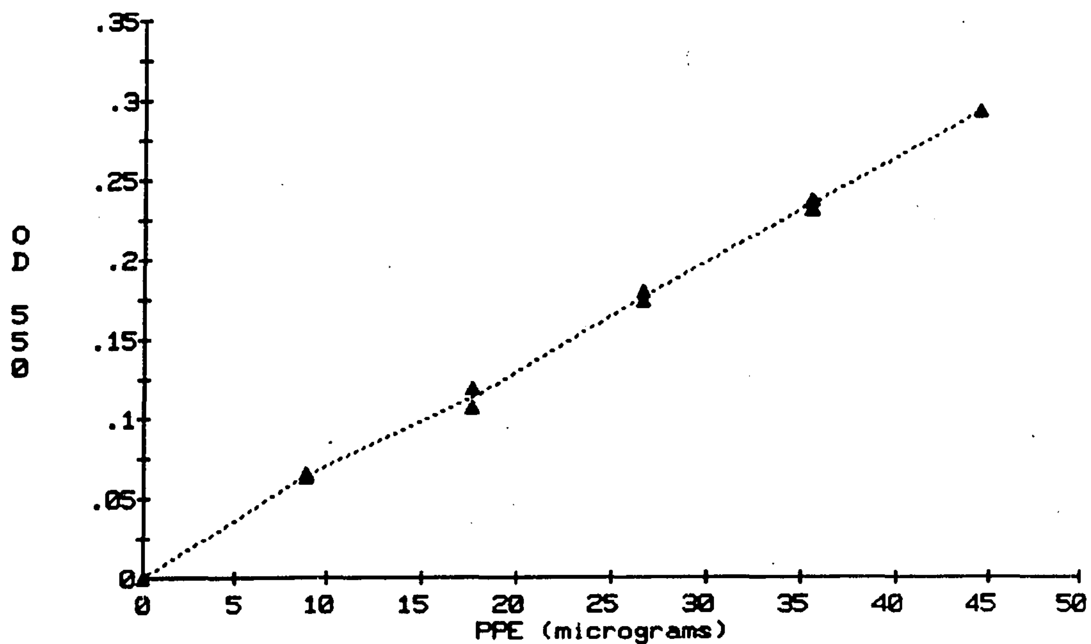


Fig. 12. PPE standard curve using elastin-rhodamine.

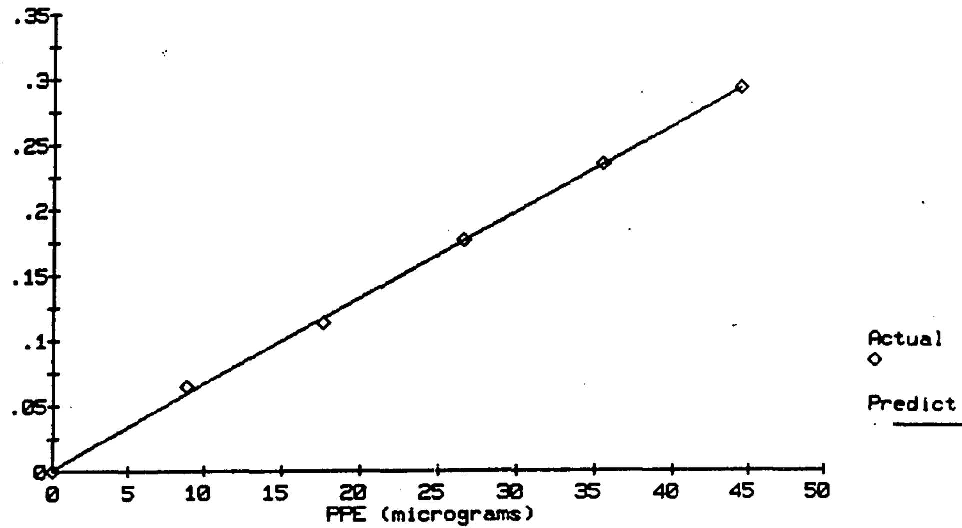


Fig. 13. Linear regression plot of PPE standard curve using elastin-rhodamine.

Mean of duplicate determinations plotted.

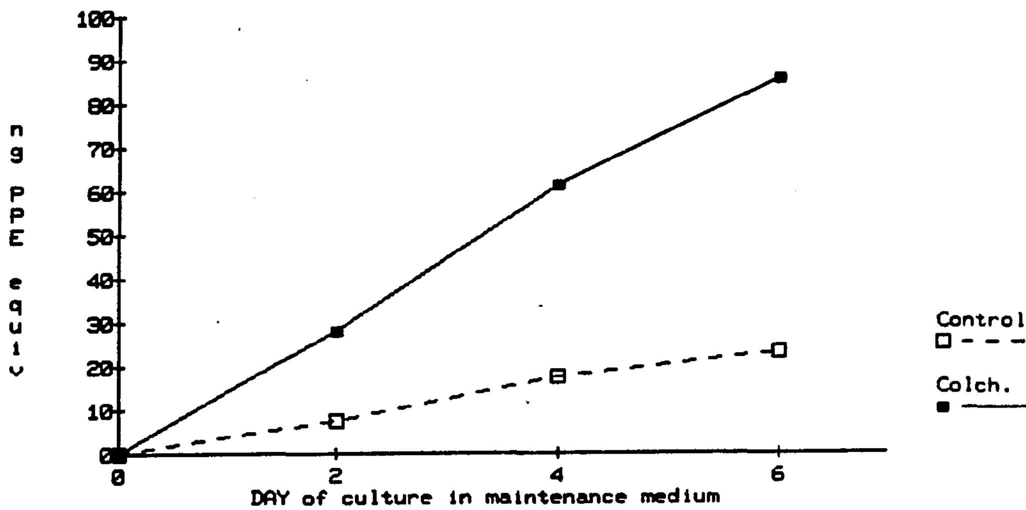


Fig. 14. Elastase secretion by alveolar macrophages during 6 days in culture.

Total accumulated elastase activity in 0.5 ml of conditioned medium of 1.7×10^6 DBA/2 alveolar macrophages assayed using HRP-elastin.

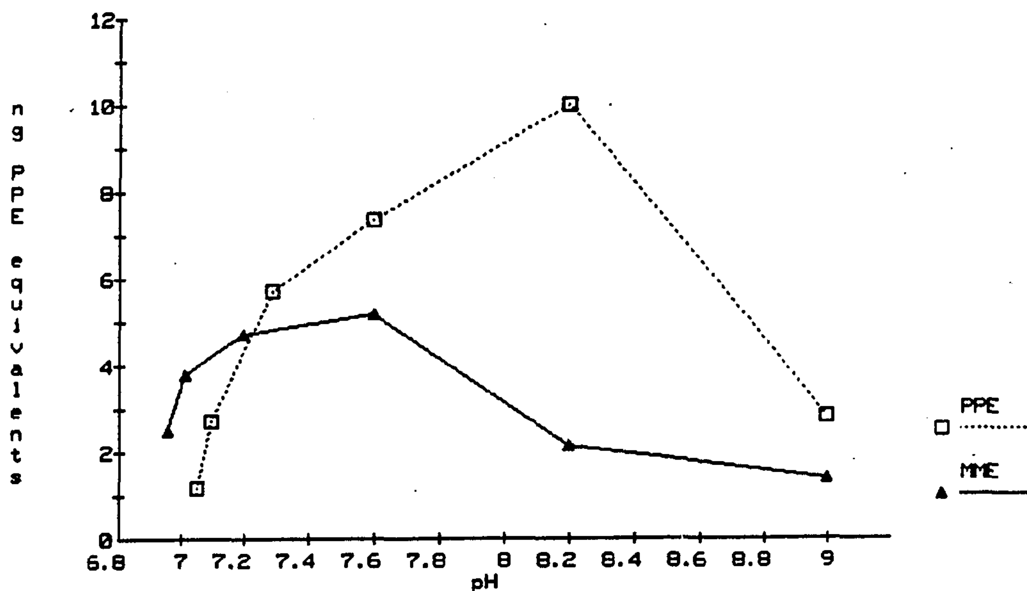


Fig. 15. Porcine pancreatic elastase and murine macrophage elastase pH profile.

MME = porcine pancreatic elastase; 10 ng activity at pH 8.2.

MME = dialyzed conditioned medium of DBA/2 alveolar macrophages.

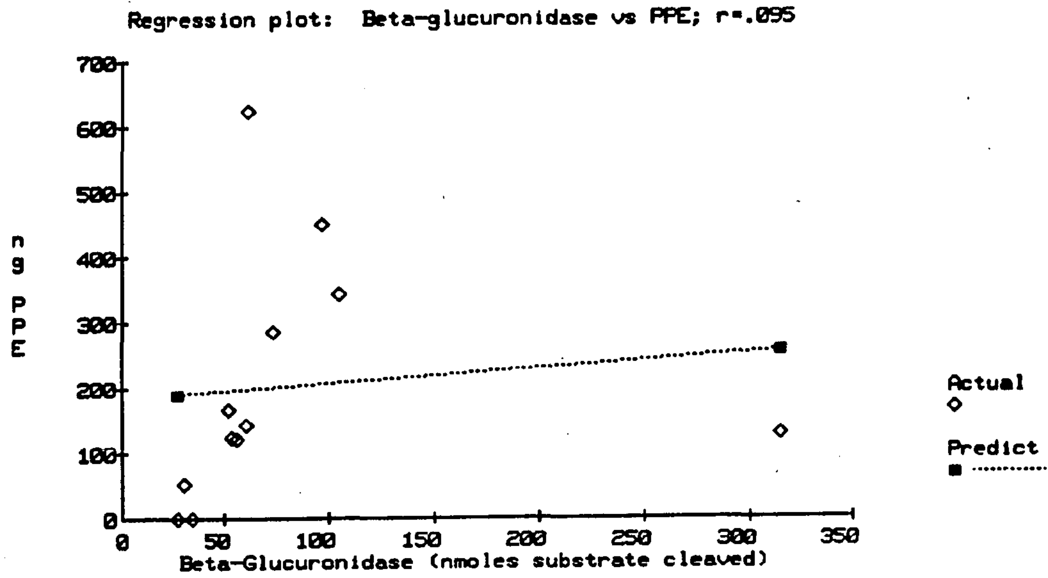


Fig. 16. Regression plot of beta-glucuronidase vs elastase activity in macrophage conditioned medium.

Alveolar macrophages were maintained in the presence of the modulating agents listed on Table 9.

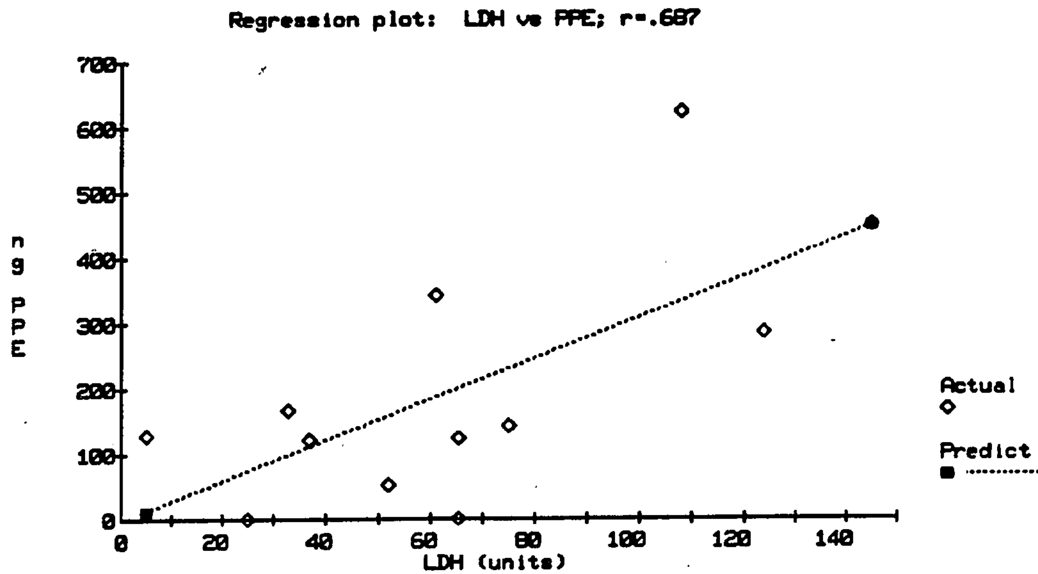


Fig. 17. Regression plot of LDH vs elastase activity in macrophage conditioned medium.

Alveolar macrophage cultures were maintained in the presence of the modulating agents listed on Table 9.

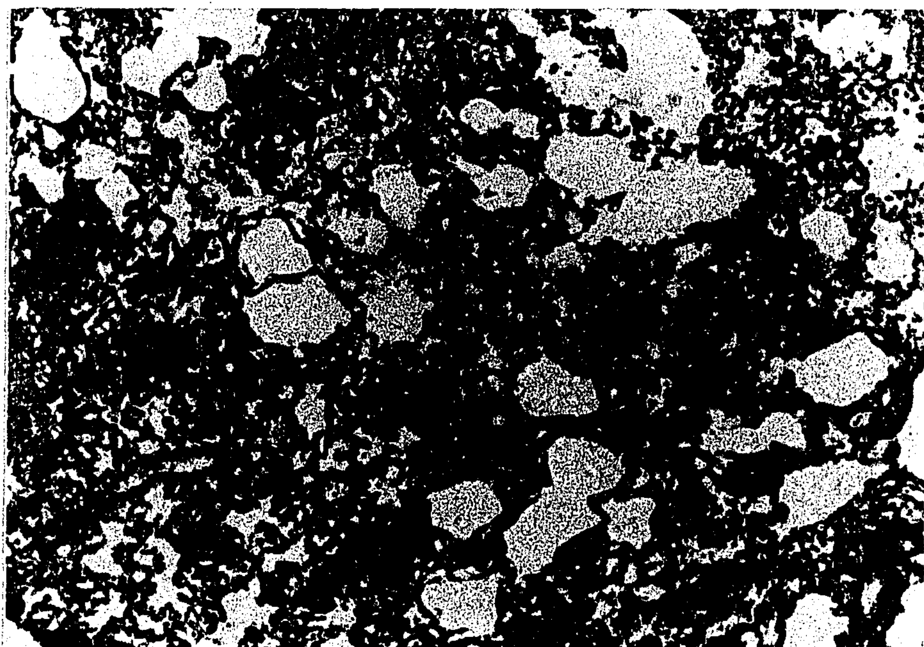


Fig. 18. Pulmonary granulomatous response to i.v. BCG emulsion.

Lung of C57BL/6 mouse, 4 weeks after i.v. injection of 300 µg emulsified BCG.

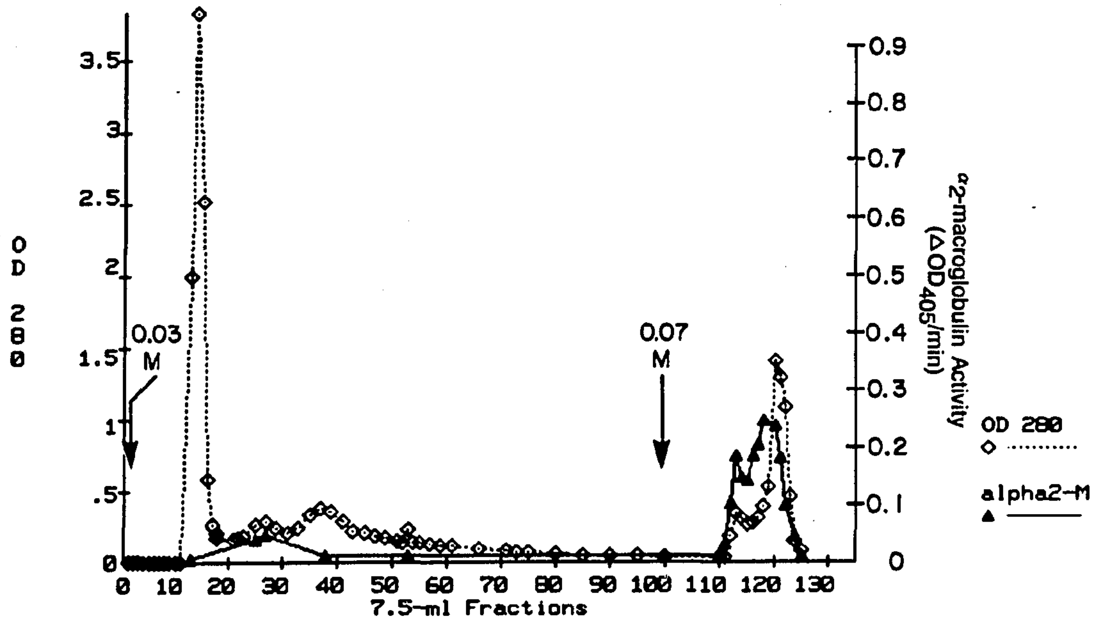


Fig. 19. Alpha$_2$-M purification: stepwise elution of hamster serum on Whatman DE-52.

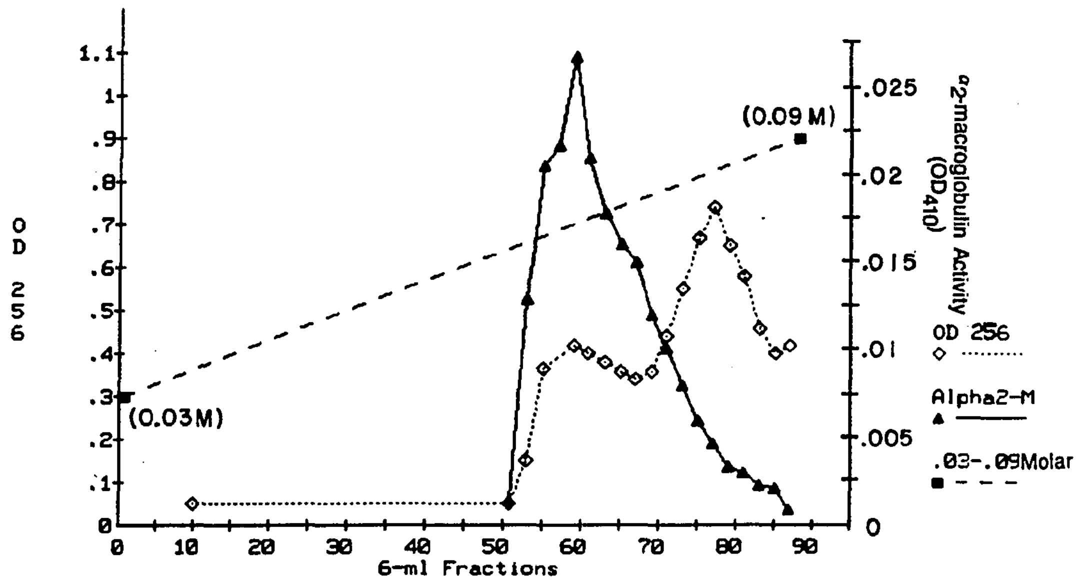


Fig. 20. Alpha$_2$-M purification: gradient elution on DE-52 of 112-124 from stepwise elution.

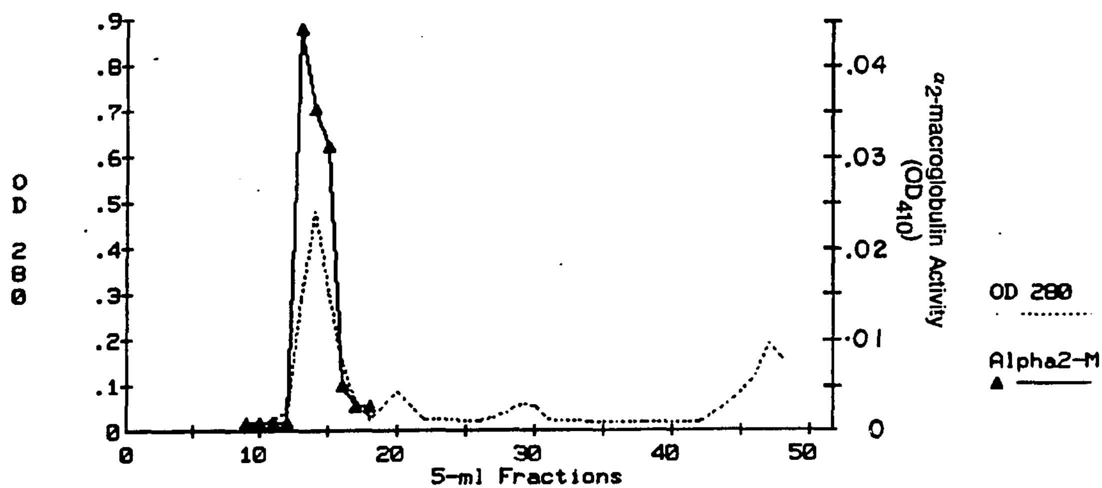


Fig. 21. Alpha$_2$-M purification: 52-65 from gradient DE-52 fractionated on Sephacryl S-300.

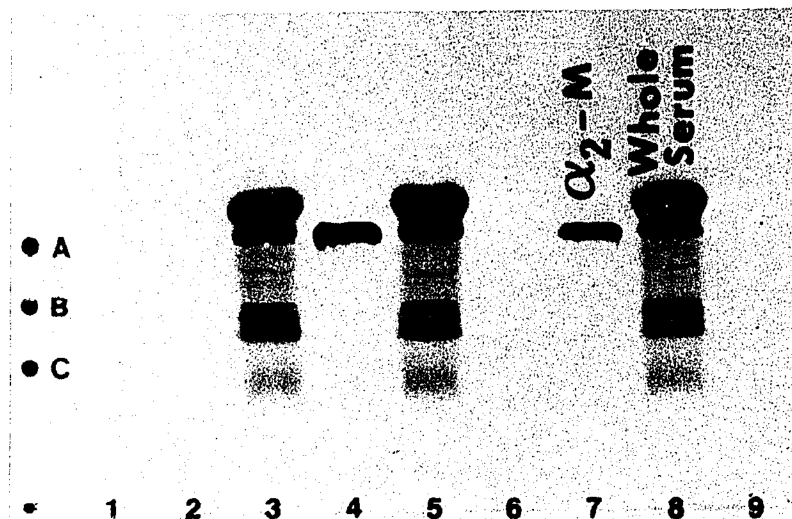


Fig. 22. Agarose electrophoresis of purified hamster alpha$_2$-macroglobulin.

Application on bottom; anode on top.

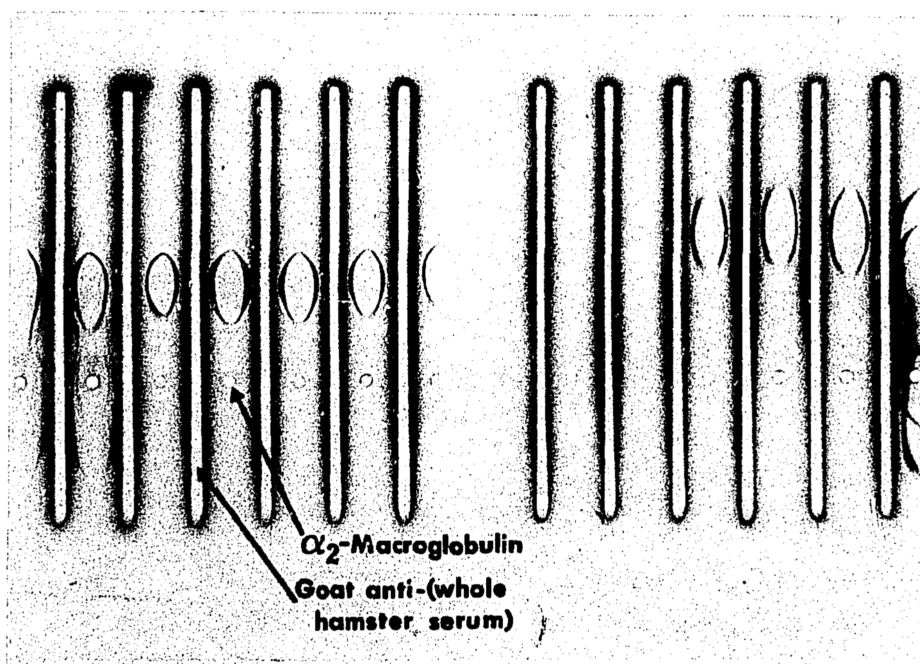


Fig. 23. Immunoelectrophoresis of purified hamster alpha$_2$-macroglobulin.

Hamster alpha$_2$-macroglobulin in well; goat anti-(whole hamster serum) in trough.

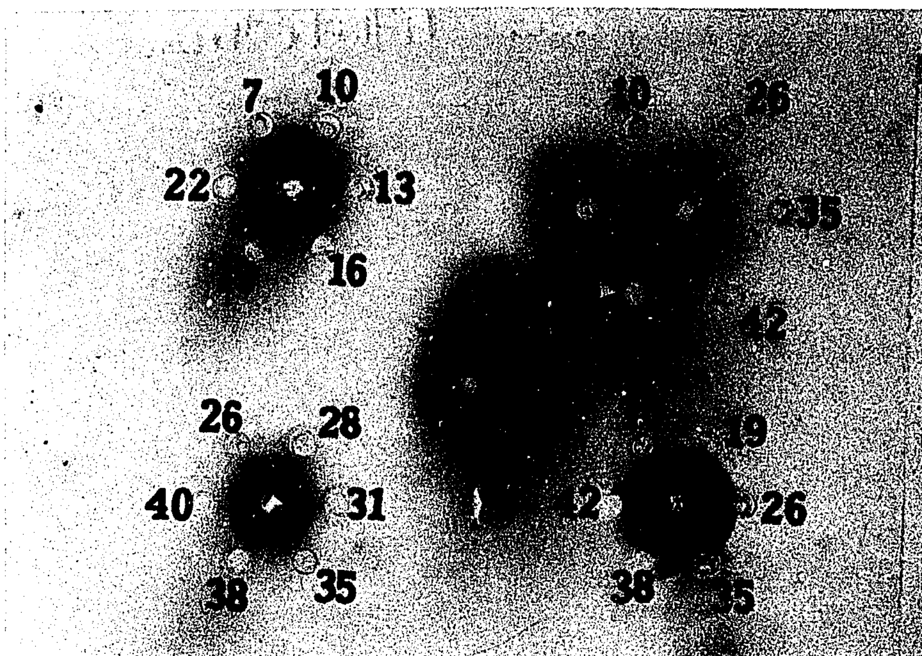


Fig. 24. Double radial immunodiffusion of fractionated rabbit anti-(hamster α_2-M).

Numbers correspond to fractions from <u>Ascaris</u> affinity column (see <u>Fig. 29</u>).

A = Purified antiserum from DEAE-Sephacel (see Fig. 28).

S = Whole hamster serum.

G = Goat anti-(rabbit IgG).

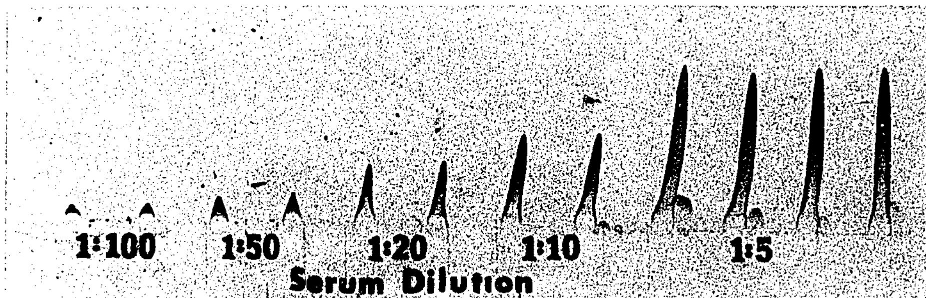


Fig. 25. Rocket immunoelectrophoresis of α_2-M in a serial dilution of serum.

2% (v/v) hamster α_2-M incorporated in 1% agarose.

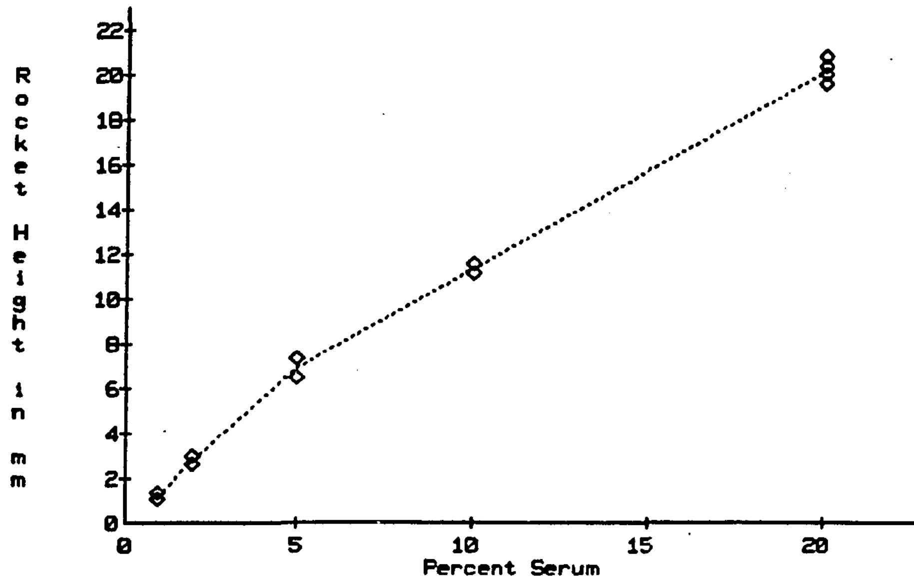


Fig. 26. Standard curve of rocket immuno-electrophoresis of serum α_2-M.

(See Fig. 25.)

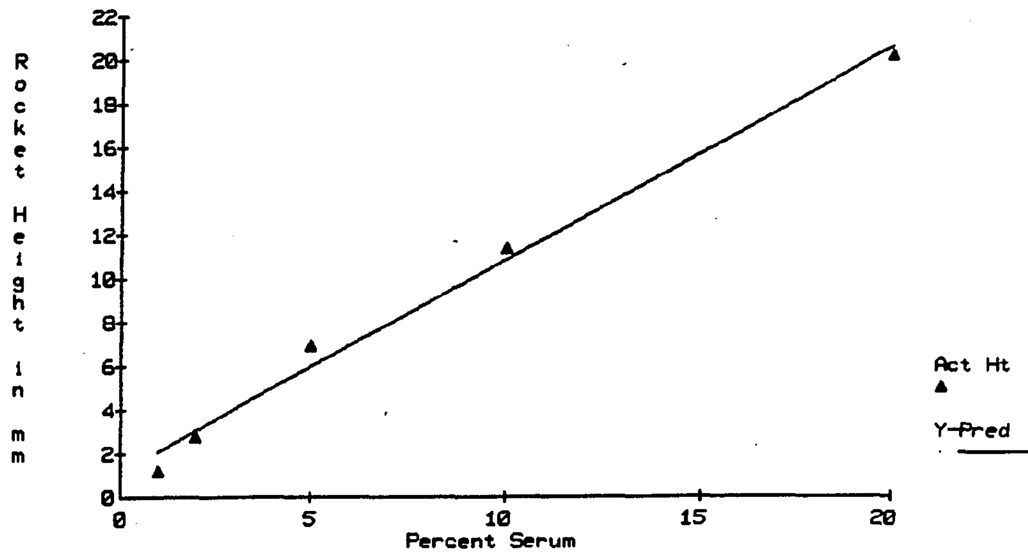


Fig. 27. Regression plot of standard curve of rocket immunoelectrophoresis of serum α_2-M.

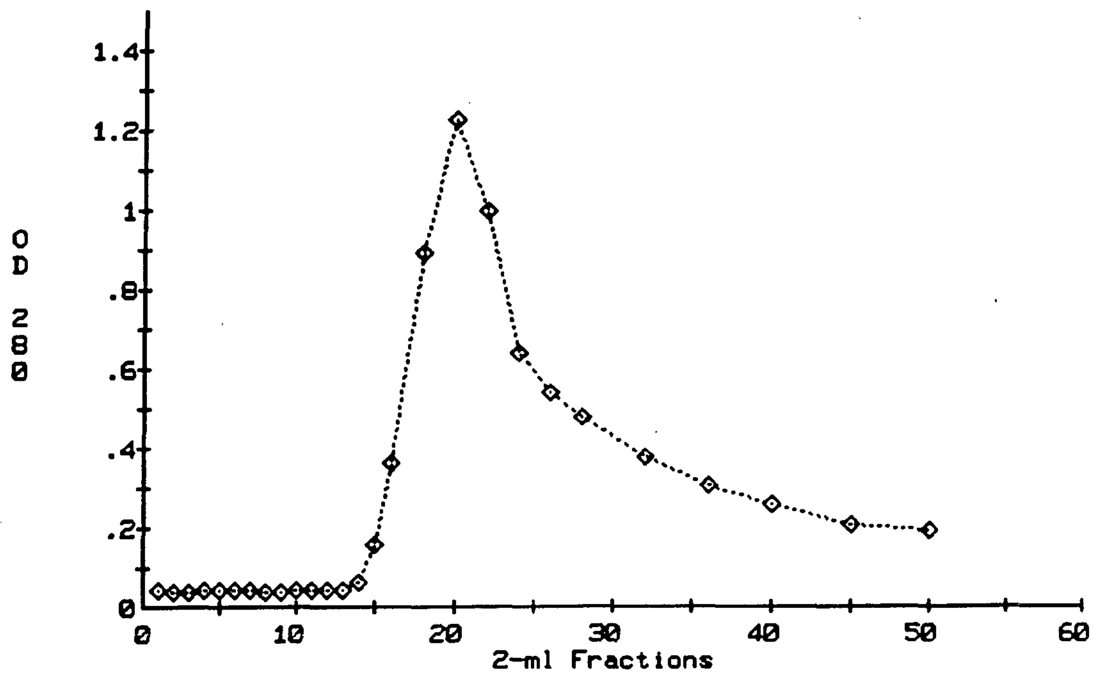


Fig. 28. Isolation of IgG from rabbit anti-(hamster α_2-M).

Elution with 0.0175 M phosphate buffer, pH 6.8.

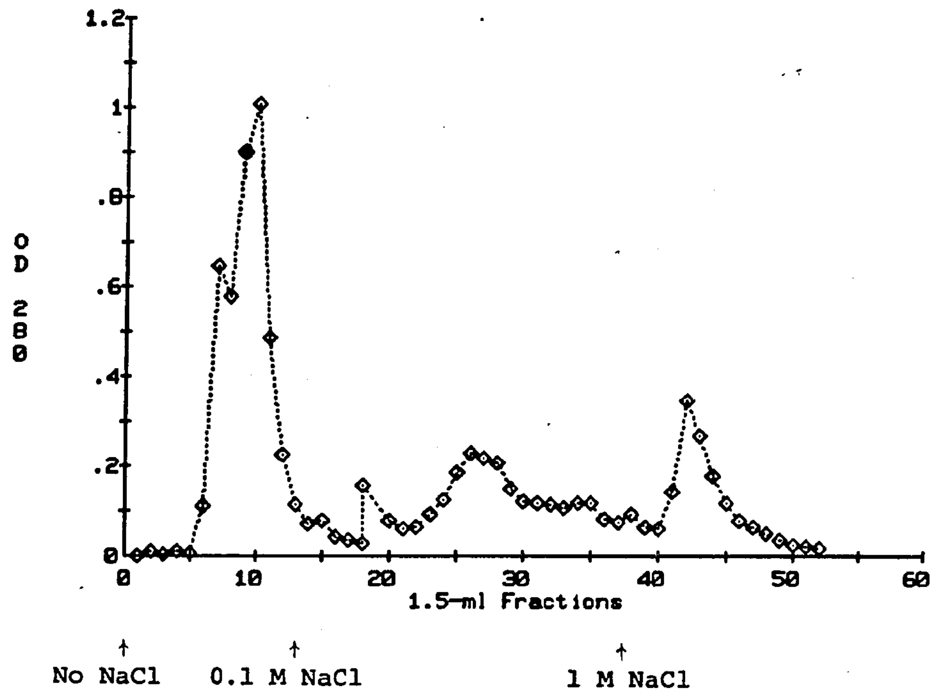
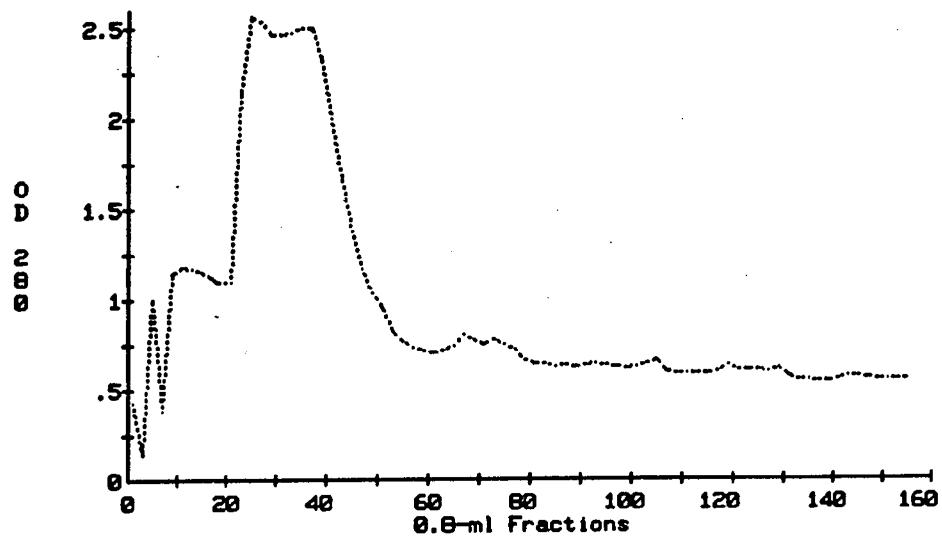


Fig. 29. Fractionation of IgG on Ascaris affinity column.

Fractionation of P388D1 CM on Elastin-Sepharose



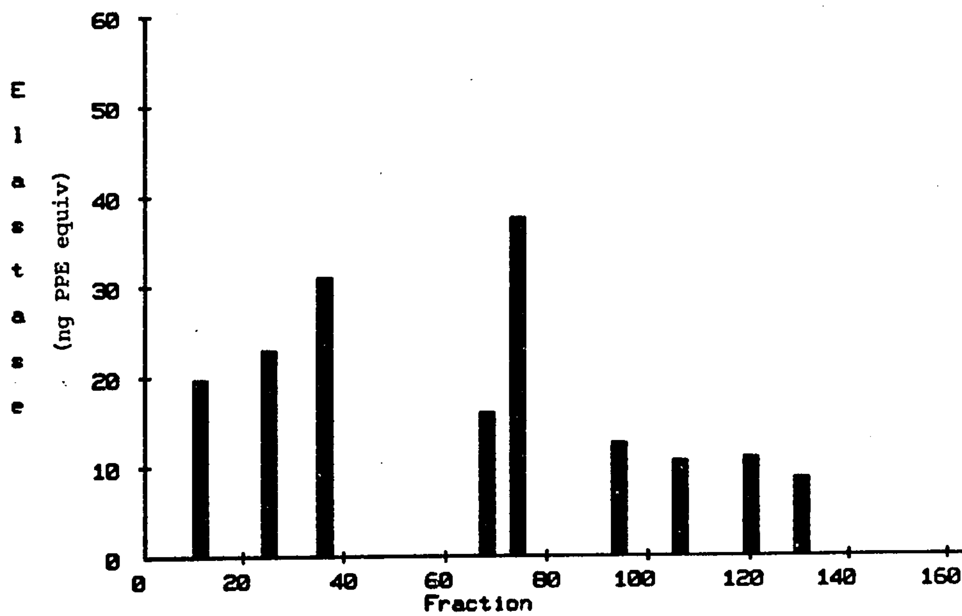


Fig. 30. Fractionation of $P388D_1$ conditioned medium on elastin-Sepharose.

a) OD_{280}.
b) Elastase activity.

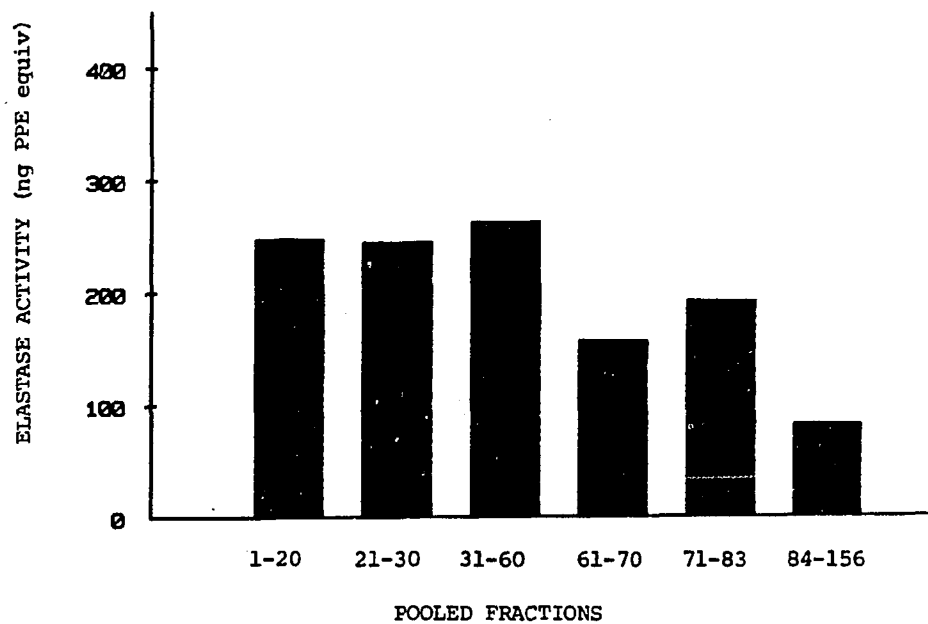


Fig. 31. Total $P388D_1$ elastase activity in pooled fractions from elastin-Sepharose column.

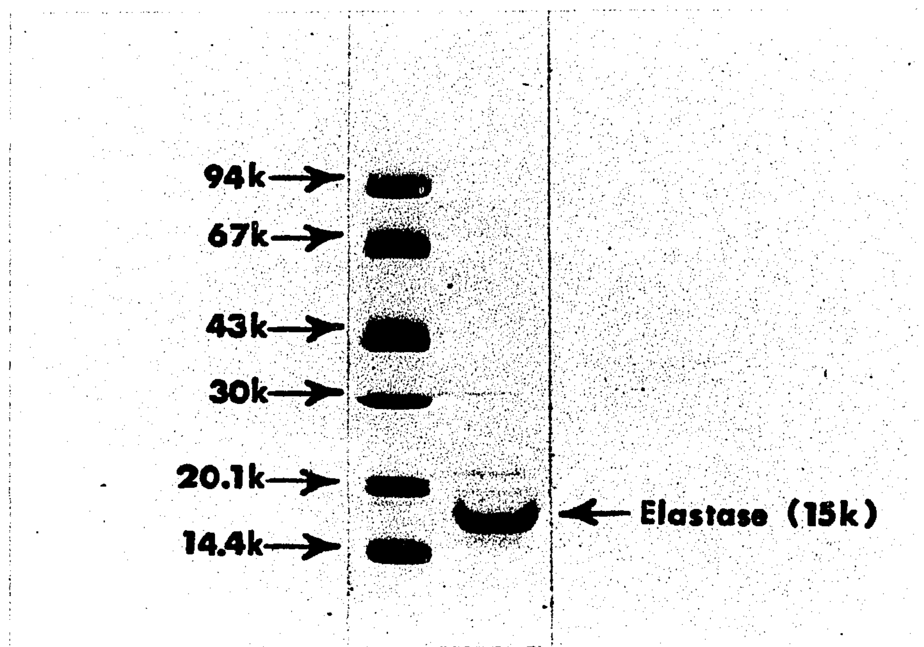


Fig. 32. SDS-polyacrylamide gradient electrophoresis of purified P388D$_1$ macrophage elastase.

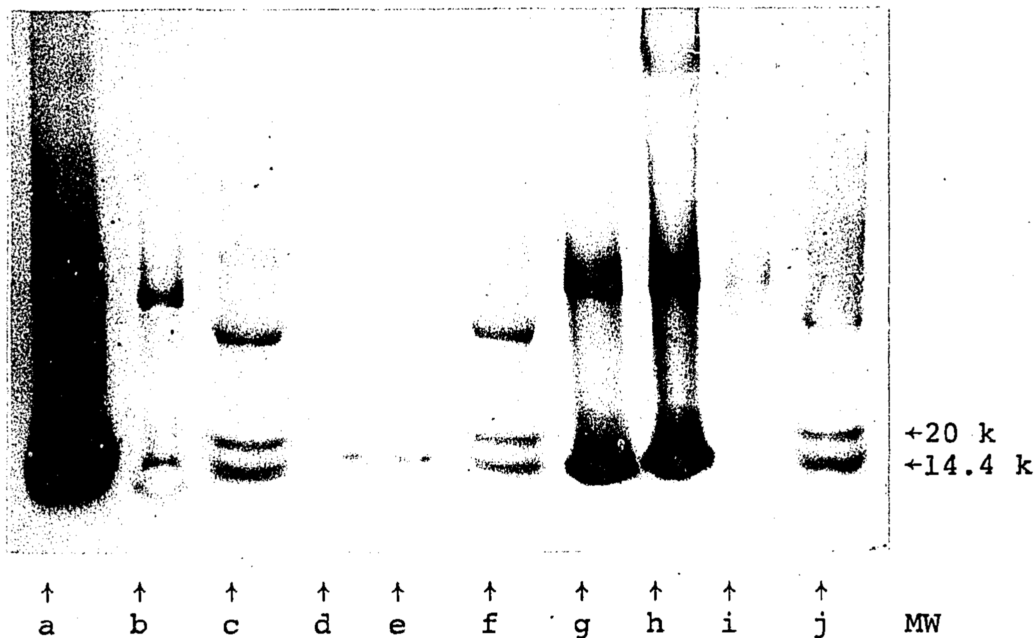


Fig. 33. SDS electrophoresis of pooled peaks from elastin-Sepharose column.

(See Fig. 30, 31.)

a = Crude $P388D_1$ conditioned medium.

b = Pooled fractions 84-156.

c, f, j = Low molecular weight standards.

d = Pooled fractions 71-83.

e = Pooled fractions 61-70.

g = Pooled fractions 31-60.

h = Pooled fractions 21-30.

i = Pooled fractions 1-20.

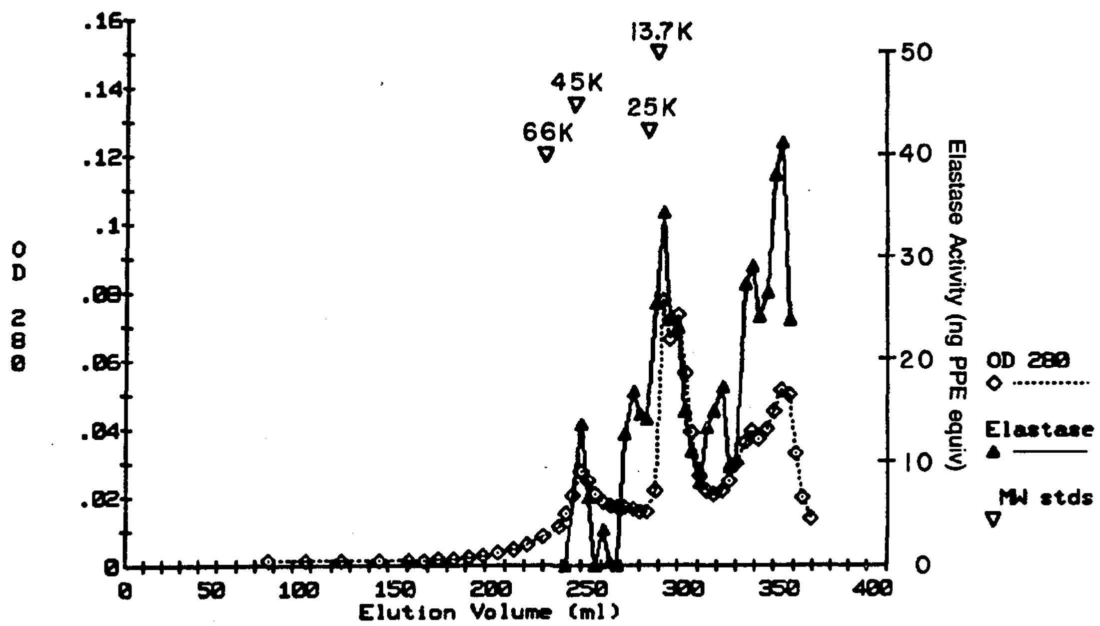


Fig. 34. P388D₁ elastase purification: Fractogel HPLC of gractions 61-70 from elastin-Sepharose.

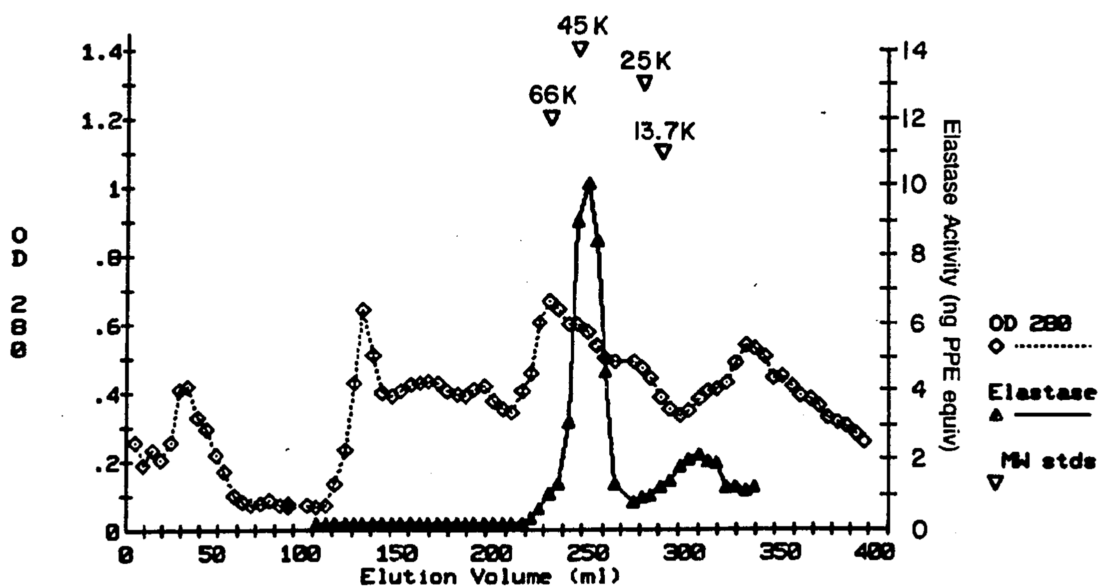


Fig. 35. P388D₁ elastase purification: Fractogel HPLC of fractions 31-60 from elastin-Sepharose.

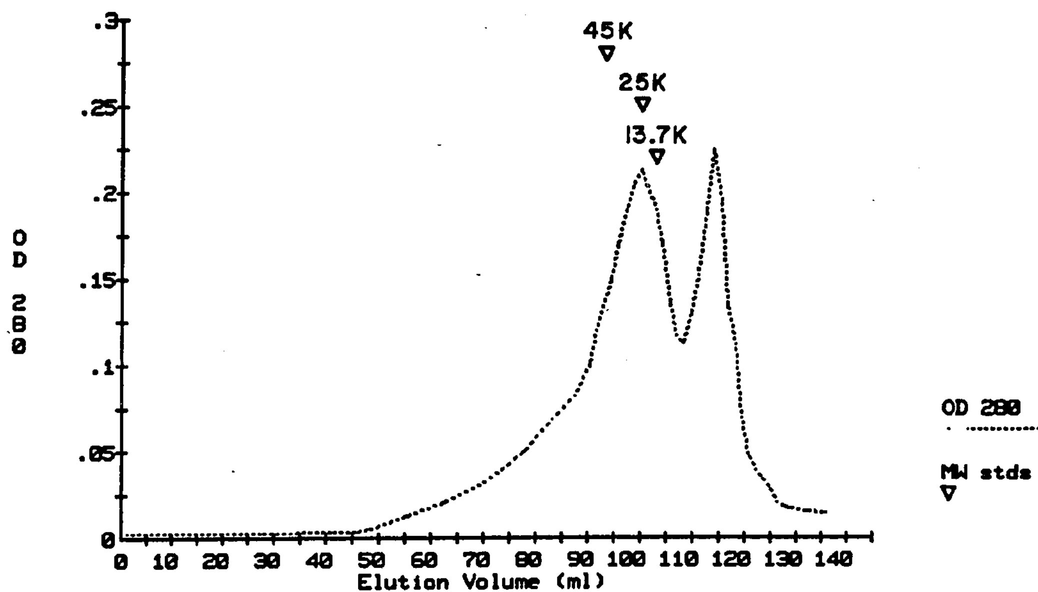


Fig. 36. P388D$_1$ elastase purification: Fractogel HPLC of fractions 71-156 from elastin-Sepharose.

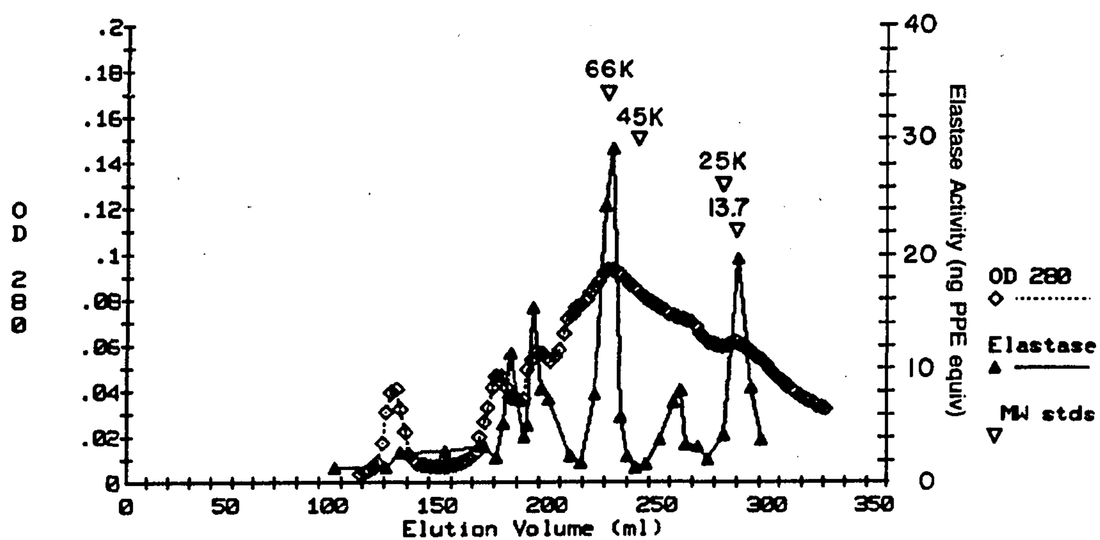


Fig. 37. P388D₁ elastase purification: Fractogel HPLC of 45-55% ammonium sulfate fraction.

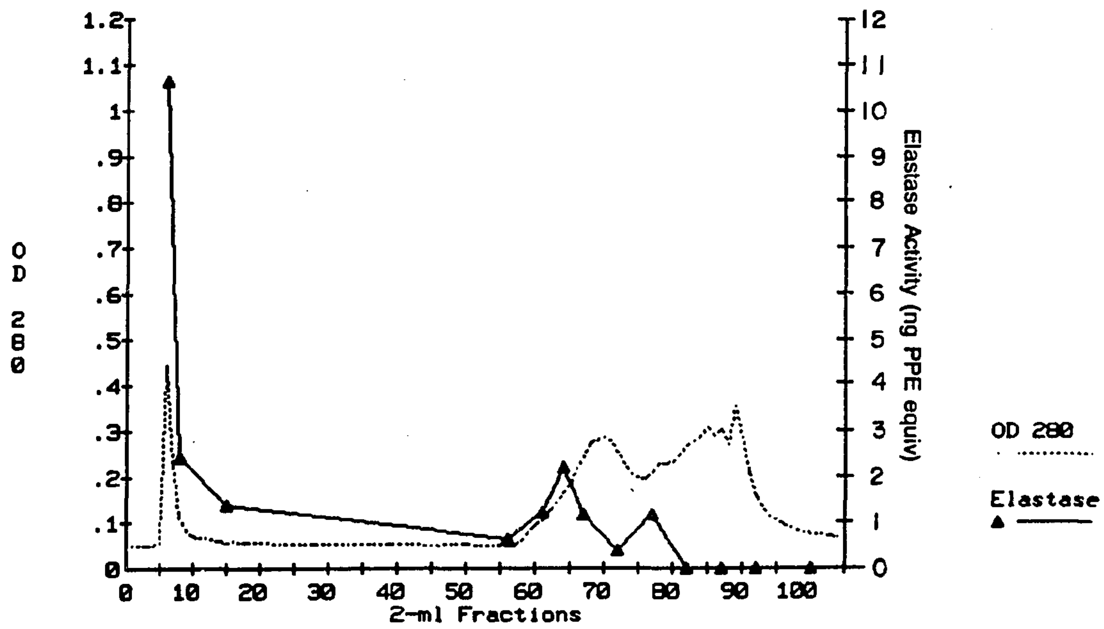


Fig. 38. $P388D_1$ elastase purification: fractionation of conditioned medium on DEAE A-25.

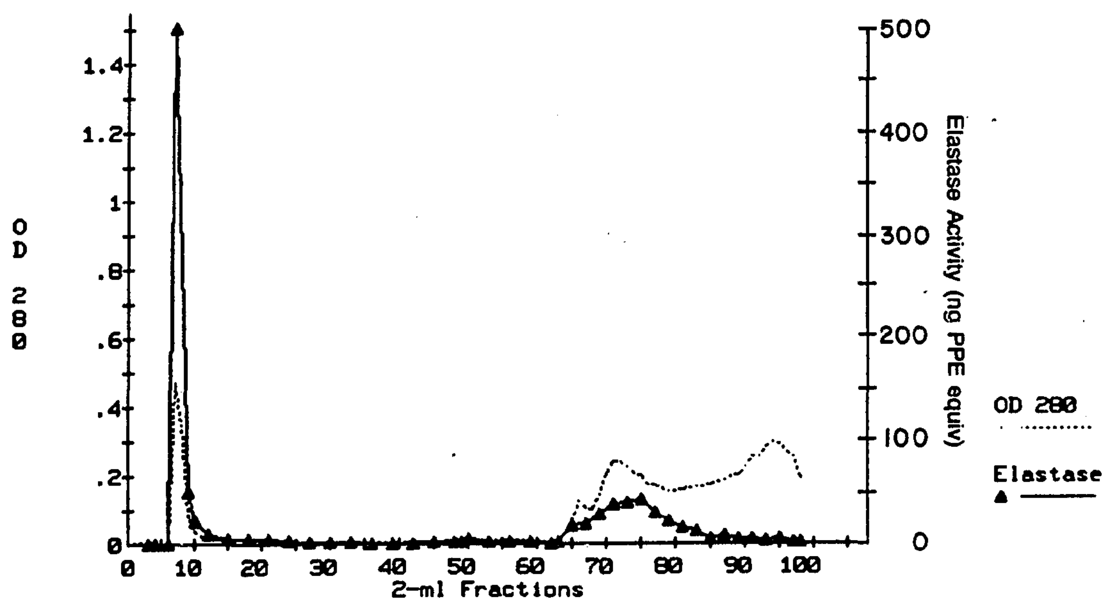


Fig. 39. DBA/2 peritoneal macrophage elastase purification: fractionation of conditioned medium on DEAE A-25.

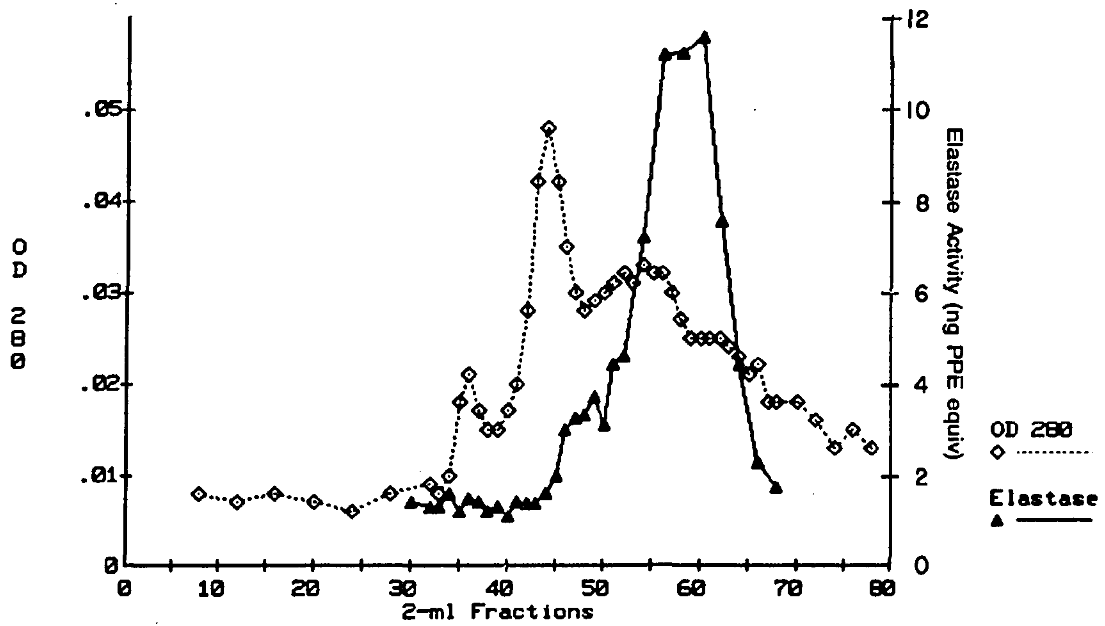


Fig. 40. DBA/2 peritoneal macrophage elastase purification: fractions 7-12 from DEAE A-25 fractionated on AcA54.

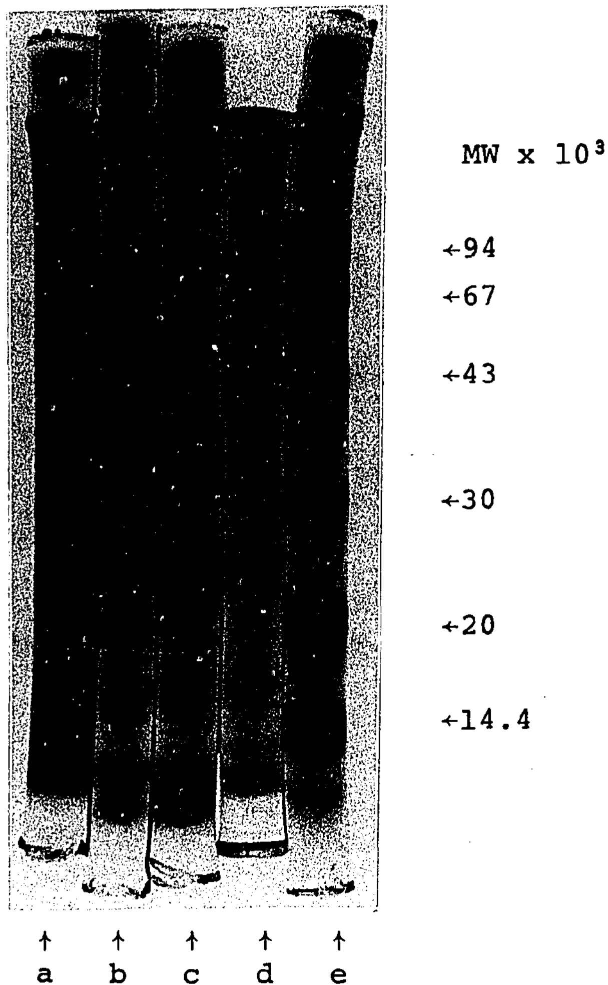


Fig. 41. SDS-PAGE of ACA54 fractions from application of DBA/2 peritoneal macrophage DEAE void peak.

(Refer to Fig. 40.)

a = Pooled fractions 33-39.

b = Pooled fractions 40-45.

c = Pooled fractions 47-56.

d = Pooled fractions 57-61.

e = Low molecular weight standards.

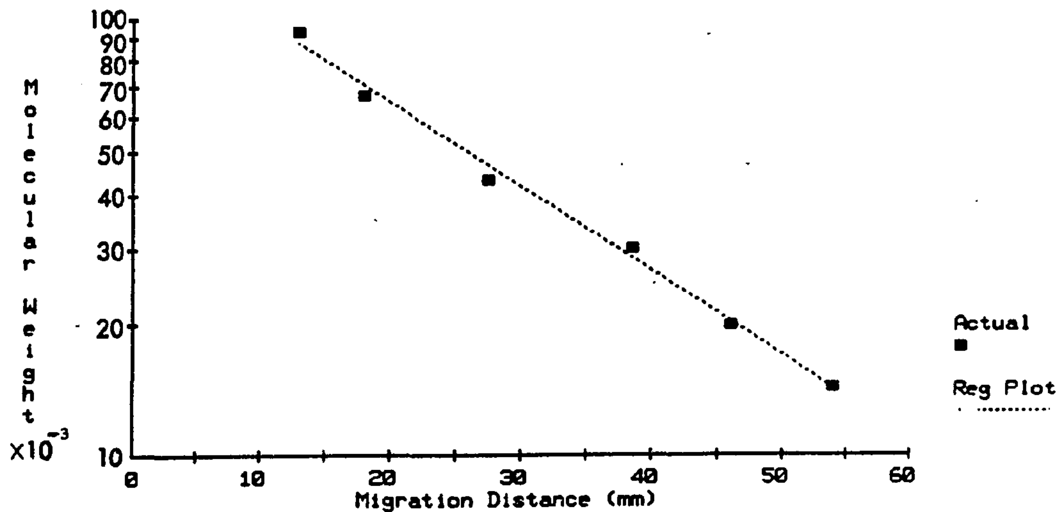


Fig. 42. Regression plot of protein standards used for molecular weight determination by SDS-PAGE.

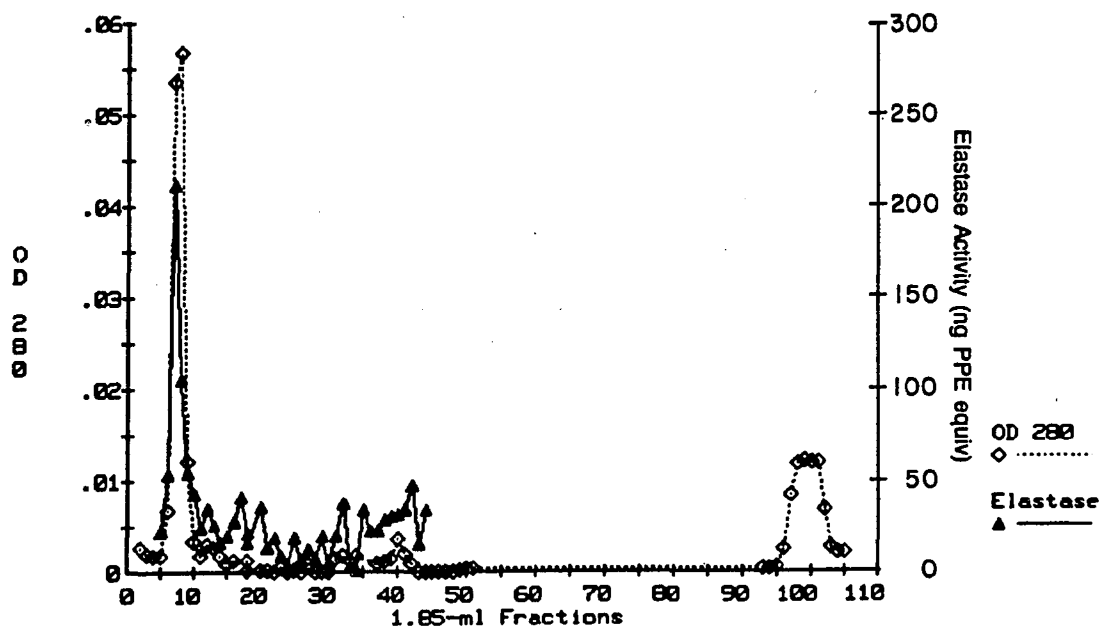


Fig. 43. DBA/2 peritoneal macrophage elastase purification: fractions 51-64 from AcA54 fractionated on Sp-Sephadex C-25.

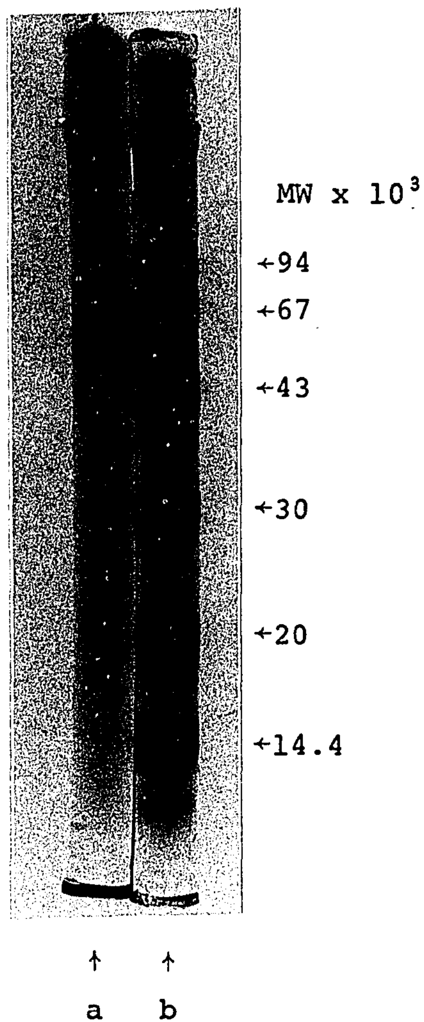


Fig. 44. SDS-PAGE of fractions retained on SP-Sephadex C-25.

(See Fig. 43.)

a = Pooled fractions 18-47.

b = Low molecular weight standards.

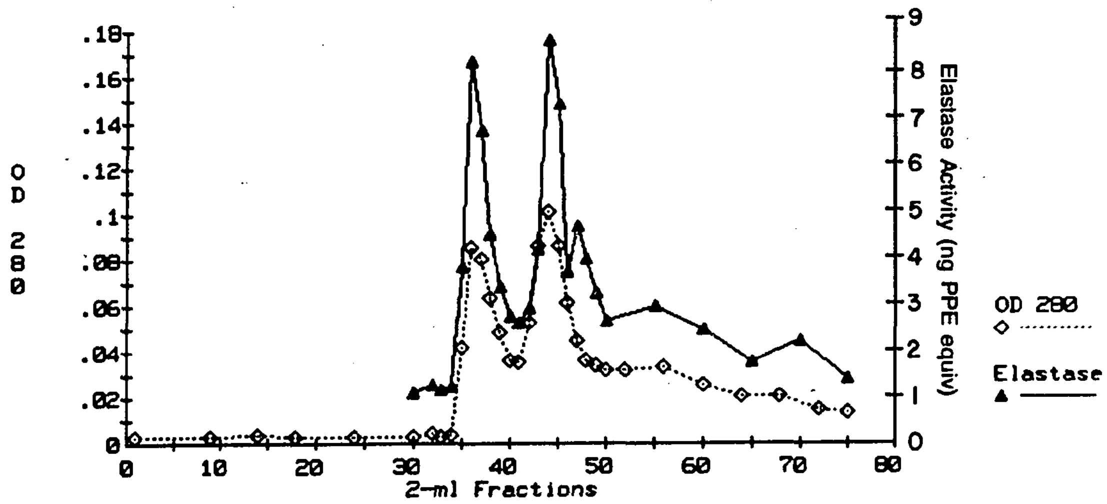


Fig. 45. DBA/2 peritoneal macrophage elastase purification: fractions 64-81 from DEAE A-25 fractionated on AcA54.

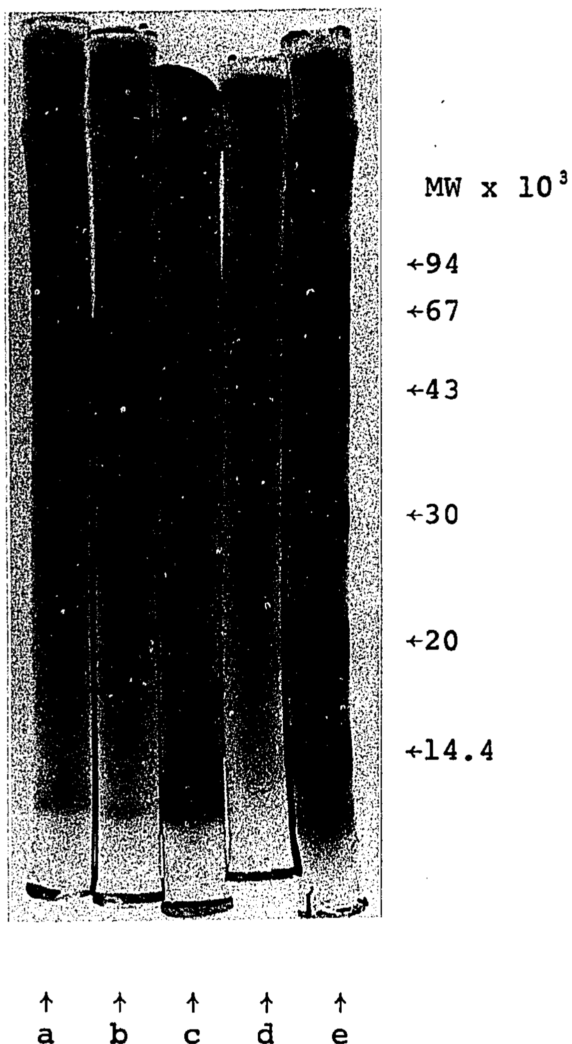


Fig. 46. SDS-PAGE of fractions from DBA/2 DEAE gradient peak application to AcA54 column.

(Refer to Fig. 45.)

a = Pooled fractions 35-39.

b = Pooled fractions 42-49.

c = Pooled fractions 51-70.

d = Pooled fractions 71-85.

e = Low MW standards.

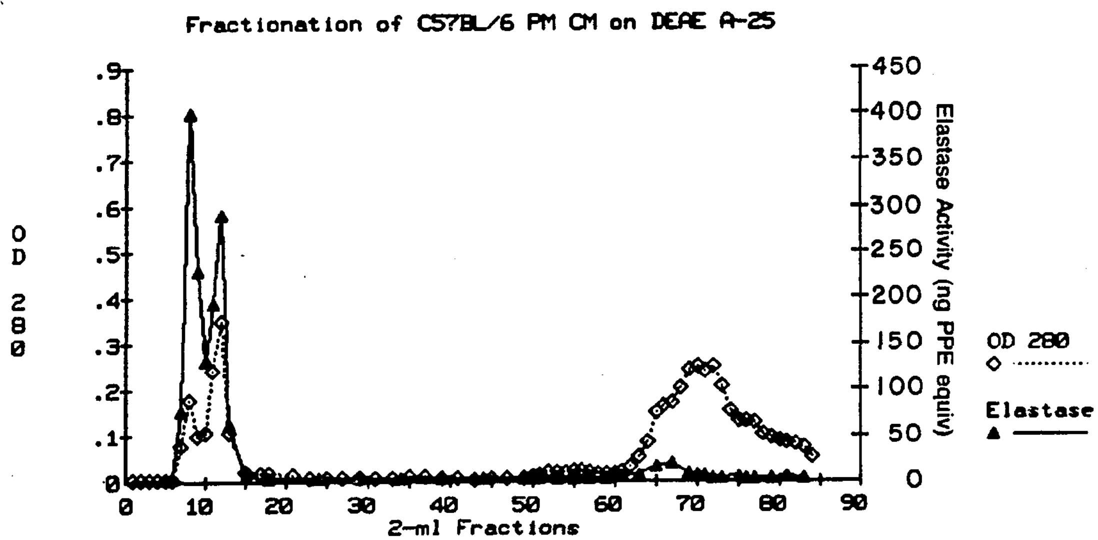


Fig. 47. Purification of C57BL/6 peritoneal macrophage elastase: fractionation of conditioned medium on DEAE A-25.

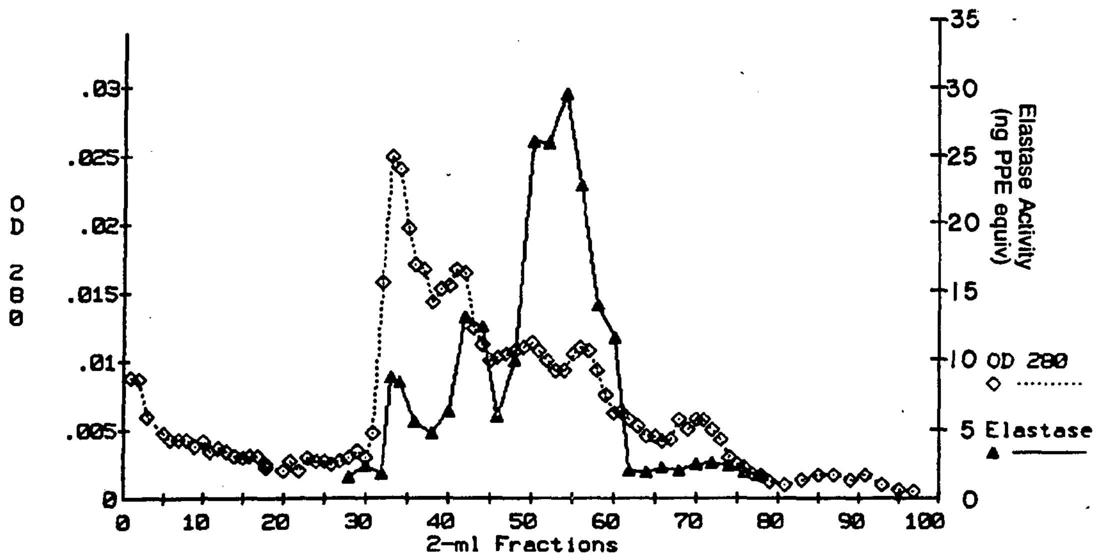


Fig. 48. Purification of C57BL/6 peritoneal macrophage elastase: fractions 7-13 from DEAE A-25 fractionated on AcA54.

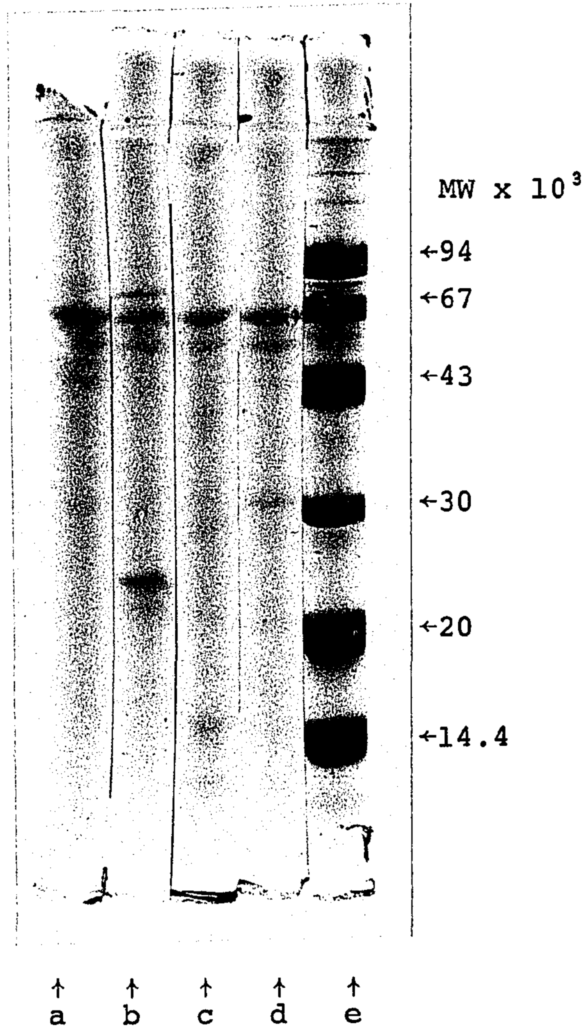


Fig. 49. SDS-PAGE of fractions from C57BL/6 DEAE void volume peak application to AcA54 column.

(Refer to Fig. 48.)

a = Pooled fractions 33-34.

b = Pooled fractions 42-44.

c = Pooled fractions 49-52.

d = Pooled fractions 53-56.

e = Low MW standards.

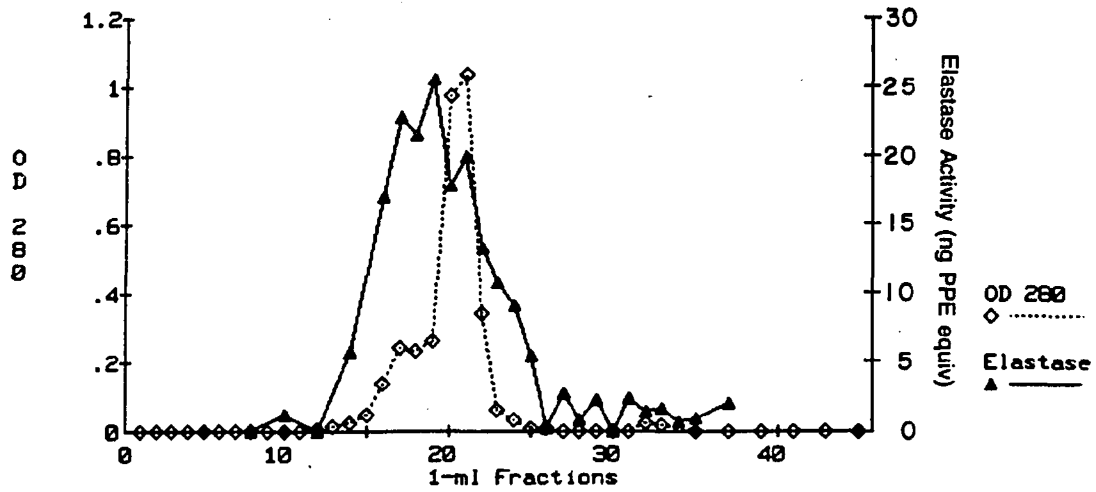


Fig. 50. Purification of DBA/2 alveolar macrophage elastase: fractionation of conditioned medium on elastin-Sepharose.

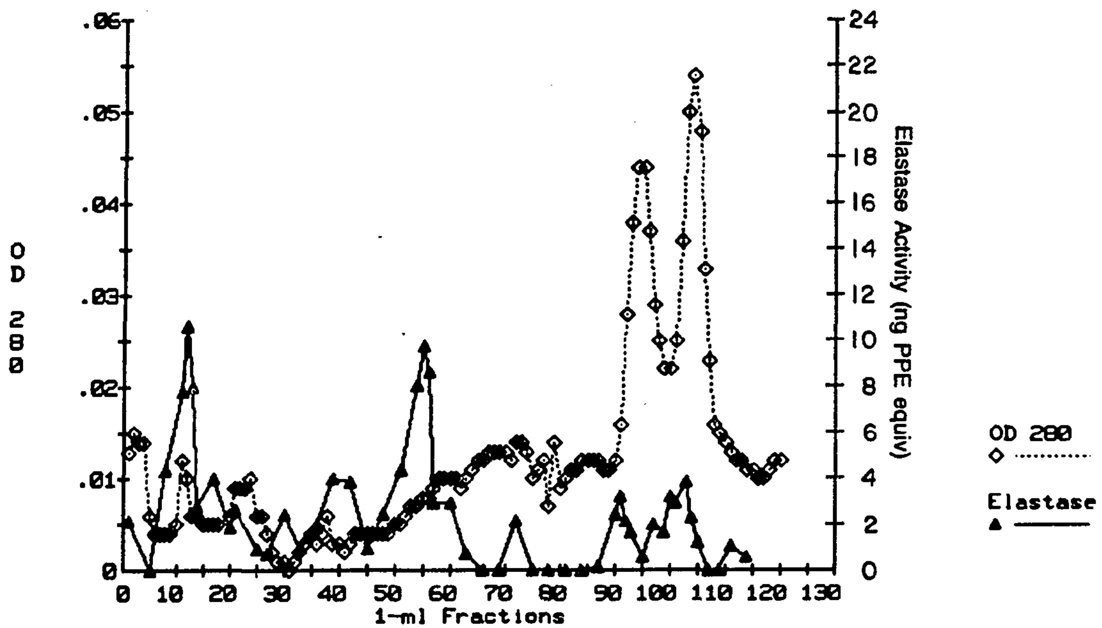


Fig. 51. Purification of DBA/2 alveolar macrophage elastase: fractionation of conditioned medium on DEAE A-25.

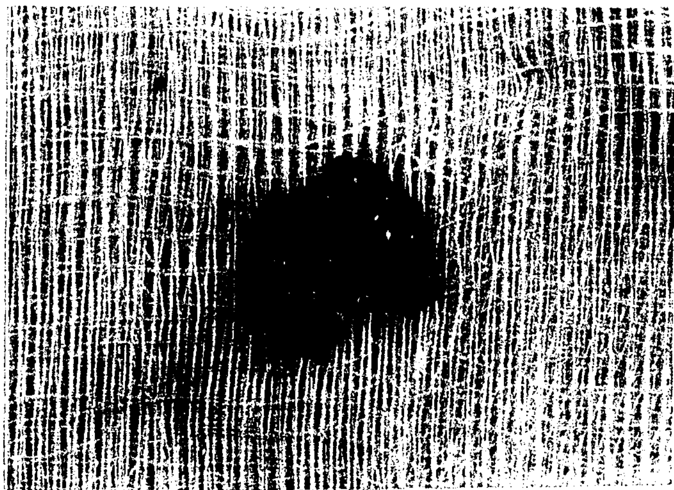


Fig. 52. Acute effects of 10 U PPE intratracheally instilled into DBA/2 mice.

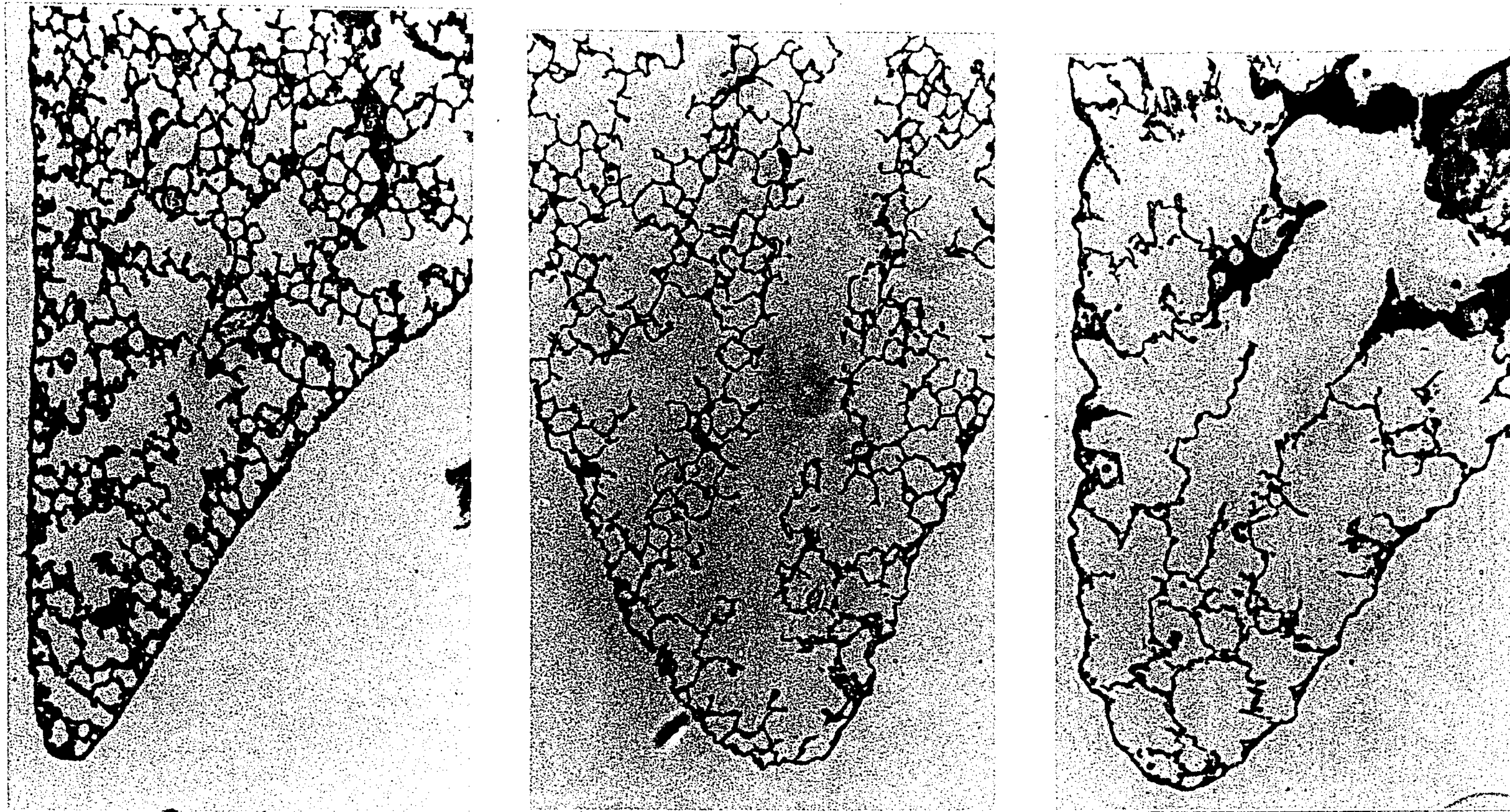


Fig. 53. Alveolar destruction by intratracheal instillation of elastase into DBA/2 mice.

a) Control; 2 wk post-instillation.

b) Crude macrophage elastase (0.5 μg PPE equiv); 2 wk post-instillation.

c) PPE (5 U; 40 μg); 48 h post-instillation.

DISCUSSION

I. ELASTASE ASSAYS

Elastase is frequently assayed by a simple radial diffusion procedure using SDS-treated elastin suspended in agar (82). This method is useful for detecting low-level elastase activity, but results of assays on complex biological material are often difficult to interpret since, in addition to enhancing enzyme-substrate interaction (45), SDS may dissociate complexes of macrophage elastase with endogenous antiproteases (5), therefore contraindicating the use of this assay for inhibitor studies. Non-SDS-treated elastin is a less sensitive substrate and cannot be used to detect elastase in crude macrophage conditioned medium without concentration, a procedure difficult to perform quantitatively with small sample volumes. Likewise, the 30-ng detection limit, coupled with the limitation of the 10-μl/well sample volume, generally precluded the use of this assay for monitoring elastase in column fractions, which most frequently were more dilute than 3 μg/ml.

Assays using SDS-treated radiolabeled elastin retain the problems associated with the SDS. In addition, tritiated SDS-elastin has been reported to be susceptible to cleavage by non-elastase proteases (19). Tritiated non-

SDS-elastin, with a 15-h incubation, was suitable for detecting as little as 5 ng of elastase activity (Fig. 8; p 104). In our present studies it was used primarily to monitor elastase during purification procedures.

The only assays which reliably detected macrophage elastase in crude conditioned medium employed [^{125}I]-elastin coupled to Sepharose beads (75), and peroxidase-labeled elastin (78). The radioiodinated elastin procedure offers subnanogram sensitivity, but uses soluble α-elastin rather than native elastin, and the specificity has therefore been questioned (88). However, by switching from the amino-containing Tris, to Hepes assay buffer, nonspecific deiodination was not a problem with freshly prepared [^{125}I]-α -elastin-Sepharose, and substrate cleavage by trypsin was negligible (Table 3; p 80). Reproducibility, both in terms of replicate samples and replicate assays (Fig. 7; p 113), was improved by normalizing for variablity in total substrate radioactivity per tube by expressing the results as the percent release of [^{125}I], rather than the absolute counts per minute. Use of the [^{125}I] label instead of [^{3}H] avoids the need for scintillation cocktails but presents a potentially greater radiation hazard. The major detraction of the substrate, partly owing to the relatively short half-life of [^{125}I], was a pronounced fall-off in sensitivity after storage exceeded 1 to 2 months.

The HRP-elastin substrate was used for the majority of studies reported in this thesis. Its sensitivity equals that of [^{125}I]-elastin-Sepharose, and does not diminish after long-term storage at -70°C. Nonspecific proteolytic cleavage was reduced by extensive preliminary incubation in the presence of trypsin and chymotrypsin. The subtrate subsequently exhibited only slight degradation by trypsin, and since collagenase is activated by trypsin (12), this likewise indicates substrate resistance to degradation by macrophage collagenase. The assay does require either the elimination of, or correction for, endogenous peroxidase activity, but this was absent from macrophage conditioned medium (Table 4; p 81). Likewise, phenol red pH indicator, present in all commercial tissue media, and other colored reagents which could potentially interfere with colorimetric analysis, must be omitted, compensated for, or removed (e.g. through dialysis) from samples. Another disadvantage of the assay is interference by protein precipitation which may result at the low pH required for the secondary reaction of peroxidase with ABTS. This problem was only encountered when attempting to evaluate the elastase inhibitory potential of mouse serum. Regardless, the precipitate can be removed through centrifugation, although this further complicates what already is a tedious, multistep assay. Perhaps due to the numerous steps involved and the coupled reaction, standard curves for assays performed on

different days were variable. However, the precision of replicate determinations for a given assay was within 5 percent.

II. EVIDENCE FOR A DISTINCT MACROPHAGE ELASTASE

Elastase activity was detected in the conditioned medium of cultured alveolar macrophages from both hamsters and mice (Table 5; p 82). Despite the lower yield of recoverable macrophages compared to hamsters, most studies used murine macrophages because the amount of elastase produced per cell is greater in mice. Furthermore, murine macrophages from inbred strains could be pooled for *in vitro* study.

Our experimental evidence supports the concept that alveolar macrophages produce an elastase via de novo protein synthesis, which is characteristically distinct from neutrophil elastase. The elastase associated with cultured alveolar macrophages, in corroboration with previous characterizations of macrophage elastase (5, 99), appears to be a metalloproteinase, completely inhibitable by EDTA. The metal dependency of the enzyme was confirmed by partially restoring EDTA-suppressed enzyme activity though subsequent addition of zinc and calcium. Macrophage elastase is resistant to inactivation by serine antiproteases such as phenyl methane sulphonyl fluoride (PMSF) and tetrapeptide chloromethylketones (Table 6; p 83). In contrast, neutrophil elastase is a serine protease, readily inhibitable by PMSF, AAPACK, and endogenous antiproteases such as α_1-PI

and α_2-M (107). EDTA only partially inhibits neutrophil elastase activity (107).

The pH dependency of macrophage elastase also appears to be different from neutrophil elastase. The pH profile of murine alveolar macrophage elastase demonstrated optimal activity occurred at pH 7.6 (Fig 15; p 121). This contrasts with the pH 8.2 optimum of neutrophil elastase, reported under similar conditions (105), and therefore suggests the observed activity is due to a separate enzyme.

Significant to the present concept of the pathogenesis of emphysema, which attributes alveolar destruction to unrestrained proteolytic attack by endogenous elastase, mouse alveolar macrophage elastase is not inhibited by α_1-PI, whereas it is inhibited by α_2-M (Table 6; p 83). Although the literature concurs that macrophage elastase retains full activity in the presence of α_1-PI, controversy exists regarding the enzyme's interaction with α_2-M. Discrepant results may be attributable to assay diferences, as White et al (107) used an SDS-elastin substrate, and Banda and Werb (5) subsequently reported that in the presence of SDS, macrophage elastase resists inactivation by α_2-M. Use of heterologous antiproteases, and differences in source and purity may also affect results.

The elastase in macrophage conditioned medium could theoretically be attributable either to the presence of

contaminating neutrophils or to macrophage release, rather than to the secretion of a distinct macrophage elastase. Campbell and Wald (11) studied the release of [^{125}I]-labeled human neutrophil elastase following receptor-mediated endocytosis by human alveolar macrophages. Under these *in vitro* loading conditions, the macrophages released enzymatically active neutrophil elastase. However, release followed exponential decay kinetics and was virtually complete 48-h post-instillation. In contrast, following an initial 48-h incubation, after which neutrophil elastase contamination would presumably be minimal, discharge of elastase by cultured mouse alveolar macrophages continued over a 6-day period (Fig. 14; p 120). This would be expected only if elastase were synthesized by the macrophages. Furthermore, cycloheximide completely abolished elastolytic activity in macrophage cultures. This requirement of protein synthesis for elastase expression indicated the macrophage was not acting merely as a vector for neutrophil elastase.

To further demonstrate that the observed elastase from macrophage cultures was not neutrophil-derived, a continuous macrophage cell line, $P388D_1$, was grown. Elastase activity, which in the pure culture could not possibly be attributable to neutrophils, was measurable in the conditioned medium (Table 5; p 82), and therefore presumably represented macrophage elastase synthesized and secreted by

the $P388D_1$ cells.

Previous reports (5, 99) indicated that dialysis of peritoneal macrophage conditioned medium increases the apparent elastase activity. No similar increase was noted following dialysis of either alveolar macrophage or $P388D_1$ conditioned medium (Table 5; p 82). This suggests the absence of a dissociable, dialyzable elastase inhibitor. However, there is non-dialyzable antiprotease activity in the $P388D_1$ conditioned medium, capable of inhibiting both pancreatic elastase and trypsin (Table 7; p 84). In addition, the failure of concentration to provide a commensurate increase in elastase activity (Table 5; p 82), suggests that an endogenous elastase inhibitor secreted by the cultured cells may be present, and that increased concentration favors the kinetics of inactivation.

III. MODULATION OF ALVEOLAR MACROPHAGE ACTIVITY

Purification of mouse alveolar macrophage elastase is hindered by the extremely limited amount of starting enzyme available from cultured macrophages. In an effort to facilitate purification, methods were attempted to increase either the number of recoverable macrophages or the elastase secreted per cell.

Myrvik and Leake (64) reported an 80-fold increase in macrophages lavageable from BCG-injected rabbits. Likewise, i.v. injection of an oil-in-water emulsion of BCG produced a large chronic granulomatous reaction in mice, particularly those with a C57 background (2). In this laboratory, when C57BL/6 mice were injected with BCG, the observed 4-fold increase in spleen weight (Table 15; p 92) confirmed the effectiveness of the inoculation. However, despite the appearance of an intense pulmonary granulomatous response (Fig. 16; p 122), there was only a 2-fold increase in the number of macrophages collected by lavage (Table 15; p 92). Approximately two-third of the cells in the lavage fluid were lymphocytes and neutrophils, whereas these cells normally comprise less than 5% of the cell population. Therefore, to avoid the risk of contamination of macrophage cultures with other cell types, injection with BCG was abandoned.

The unimpressive increase in macrophage yield in view of the apparently large cellular influx prompted a re-evaluation of our collection procedure, which had consisted of 6 serial lavages. In normal mice, twice as many macrophages could be obtained per animal by performing 18 lavages (Table 16; p 93). Still more macrophages were recoverable from lung minces (Table 17; p 94), but cell collection proceeded by lavage because this provided superior macrophage viability and was less traumatizing to the cells than the mincing procedure.

Similar to the results obtained with BCG, exposure of mice to NO_2 (Table 8; p 85) or to cigarette smoke, and intratracheal instillation of charcoal (data not shown), all increased the potential macrophage elastase yield by recruiting an alveolar macrophage population, but promoted a large neutrophil influx as well. In control animals, neutrophils comprise less than 10% of the cell population of lung lavages, and these contaminating cells are eliminated by rinsing them from the culture wells after allowing the macrophages to attach. With these experimental treatments, however, the percentage of neutrophils rises dramatically, which increases the potential for contamination with neutrophil elastase. The high proportion of neutrophils also interferes with plating macrophages out as a confluent monolayer, thereby diminishing the cell-to-cell interaction which may be necessary for optimal elastase

secretion. For these reasons, the macrophages for the present studies were obtained from untreated animals.

The preliminary exposure to nitrogen dioxide did not activate the macrophages to a state of heightened elastase secretion, at least one which persisted in vitro (Table 8; p 85). Elastase secretion also did not appear to be promoted either by interferon or bacterial endotoxin, both of which are capable of in vivo macrophage activation, as assessed by increased cytotoxic and tumoricidal potential (1,10). The conditioned medium from cultured macrophages recovered from mice which received intratracheal instillations of lipopolysaccharide or the synthetic double stranded RNA, polyinsinic: polycytidylic acid, which induces interferon (74), actually exhibited diminished elastase activity (Table 8; p 85).

Unlike peritoneal macrophages, which are activated to secrete elevated levels of elastase when presented with a phagocytic challenge, alveolar macrophage elastase secretion is refractory to phagocytosis (105). The only agents shown previously to increase mouse alveolar macrophage elastase in vitro are cytoskeleton-disrupting drugs (106). To determine whether the state of microtubule assembly affects elastase secretion, DBA/2 alveolar macrophages were maintained in the presence of a variety of microtubule-active agents (37). Colchicine, a drug which depolymerizes

microtubules, produced a 3.5-fold increase in elastase activity in macrophage conditioned medium (Table 9; p 86). Nocodazole, a chemically unrelated but functionally similar drug (21), increased secretion almost 3-fold. High levels of intracellular calcium can also cause microtubular disaggregation (98). However, calcium ionophore A23187, which theoretically equilibrated the cultured macrophges with the calcium in the maintenance medium, completely blocked elastase activity. Since disruption of microtubules was not histologically confirmed, it is possible either that A23178 resulted only in a partial depolymerization which was inadequate to potentiate elastase secretion, or that microtubule disaggregation is unrelated to elastase secretion. If depolymerization of microtubules does increase the release of alveolar macrophage elastase, it may be predicted that promoters of microtubule assembly would diminish elastase secretion. However, neither taxol nor PMA, both of which stabilize and increase the number of microtubules (79, 80), reduced elastase secretion (Table 9; p 86) even though PMA has been reported to enhance secretion in peritoneal macrophages (19).

The results seen with colchicine may therefore not be due entirely to its microtubule effect. For example, colchicine is known to increase intracellular cyclic AMP (35). Theophylline likewise leads to an elevation of cAMP, and has been demonstrated to promote elastase secretion from

peritoneal macrophages (23). A similar effect was observed with the DBA/2 alveolar macrophages (Table 9; p 86). However, although we used 2 mM theophylline, the macrophages were not maximally stimulated, since 2mM theophylline and 2μM colchicine together released more elastase than either agent alone. The drugs might therefore act on different sites of the alveolar macrophage.

Both A23187 and PMA also increase cAMP, and produce a respiratory burst in macrophages (66, 44, 31). If either of these processes were implicated in elastase secretion, one would have expected the conditioned medium from macrophages maintained in these agents to have elevated elastase levels. However, PMA was without effect, and A23187, despite increasing metabolic activity, as measured by glucose utilization (Table 10; p 87), completely suppressed elastase secretion, indicating elastase release is not an invariable consequence of cell activation.

If the elastolytic activity observed in the conditioned medium represented the release of previously ingested neutrophil elastase, its activity would not be completely dependent upon protein synthesis, and cycloheximide would not have completely abolished elastase activity (Table 9; p 86) at a dose which was not cytotoxic (Table 12; p 89). However, there still may be a small intracellular pool of presynthesized elastase, since approximately

12% of the elastase released with colchicine is still noted with colchicine in the presence of cycloheximide (Table 9; p 86). Presumably, colchicine can stimulate release of a small amount of elastase which is within the macrophages, by a process that does not require active protein synthesis. In addition, a correlation coefficient of 0.687 between elastase secretion and LDH release might suggest there is stored elastase released upon cell death. The highest levels of LDH were found in the conditioned medium from colchicine- and from theophylline-treated macrophages. Total intracellular LDH was not determined, so it is not known whether this represented increased production of LDH, or a sublethal cytotoxic response. However,the fact that colchicine and theophylline together produced higher levels of elastase secretion than either agent alone, whereas these same agents released greater quantities of LDH individually than in combination, argues against a non-specific mechanism for elastase release. In addition, the magnitude of the two responses differed greatly: elastase secretion from macrophages maintained in colchicine plus theophylline was 500% of the control value, while LDH was 165% of control. Furthermore, nocodazole had no effect on LDH release whereas it was a potent promoter of elastase secretion. These observation make it unlikely that treatments associated with elevated elastase activity are simply eliciting the discharge of intracellularly stored enzyme.

The most direct evidence for immediate post-synthetic secretion of macrophage elastase without intracellular storage (33) is the exceedingly low levels of the enzyme detectable in cell lysates (57). Alternatively, the failure of cell lysates to express elastase activity may be a consequence of enzyme inactivation by cytosolic antiproteases. To determine whether macrophage elastase may be lysosomally associated, macrophages were maintained in medium containing methylamine, which initiates exocytosis of lysosomal enzymes (73). Despite inducing a 6-fold increase in the marker lysosomal enzyme, beta-glucuronidase (Table 11; p 88), methylamine had no effect on elastase secretion (Table 9; p 86). The correlation coefficient of 0.095 (Fig. 17; p 123) indicated a very weak relationship between elastase and β-glucuronidase secretion. However, among the agents which promoted elastase secretion, theophylline increased β-glucuronidase approximately 40%, and both microtubule disrupting drugs, colchicine and nocodazole, produced 80% increases. The results seen with colchicine and theophylline parallel their effects on LDH secretion, and may therefore represent cell death. White et al reported no increase either in LDH or β-glucuronidase in macrophage cultures incubated with colchicine for 24 h (106), as opposed to the 60-h incubation used for this study. However, colchicine has been reported to increase acid hydroxylase secretion by cultured mouse peritoneal

macrophages (91). McCarthy et al (61) have suggested that different lysosomotropic agents may produce qualitatively different responses in macrophages. It is possible, therefore, that colchicine and theophylline induced exocytosis both of β-glucuronidase and of elastase-containing lysosomes, while methylamine only promoted β-glucuronidase release. If this were the case, macrophage secretion of both enzymes would be similarly affected in culture with colchicine and theophylline together. However, this treatment produced a 5-fold increase in elastase, despite only a 15% increase in β-glucuronidase. Macrophage elastase therefore does not appear to be lysosomally stored.

Endogenous α_2-M is produced by cultured alveolar macrophages (104) and colchicine blocks this secretion (103). Since α_2-M inhibits macrophage elastase (Table 6; p 83), it was possible that the apparent increase in elastase activity seen with various agents might be due to an increase in free elastase, secondary to diminished α_2-M production. To test this hypothesis, antiserum to α_2-M was developed with the aim of selectively inactivating α_2-M _in vitro_. However, the partially purified IgG from rabbit antiserum failed to completely inactivate α_2-M in hamster serum (Table 20b; p 97), and was therefore presumed to be unsuitable for this use in macrophage cultures.

The alternative approach to α_2-M inactivation was to

culture the macrophages in the presence of methylamine. Primary amines, in addition to their lysosomotropic action, completely inhibit antiprotease reactivity of α_2-M (96). Despite the presumed inactivation of α_2-M in macrophage cultures with methylamine treatment, elastase activity was not increased (Table 9; p 86). This suggests that the elevated levels of macrophage elastase produced by colchicine treatment are not a consequence of reduced secretion of α_2-M, and in fact, conditioned medium from colchicine-treated macrophages expressed elevated apparent functional α_2-M activity when assayed with chromozym-TRY (Table 13; p 90). However, the conditioned medium of methylamine-treated macrophages also expressed increased functional α_2-M activity (Table 13; p 90). Possibly, methylamine-treated α_2-M can form complexes with trypsin (97), which would invalidate the results obtained with the chromozym-TRY substrate, which actually measures the residual amidolytic activity of α_2-M-bound trypsin. Alternatively, through its lysosomal action, methylamine may have promoted the discharge either of α_2-M or of an α_2-M-protease complex which was reactive toward the synthetic substrate. It is therefore uncertain whether the objective of α_2-M inhibition was achieved with methylamine, in which case the effect of altered α_2-M levels on the expression of macrophage elastase likewise remains inconclusive.

In summary, protein synthesis is required for

sustained release of macrophage elastase, and very little of the enzyme appears to be stored in lysosomes. We have reported for the first time that nocodazole and theophylline promote elastase secretion by cultured mouse alveolar macrophages. In addition, we have elicited a 5-fold increase in elastase secretion by using theophylline and colchicine in combination. A common action of these drugs is elevation of cAMP, yet other agents which alter cAMP are without parallel effect on elastase. Macrophage stimulation with colchicine and nocodazole indicates elastase release is promoted by microtubule disruption. Basal elastase release is not however reduced by stabilizing the microtubules, according to the results obtained with taxol. The effects of A23187 and PMA indicate that alveolar macrophage elastase secretion is not increased either by calcium influx or by general cell activation. Distinct biochemical properties among different populations of macrophages was exemplified by the failure of PMA to increase the elastase secretion of alveolar macrophages.

IV. PURIFICATION PROCEDURES

A) $P388D_1$ ELASTASE

The continuous $P388D_1$ cell line possesses many of the characteristics of normal macrophages (51), including the secretion of neutral proteases (100). The availability of large numbers of cells, and the absence of contaminating cell types provided the rationale for the purification of $P388D_1$ elastase.

From ten liters of $P388D_1$ conditioned medium, the single-step isolation procedure of White et al (107), using elastin linked to agarose, yielded total elastase activity equivalent to little more than 1 microgram of PPE (Fig. 31; p 137). Thirteen percent of this activity was contained in a pool which was slightly retarded in its passage through the affinity column, and appeared homogeneous on electrophoresis, with a molecular weight of 15k. Subsequent fractionation by gel filtration HPLC revealed an apparent heterogeneity, as elastase activity was distributed among three peaks (Fig. 34, p 140; Table 23, p 100) with an activity ratio of 1:7:10. In addition to a peak which eluted at MW 15k, there was an earlier and later peak, 40k and <10k respectively, when compared to molecular weight standards. This suggests there may be three forms of $P388D_1$ elastase. Since the material applied to the

Fractogel column was retained on a 10,000 MW cut-off membrane, and the electrophoresis gel revealed no band below 15k, it appeared, with respect to the third peak, that separation on the HPLC column was not due entirely to molecular sieving.

An analogous situation was reported by Banda and Werb (5) during the purification of peritoneal macrophage elastase by gel filtration. They recovered three forms of elastase from an Ultrogel AcA54 column: form A eluted at an apparent MW of 57k, form B at 25k, and form C at 7-8k. However, electrophoresis revealed forms B and C had a true molecular weight of 22k, and form A, a molecular weight of 42k. Thus, although the absolute molecular weights differ, both P388D$_1$ elastase and peritoneal macrophage elastase appear to exist in multiple forms: two forms of lower, equivalent molecular weight, and one higher molecular weight species.

The late-eluting fractions from the affinity column were pooled, and they separated on Fractogel only into the counterparts of peaks 2 and 3. The final peak had the higher activity: 1/70 that of PPE. Other elastase-containing pools from the elastin-Sepharose column which were fractionated by HPLC expressed activity at the same elution volumes, although quantitatively they partitioned differently. In all cases, the elastase activity recovered

from the Fractogel column was at least an order of magnitude greater than what was applied. Banda and Werb similarly recovered 800% of the starting activity of peritoneal macrophage conditioned medium after purification (5). Most of the peritoneal macrophage elastase increase was realized after dialysis, suggesting the existence of either 1) a dissociable, dialyzable endogenous inhibitor which can mask elastase activity, or 2) an activatable proelastase.

Concentrated $P388D_1$ conditioned medium, however, exhibited no increase in elastase activity after dialysis (Table 5; p 82). To look for evidence of a $P388D_1$ proelastase which might be activated through proteolytic cleavage, trypsin and PPE were each added to $P388D_1$ conditioned medium. Rather than provide support for the existence of an activatable elastase zymogen, however, most of the added protease was inactivated (Table 7; p 84). It appears therefore that the conditioned medium contained an excess of an endogenous antiprotease.

The $P388D_1$ elastase was purified with the hope that an antiserum to it could be produced that would cross-react with alveolar macrophage elastase. Although there is an apparent molecular weight disparity between $P388D_1$ and alveolar macrophage elastase (15k vs 23k, respectively), both may still prove to be antigenically identical. If so,

the antiserum could form the basis both of a radioimmunoassay and an affinity isolation procedure for alveolar macrophage elastase. It would also justify the use of the more easily obtainable cell line elastase in studies planned to evaluate the potential of macrophage elastase to induce lung injury.

B) PERITONEAL MACROPHAGE ELASTASE

Peritoneal macrophage elastase was purified from the conditioned medium of inflammatory macrophages obtained from DBA/2 mice, following the procedure of Banda and Werb (5). An inbred strain was used to permit pooling of the collected macrophages for culture; strain DBA/2 was specifically selected 1) because this was the original host of the P388 lymphoma from which the $P388D_1$ cell line was derived (20), providing therefore the most appropriate source for an eventual comparison of the two elastases, and 2) because White et al (107) have previously partially purified DBA/2 peritoneal macrophage elastase by an alternative procedure.

Intraperitoneal injection of sterile irritants such as thioglycollate, induces an inflammatory exudate in the peritoneal cavity of mice which contains a high yield of macrophages. These macrophages secrete significantly more elastase than resident macrophages (105). Routinely, 15-30

million macrophages are reportedly harvested 4-7 days after thioglycollate injection (63). The time course for a macrophage response in DBA/2 mice appears, however, to be accelerated, and the yield less pronounced. Four days after thioglycollate administration, the cellular composition of the peritoneal lavage fluid was indistinguishable from baseline; the optimal response--only 7.5 million macrophages--appeared at day 3 (Table 18; p 95). This seems to be due to strain variation in responsiveness to thioglycollate since, after identical treatment, C57BL/6 mice of the same age and weight exhibited the anticipated high yield of over 25 million macrophages (Table 19; p 96). Similarly, Allen et al (2) reported only certain strains of mice developed a BCG-induced pulmonary inflammatory reaction: mice with a C57 background were responders and DBA/2 mice were nonresponders.

The culture medium from DBA/2 mice was subjected to anion exchange chromatography and, as reported by Banda and Werb (5), a peak of elastase activity appeared at the void volume, and a second peak eluted with low salt. Each of these peaks was subsequently resolved by gel filtration into three elastase-containing peaks. Banda and Werb (5) referred to the three peaks they obtained as forms A, B, and C: from A eluted as a 57k MW protein; form B eluted at a MW of 25k and appeared as 22k on SDS-electrophoresis; and form C, also 22k on electrophoresis, had an anomalous 6-7k

expected MW on the basis of its elution volume. After chromatography of their void volume pool, form B comprised 81% of the recovered elastase. Similarly, 82% (Table 24a; p 101) of the elastase activity isolated from our DBA/2 culture medium void volume pool, eluted as a 25k MW protein (Fig. 40; p 146), and had an actual MW of 23k (Fig. 41; p 147). However, this was the final peak off the AcA54 column. A similar AcA54 pattern was seen with the DEAE void pool from C57BL/6 conditioned medium (Fig. 48; p 154), as the third peak contained 74% of the elastase activity (Table 24a; p 101).

An additional difference noted between our results and those of Banda and Werb (5), was our failure to detect the 23k MW protein in SDS gels from the AcA54 application of the second peak off the DEAE column. A band of 27k MW was apparent, however, and is consistent with the molecular weight reported by White et al for DBA/2 macrophage elastase (107). The predominant protein appeared however to be in the range of 57-60 k MW, both on the basis of elution volume and SDS-electrophoresis (Fig. 46; p 152). The form A elastase of Banda and Werb (5) likewise eluted as a 57k MW protein. It is possible this 57-60k doublet represents macrophage collagenase, as most mammalian collagenases have a molecular weight within this range (12). Like macrophage elastase, collagenase is a metalloproteinase, and has a similar inhibitor profile (101). The observed elastolytic

activity is, however, unlikely due to collagenase, since, in addition to the reported resistance of elastin (31) and α-elastin substrates (75) to collagenase, even degradation of collagen by macrophage collagenase requires activation by such agents as mercurials or trypsin.

In summary, the elution profile of crude macrophage elastase demonstrates that the enzyme exists in multiple forms. Enzyme characterization as a metallo- rather than serine proteinase was confirmed by the inhibition of the activity of each form by EDTA, and the resistance of each form to inactivation by PMSF (Table 25; p 102). Although each elastase peak from the AcA54 column revealed a 21-27k MW band on SDS electrophoresis, only the final peak was purified as a preparation devoid of higher molecular weight species. The most persistent contaminant, a 57-60k MW protein, may be macrophage collagenase.

C) ALVEOLAR MACROPHAGE ELASTASE

The alveolar macrophage elastase from DBA/2 mice was fractionated, like peritoneal macrophage elastase, into two major peaks on DEAE-Sephadex. On the basis of the elastase yield from 35 mice it was estimated that several hundred mice would have to be lavaged to obtain a sufficient number of macrophages to complete a purification procedure, which was therefore not realizable at this time.

D) HAMSTER α_2-MACROGLOBULIN AND RABBIT ANTISERUM

Although α_2-M is regarded as an inhibitor of virtually all endoproteinases (7), there are reports that macrophage elastase is not inhibited by α_2-M (103). We modified the rat α_2-M purification procedure of Hayashida et al (32), using sequential anion exchange and gel filtration chromatography, to obtain highly purified α_2-M from turpentine-injected hamsters, with the aim of helping to resolve this issue. Turpentine markedly elevates rat serum α_2-M levels (67), but failed to increase hamster α_2-M at 48 h. Dosage and time-course studies are therefore indicated to determine whether turpentine can produce an α_2-M acute-phase response in hamsters, which would provide additional starting material for purification purposes.

The observed 11.3 U/ml α_2-M activity of hamster serum is comparable to the 10.2-10.9 U/ml value for humans (18), although there is a report that hamster serum α_2-M may be more than 3 times the human level (89). However, on the basis of the 21-fold enrichment of α_2-M obtained during purification, hamster α_2-M would comprise approximately 5% of the total serum protein, which is consistent with the percentage contribution of human α_2-M. The purified hamster α_2-M was subsequently shown to inhibit mouse alveolar macrophage elastase (Table 6; p 83). Therefore, in addition to the neutrophil elastase/α_1-PI couplet, macrophage

elastase/α_2-M may constitute another facet of protease-antiprotease balance necessary for the maintenance of normal lung architecture.

Antiserum developed to the α_2-M was evaluated for its ability to selectively deplete functional levels of hamster α_2-M. However, rabbit α_2-M in the antiserum had to be eliminated. Anion exchange chromatography succeeded in isolating anti-α_2-M from contaminating rabbit α_2-M. This material could substantially, but not completely, reduce functional activity of hamster α_2-M. Subsequent affinity chromatography provided too poor a yield to evaluate its effectiveness at further purification of the anti-α_2-M IgG.

V. *IN VIVO* INSTILLATIONS

A) INSTILLATION OF CRUDE ALVEOLAR MACROPHAGE ELASTASE INTO MICE

The current accepted hypothesis of emphysema maintains the disease results from the release of elastolytic enzyme from lung inflammatory cells. The two primary cells implicated in the disease process are the neutrophil and the alveolar macrophage. Whereas neutrophil elastase has been purified and is recognized as an important pathologic agent in the degradation of lung matrix elastin (44), alveolar macrophage elastase has never been purified and completely characterized. It has therefore been difficult to establish a causal role for this enzyme in lung disease.

The actual objective of this research project was to discern if, and to what extent, macrophage elastase contributes to the pathogenesis of emphysema. Although this would be best resolved by evaluating, in a dose-response manner, the ability of purified alveolar macrophage elastase to produce *in vivo* lung degradation, it has not proved feasible to obtain sufficient crude elastase to complete the purification protocol. Nevertheless, there are no direct *in vivo* demonstrations of any macrophage secretory product causing lung injury, and this would represent a significant finding. We therefore instilled mice intratra-

cheally with concentrated alveolar macrophage conditioned medium. Each mouse received the elastolytic equivalent of 0.5 μg of PPE.

After two weeks, the lungs of the experimental animals were unremarkable on gross examination. There was no evidence of intra-alveolar hemorrhage on histologic sections, and lung structure appeared generally uniform. The overall impression was confirmed by measuring Lm, which although slightly greater than controls (47.2 vs 45.2 μm), was not significantly different (Table 29a; p 106), and was consistent with recently reported normal values (38, 87). However, in three of the five experimental mice, the basal lobes had focal regions of alveolar disruption of distal airways (Fig. 53b; p 159), which was evidence of a gravity-dependent emphysema. In addition, the percentage of individual lung fields of experimental animals with high Lm values was considerably although again not statistically, greater than controls (Table 29b; p 106).

This laboratory has been among the most vocal critics of prejudicial selection of lung fields in concluding the existence of emphysema, and we are cautious not to overinterpret the results of this study. It appears the macrophage elastase instilled was close to the threshold dose for producing emphysema. Definitive conclusions will require the administration of higher elastase challenges,

and subjective impressions must be corroborated by the Lm data.

B) INSTILLATION OF PANCREATIC ELASTASE INTO MICE

A single endotracheal instillation of 25 U of PPE into hamsters produces experimental emphysema within 24 hours, accompanied by a 70% reduction in lung elastin (52). Thereafter, the emphysema progressively worsens despite the gradual restoration of normal elastin content (52). Explanations for the chronicity of the disease include: retention of enzymatically active elastase either complexed with α_2-M (80, 81) or internalized and subsequently release by alveolar macrophages (11); the influx of inflammatory cells in response to the acute injury, and their secretion of endogenous elastases (52); alterations in connective tissue biosynthesis (52); or, susceptibility of the weakened lung architecture to mechanical respiratory deformation (46).

Recent experiments with mice (38, 87) have used as little as 5 U of PPE to induce emphysema. However, the lesion was assessed at 2-4 wk after instillation, by which time secondary factors, such as influx of inflammatory cells, may have contributed to the extent of lung damage. Similarly, we have evaluated Lm in mice 2 wk post-instillation of macrophage conditioned medium. To ensure that the lung injury arose as a direct consequence of the instilled protease, it would be preferable to evaluate the

lesion at 48 h.

With the aim of determining the *in vitro* elastolytic equivalent capable of inducing an acute significant increase in Lm in our specific test animal, we instilled 5 U of PPE into DBA/2 mice. After 48 h the lungs appeared coarsened on gross examination, with a few scattered bubbly patches, characteristic of emphysema. Microscopically, diffuse panlobular airway enlargement was seen in two animals, and a more restricted lesion was observed in the other three. The enzyme may not have been uniformly dispersed, but in studies using drawing ink, the instillation procedure effectively distributed the tracer to all lung regions. The difference in Lm between experimental and control animals was highly significant (Table 28; p 111).

On the basis of the Lm data, 5 U of PPE may be in considerable excess of the minimum elastase challenge necessary to produce a quantifiable lesion. Studies to determine the threshold dosage of PPE are important to provide an indication of what minimum *in vitro* elastolytic equivalent of alveolar macrophage elastase would have a reasonable possibility of inducing emphysema. As demonstrated by Senior et al (83) however, who encountered much milder emphysema using leukocyte as opposed to pancreatic elastase, *in vitro* elastolytic equivalence may not be predictive of *in vivo* pathologic potential.

C) ELASTOLYTIC AND HEMORRHAGIC ACTIVITY OF PORCINE PANCREATIC ELASTASE IN HAMSTERS

Ten units of highly purified porcine pancreatic elastase (Elastin Products) or a SLAPN dosage-matched equivalent of a less pure preparation (Sigma) were instilled into hamsters. After 48 h, lung sections revealed that only the higher purity enzyme caused widespread alveolar destruction (Table 27a; p 104), and only the less pure elastase produced extensive hemorrhage (Table 27b; p 104). That two different grades of elastase, matched to provide equivalent enzyme dosage as determined on SLAPN substrate, do not produce equivalent *in vivo* elastolytic effects, emphasizes the problems inherent with *in vitro* enzyme assays, particularly those involving synthetic substrates. Possible explanations which may account for the apparently blunted *in vivo* activity of S-PPE include: enzyme impurities potentiated the activity of elastase on SLAPN without having a similar effect on native elastin; an impurity itself cleaved the synthetic substrate; an impurity unreactive toward SLAPN interfered with *in vivo* elastolysis; or, the increased hemorrhage produced by the cruder elastase raised the local concentration of elastase inhibitors, which inactivated the enzyme. Even the use of native elastin substrates does not eliminate these problems. Rinderknecht et al (76) reported that although trypsin and chymotrypsin are themselves without elastolytic effect, they enhance elastase activity.

Potentiation was speculated to arise either by inducing conformational changes of the elastin substrate which exposed additional binding sites, or from the interaction with elastase itself. *In vivo*, nonspecific proteases may damage the alveolar epithelium and basement membrane and promote access of elastolytic enzyme into the lung interstitium (31).

When Senior et al (83) compared the *in vivo* effects of leukocyte elastase and pancreatic elastase, they found that at a dosage with similar capacity to degrade elastin *in vitro*, pancreatic elastase caused a much more severe emphysema when instilled into hamsters; conversely, the leukocyte elastase generated a greater hemorrhagic response. They proposed that as a consequence of the hemorrhage, leukocyte elastase may have been inactivated by an influx of enzyme inhibitors. The hemorrhage may have resulted from the activity of the enzyme on a substrate other than elastin. Leukocyte elastase may cause vascular injury directly or via complement activation and kinin generation (83).

Similarly, an impurity in cruder preparations of elastase may either enhance the hemorrhagic activity of the enzyme, or itself constitute a hemorrhagic component with endothelial junction sensitivity, or anoxia-promoting or complement-activating capability. The observed inverse

relationship between hemorrhage and emphysema suggests that pulmonary hemorrhage resulting from elastase-induced small vessel injury, rather than promoting emphysema via the entry of circulating proteases, may have a protective effect attributable to antiprotease influx.

CONCLUSION

Elastolytic enzymes are implicated in the pathogenesis of emphysema. Although the prevailing theory maintains that the neutrophil is the major source of elastase which has access to the lung parenchyma, macrophages are also believed to secrete elastase. The current investigation provides additional support for a distinct macrophage-derived enzyme that degrades elastin *in vitro*, and may contribute to elastolysis *in vivo*.

Since the anticipated elastolytic activity of macrophages was very low, a preliminary comparison of several elastase assays was performed to determine which would provide optimal sensitivity and specificity. Only the HRP-elastin and radioiodinated elastin substrates were able to detect elastase in the range necessary for some of our studies. By adjusting the reaction time, 0.5-100 ng of porcine pancreatic elastase activity could be assayed with either substrate, both of which were resistant to non-specific cleavage by trypsin. The assay using radioiodinated elastin is the more convenient procedure, but substrate sensitivity deteriorates upon storage for more than 1-2 months. In contrast, the HRP-elastin substrate is stable for at least 2 years, but the assay is quite tedious. In addition, colored samples and endogenous peroxidase can

interfere with the results. However, after eliminating phenol red from the culture media, and ascertaining there was no peroxidase activity in our macrophage cultures, the HRP-elastin was used for most of the studies reported herein.

The sensitivity of the assay procedure permitted the unequivocal demonstration of elastase activity in cultures of murine alveolar macrophages. The enzyme activity was inhibited by EDTA, then partially restored upon subsequent addition of divalent cations, which supports the characterization of macrophage elastase as a metalloproteinase (5). In contrast with neutrophil elastase, macrophage elastase activity was unaffected both by such inhibitors of serine proteases as phenyl methane sulphonyl fluoride and tetrapeptide chloromethylketones, and by endogenous α_1-PI. Macrophage elastase activity was, however, inhibited by α_2-M. It has been proposed that apparent macrophage elastase activity may merely represent secretion of ingested neutrophil elastase. Their dissimilar inhibitor profile argues against this, as does the requirement for de novo protein synthesis, as deduced from the inhibition of elastase secretion by cycloheximide, and the observed continuous discharge of enzyme over a 6-day culture period. Furthermore, the P388D$_1$ macrophage cell line, in which neutrophil contamination is not a consideration, expresses elastase activity.

Once convinced of the existence of alveolar macrophage elastase, we evaluated the effect of various treatments aimed either at 1) increasing _in vivo_ the number of macrophages recoverable by bronchoalveolar lavage, or 2) increasing _in vitro_ the elastase secretion per cell. _In vivo_, both nitrogen dioxide and BCG did improve the macrophage yield but they did not stimulate elastase secretion _ex vivo_, and they promoted a neutrophil influx as well. Cautious to avoid potential contamination with neutrophils, or macrophage ingestion of neutrophils, we used alveolar macrophages from normal animals for subsequent studies.

In vitro, colchicine has previously been shown to promote alveolar macrophage elastase secretion (106), and we noted a 3.5-fold increase. Nocodazole, although chemically unrelated to colchicine, likewise disaggregates microtubules and produced a 3-fold rise in elastase activity, thereby suggesting an association between microtubular disruption and elastase secretion. However, since taxol, which stabilizes microtubules, did not significantly alter elastase activity, secretion need not be dependent on tubule depolymerization. Colchicine can also elevate intracellular cAMP levels (35). Since theophylline more than doubled the elastase secretion of cultured alveolar macrophages, there is support for a cAMP-mediated mechanism of elastase discharge. Microtubule disrupting agents may increase cAMP as a result of the removal of cytoskeletal

constraints. The increased membrane fluidity could facilitate the physical association of the adenylate cyclase subunits, thereby activating the catalytic unit. However, stimulation by colchicine or theophylline alone is submaximal, since together a 5-fold increase is observed. This additive response suggests these agents may be acting at two different loci (e.g. a microtubule protein, and adenylate cyclase). Although calcium stimulates elastase secretion by neutrophils, it appears not to mediate macrophage elastase secretion, since calcium ionophore A23187 completely suppressed elastase activity.

Colchicine could act to stimulate synthesis and/or secretion. Whereas cycloheximide completely eliminates basal elastase secretion in cultured macrophages, it failed to suppress 12% of colchicine-stimulated elastase activity. This suggests the existence of a relatively small intracellular pool of potentially releasable macrophage elastase. The enzyme does not, however, appear to be stored in lysosomes, since methylamine, which promotes discharge of lysosomal enzymes, did not elevate elastase activity in macrophage cultures.

Colchicine was used to promote alveolar macrophage elastase activity in experiments designed ultimately to obtain sufficient purified enzyme to create an animal model of macrophage elastase induced emphysema. However, the

amount of elastase recovered during anion exchange chromatography was inadequate to continue further purification, and the projected several hundred animals needed to obtain sufficient material made it untenable to proceed with this aspect of the study. Rather, we decided 1) to purify elastase from the $P388D_1$ cell line and from peritoneal macrophages, and 2) to evaluate the in vivo elastolytic potential of crude, concentrated alveolar macrophage conditioned medium. Elastolytic activity equivalent to 1 μg of porcine pancreatic elastase was recovered from crude $P388D_1$ conditioned medium applied to an α-elastin-agarose column; 13% of this activity appeared as a homogeneous 15k MW protein on electrophoresis. Subsequent gel filtration HPLC, however, resolved 3 separate components, and the sum of the total elastase activity eluted was over 10-fold greater than the initial activity. Banda and Werb (5) similarly recovered 800% of their starting activity during purification of peritoneal macrophage elastase, and suggested the purification may remove a dissociable endogenous inhibitor of macrophage elastase. Alternatively, the amplified recovery might be explained by an activation of a proelastase, which has yet to be identified. However, trypsin failed to increase the elastolytic activity of $P388D_1$ crude conditioned medium. We did not investigate whether plasmin can activate latent macrophage elastolytic activity, which has just been reported (13).

Purification of peritoneal macrophage elastase confirmed the apparent heterogeneity reported by Banda and Werb (5). Two of the three forms had a molecular weight of ~23k, but only one was purified without contamination by a persistent 57-60k MW protein, which may represent macrophage collagenase. Enzymatic activity of each form was completely suppressed by EDTA, whereas at least 80% of activity was retained in the presence of phenyl methane sulphonyl fluoride, thereby establishing that the activity was not due to neutrophil elastase.

The final aspect of this project was the intratracheal administration to mice of macrophage conditioned medium containing the _in vitro_ elastolytic equivalent of 500 ng of porcine pancreatic elastase. After 2 weeks, mean linear intercept, an index of alveolar disruption, was slightly but not significantly elevated in the experimental mice, and in 3 of these 5 animals, histologic lung sections revealed focal areas of airway enlargement suggestive of incipient emphysema. However, uncorroborated by the Lm data, we are skeptical of these subjective impressions.

For the future, _in vivo_ studies must either attempt a higher elastase challenge, or perhaps reduce the animal's antiprotease defenses prior to instillation. To elucidate the mechanism of elastase release, dose-response studies on additional potential modulators of macrophage elastase

secretion, including cAMP analogues and microtubule-active drugs, are planned.

Support for the existence of a distinct alveolar macrophage-derived elastolytic enzyme is very strong. Although its enzyme activity may be extremely low, its resistance to inactivation by α_1-PI, its seclusion from potential inactivators due to its local pericellular site of action, and the possible amplification of its activity by serum or alveolar factors, all may contribute to a chronic, unsuppressed enzyme activity with the potential to degrade lung elastin. Any definitive role for macrophage elastase in the pathogenesis of emphysema awaits, however, firm demonstration that the purified enzyme can result in experimentally-induced emphysema.

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