



LEUKOCYTE ELASTASE AND ANTI- ELASTASES IN PULMONARY EMPHYSEMA

A thesis submitted by

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ABSTRACT

Although the cause of emphysema is uncertain, the preferred theory to explain the aetiology points to an imbalance in the protease-antiprotease systems within the lung with human leukocyte elastase (HLE) and α_1 -protease inhibitor (α_1 -PI) being the main candidates. However an inherited deficiency of α_1 -PI, is responsible for only a small percentage of all cases of emphysema with the most frequent form occurring in some smokers who have normal α_1 -PI levels. This thesis examines some aspects of the protease-antiprotease theory.

The aim of the first part of this thesis was to examine the proposal that there may be a reduced capacity to inhibit HLE activity in serum and bronchoalveolar (BAL) fluid from emphysema patients and susceptible normal smokers when compared to normal individuals. Comparison groups included children, adult non-smokers, and patients with an acute lung disease, adult respiratory distress syndrome (ARDS). The serum α_1 -PI concentration was significantly increased in children and in adults with emphysema and ARDS. However, no reduction in the HLE inhibitory and the α_1 -PI functional capacity of serum samples from the emphysema and smoking groups was found when compared to the other groups studied, except in the ARDS group, where both were significantly reduced. BAL samples from volunteers were obtained from normal adult non-smokers and smokers and from patients with ARDS. The α_1 -PI concentration in BAL samples from ARDS patients was increased by more than 40 fold but was only 37% functional compared to the 70% and 85% seen in non-smokers and smokers respectively. However no significant reduction in the HLE inhibitory and the α_1 -PI functional capacity of BAL samples from smokers was noted when compared to non-smokers. The presence of other HLE inhibitors in addition to α_1 -PI was demonstrated by increased inhibition towards HLE in all BAL samples. In conclusion, no evidence was found for a reduction in the ability of serum and BAL fluid from emphysema patients and normal smokers to inhibit HLE activity that could explain the increased risk of development of emphysema in smokers.

The second part of this study was to assess the effectiveness of a number of nonspecific and specific inhibitors towards HLE under varying assay conditions including the presence of lung and other surfactants. Overall heparin and the specific HLE inhibitor, ICI 200355, were found to be the most potent inhibitors of HLE using MeO-Suc-A-A-P-V-NA as substrate, although heparin is only most effective as a partial inhibitor (approx. to 25% of residual HLE activity). However, nonspecific proteins such as elastin peptides and even albumin, if present at a high enough concentration (50% inhibition seen with 6 g/l), were found to be capable of partial inhibition of HLE. The effectiveness of glycosaminoglycan (GAG) inhibition of HLE was, under certain conditions, found to be dependent on factors, such as the type of GAG, the HLE assay substrate, the degree of GAG sulphation and the size of the GAG. For example, heparin was the more effective inhibitor with the soluble substrate Suc-A-A-A-NA while heparan sulphate was more effective with insoluble lung elastin as substrate. In general, less inhibition was found with GAGs that were less sulphated and with low molecular weight GAGs (<2000). Cathepsin G, is not completely inhibited by the major protease inhibitors, α_1 -PI or α_2 -M, unlike HLE, the other major inflammatory serine protease. Pulmonary surfactant had a mild inhibitory effect on HLE activity whereas non-ionic surfactants, such as Brij 35, were found to not only activate HLE but also reduce the effectiveness of some inhibitors, particularly α_2 -macroglobulin and those GAGs which are poor inhibitors of HLE.

In the third part of this study high affinity binding fractions towards HLE were isolated from heparin to determine if heparin has some specificity of binding towards HLE. Sulphate content of the heparin fractions fell with increased binding and inhibition of HLE, which suggests that maximum inhibition and binding depend on an ordered sulphate group sequence rather than simply having the strongest polyanion. Heparinase digestion of heparin bound to HLE allowed the isolation of the most tightly bound heparin saccharide fractions that are likely to contain the high affinity binding sequences for HLE.

In conclusion, the significant findings from this study has shown that there was no reduction in HLE and α_1 -PI functional capacity in adults who smoked or who had emphysema. There was also a significant contribution of other inhibitors in addition to α_1 -PI in all BAL samples examined. In addition to α_1 -PI, a number of other compounds were shown to inhibit HLE, including heparin and heparan sulphate, which is clinically significant because of their strong inhibition of HLE and the large amounts of heparan sulphate proteoglycans and heparin that are found in the lung. This study also highlights the importance of the modifying effects of surfactants, including pulmonary surfactant, towards HLE activity and the function of various inhibitors towards HLE. Finally, a study of the interaction of heparin with HLE has revealed new information on the type and degree of specificity of the binding of heparin for HLE.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where reference has been made in the text.

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ABBREVIATIONS

α_1 -ACH	alpha-1-antichymotrypsin
α_1 -PI	alpha-1-protease inhibitor
α_2 -M	alpha-2-macroglobulin
ARDS	adult respiratory distress syndrome
BAL	bronchoalveolar lavage
COPD	chronic obstructive pulmonary disease
CT	computed tomography
cpm	counts / minute
EC	Enzyme Commission
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
FEV ₁	forced expiratory volume in 1 second
FVC	forced vital capacity
GAG	glycosaminoglycan
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HLE	human leukocyte elastase
HPLC	high pressure liquid chromatography.
K	exponential function describing a pressure volume curve
kDa	kilodaltons
k_{ass}	binding association constant.
K_i	inhibition constant; concentration of inhibitor at which half maximal enzyme velocity is achieved under the stated conditions
K_m	Michaelis constant; concentration of substrate at which half maximal enzyme velocity is achieved under the stated conditions, also indicative of the affinity of the substrate.

MeO-Suc-A-A-P-V-NA	methoxysuccinyl-(L-alanine) ₂ -L-prolyl-L-valine- <i>p</i> -nitroanilide
M/NEI	monocyte/neutrophil elastase inhibitor
M _r	molecular radius
PAGE	polyacrylamide electrophoresis.
PMN	polymorphonuclear leukocyte
PPE	porcine pancreatic elastase
SLPI	secretory leukoprotease inhibitor
Suc-A-A-A-NA	n-succinyl-(L-alanine) ₃ - <i>p</i> -nitroanilide
TIMPS	tissue inhibitor of metalloproteinases
TRIS	tris(hydroxymethyl)aminomethane
VGVPAG	valine-glycine-valine-alanine-proline-glycine (elastin hexapeptide)
VPGVG	valine-proline-glycine-valine-glycine (elastin pentapeptide)

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THE ROLE OF PROTEASES AND ANTIPROTEASES IN EMPHYSEMA - A REVIEW OF THE LITERATURE.

1.1 INTRODUCTION

Chronic obstructive pulmonary disease (COPD), of which emphysema is one component, is a major cause of morbidity and mortality. In Europe, together with asthma and pneumonia, COPD is the third most common cause of death (Siafakas et al, 1995). In the USA, COPD is currently the fourth leading cause of death (Mortality Patterns - United States, 1993). It is also a major cause of morbidity as demonstrated by the fact that more than 5.4 million Americans were estimated to have COPD in 1985, and it accounted for 13% of hospital admissions in the USA (Feinleib et al., 1989). The majority of cases occur in patients who have been or are currently smoking. Thus, prevention of the development of COPD by reducing the prevalence of smoking remains a priority. While the prevalence of smoking in developed countries, such as the USA, has fallen from 52.1% in 1965 (Smoking and health, 1964) to 25.7% in 1991 (Cigarette smoking among adults-United States, 1993), the recent large increase in smoking in many countries (MacKenzie et al., 1994) will see COPD becoming an even bigger worldwide problem. However, only a relatively small proportion of smokers develops symptomatic disease (Fletcher and Peto, 1977). As a consequence, there has been considerable interest in not only how smoking causes COPD, but also in identifying other factors (eg. genetic factors, environmental factors) that make individuals susceptible to COPD.

Pulmonary emphysema together with chronic obstructive bronchitis and some obstructive types of bronchiectasis form the group of conditions known as COPD, or alternatively as chronic obstructive lung disease (COLD) or chronic obstructive airways disease (COAD). Acronyms, such as COPD, have come into wide use because the pathophysiology of COPD is uncertain and it is difficult, with the usual clinical and epidemiological tools, to diagnose emphysema

accurately and thus to determine the relative roles of emphysema, bronchitis and asthma in causing airway obstruction. However, such acronyms are not ideal terms as chronic bronchitis and emphysema, as they are currently defined, need not necessarily be accompanied by airway obstruction. This will be discussed further in relation to emphysema (See section 1.1.1). Despite the reservation, in this thesis the term COPD is used collectively to describe the clinical groupings.

Emphysema, bronchitis and asthma share a number of epidemiological characteristics such as a higher prevalence in men, smokers, urban dwellers, and poorer social classes. There is also considerable overlap between subjects with asthma and COPD in airway responsiveness, airflow obstruction and pulmonary symptoms (see Table 1.1). The so called “Dutch hypothesis”, as first proposed by Orie et al (1961), includes asthma as one of the conditions in a single underlying lung disease mechanism that can lead to chronic airway limitation. However the theory needs to be more extensively tested before it is widely accepted.

The clinical diagnosis of COPD is usually based on the demonstration of irreversible airflow limitation after the exclusion of a few specific conditions (eg. localised disease of the upper airways, bronchiectasis, and cystic fibrosis) that can also cause fixed airway obstruction (American Thoracic Society, 1987). The airflow limitation in COPD is due to a variable combination of airway disease and emphysema. The airway disease component, often referred to as small or peripheral airway disease or chronic obstructive bronchiolitis, consists mainly of decreased luminal diameters resulting from various combinations of increased wall thickening, increased luminal mucus and changes in the lining fluid of the small airways. Emphysema, on the other hand, is defined anatomically by permanent, destructive enlargement of airspaces distal to the terminal bronchioles without obvious fibrosis (see 1.1.1. below). The relative contribution of airway disease and emphysema to airflow limitation is difficult to define *in vivo*.

At present the prognosis for patients with clinically evident COPD remains poor, particularly those with type II respiratory failure and cor pulmonale, where the 5 year survival may be less than 30 %. Some increased survival may be obtained by using continuous long-term, controlled oxygen therapy or with surgical intervention by way of lung transplantation or lung reduction. An understanding of the mechanisms of the development of COPD may allow treatment to arrest the further progress of established disease. More importantly, it could lead to treatment that prevents the development of COPD, or at least of the clinically debilitating stages of the disease.

Pulmonary emphysema is a major component contributing to the morbidity and mortality of COPD and occurs to a greater or lesser extent in all of the COPD subgroups. Despite the importance of pulmonary emphysema as a component of COPD, its pathogenesis remains uncertain. The current concepts about the pathogenesis of emphysema are based on essentially two classical studies. One report demonstrated an increase in emphysema in patients with a decrease in the antiprotease, α_1 -proteinase inhibitor (α_1 -PI) (Laurell & Eriksson, 1963) and the other the induction of emphysema in experimental rats after the intratracheal instillation of the protease, papain (Gross et al, 1964). These findings led to a simple protease-antiprotease model in which an imbalance resulted in increased proteolytic activity, which destroyed the elastic framework and the air space walls of the lung. However, while most of the experimental and epidemiological data support the case for this model, the pathogenetic mechanisms, in practice, appear to be much more complex. For example, this simple model explains the very early development of emphysema in patients with a homozygous α_1 -PI deficiency (PI Z) who smoke, but the situation is not as definitive in those deficient patients who do not smoke where emphysema may not develop for another 10-20 years or not at all, as is the case for most individuals with normal levels of α_1 -PI. Overall, it has been estimated that only 10-15% of individuals with the homozygous form of α_1 -PI deficiency develop emphysema (Knight et al., 1997). Thus the absence of α_1 -PI does not necessarily lead to the development of emphysema

and suggests that factors additional to α_1 -PI deficiency are required for the development of the disease.

A long series of experiments with animal models has demonstrated that elastolytic enzymes, introduced via the trachea, can produce emphysema (Snider et al., 1986; Evans and Pryor, 1994). The majority of the evidence suggests that human leukocyte elastase (HLE), the physiological target of α_1 -PI (Beatty et al., 1980; Longstaff and Gaffney, 1991), is the elastolytic enzyme responsible for the degradation of lung tissue in emphysema, although any of the elastolytic enzymes that can potentially be released in the lung could have a role.

The theory has been modified to explain smoking-induced emphysema as discussed later in this chapter (See section 1.5). This includes a probable increase, in the lung, of oxidants and free radicals from tobacco smoke as well from greater number of activated neutrophils and macrophages found in the lungs of smokers. These oxidants may produce tissue damage directly or by partially inactivating α_1 -PI (Evans and Pryor, 1994; Hoidal et al., 1981). The elastolytic load on the lungs is also probably increased due to the increased number of activated neutrophils (McGowan et al., 1983; Hunninghake and Crystal, 1983; Hoidal and Niewoehner, 1983a). While experiments with animal models have demonstrated that exposure to tobacco can increase the amount of emphysema in the presence of tracheally-induced elastolytic enzymes (Hoidal and Niewoehner, 1983b, Janoff, 1985), tobacco smoke, by itself, has not been shown conclusively to induce emphysema in animal models. The majority of the studies of the pathogenesis of smoking-induced emphysema in humans have simply compared smokers with non-smokers, but because of the wide variability in susceptibility of individual smokers to develop progressive COPD, most of the results have been inconsistent. Several studies have suggested that genetic factors, other than α_1 -PI type, may be involved in the susceptibility to develop COPD. Other than smoking and α_1 -PI deficiency, the other major risk factor for which there is clear evidence is occupational exposure, in particular, coal and gold mining (Oxman et

al., 1993). Until some of the other unidentified host factors for the development of COPD are identified, the exact pathogenesis of smoking-induced emphysema will continue to be unknown.

The elucidation of the mechanisms involved in the development of emphysema may depend on the use of suitable animal models such as transgenic mice in which the protease and antiprotease levels can be changed. While the expression of α_1 -PI in mice appears to be different from that which occurs in humans (Tardiff and Krauter, 1998), the different forms of genetic emphysema seen in the tight-skin, pallid, and beige strains of mice (Keil et al, 1996) lend support the notion that the pathogenesis of emphysema is multifactorial.

This chapter reviews the epidemiology of emphysema, and discusses the mechanisms proposed to explain its pathophysiology. The protease-antiprotease model is discussed and the evidence supporting the hypothesis in humans and in animal models is reviewed and evaluated. In particular, the central position in this hypothesis of the protease human leukocyte elastase (HLE) and the antiprotease α_1 -PI will be carefully considered, as the relationship between these two components is the basis for a major part of the work reported in this thesis.

1.1.1 DEFINITION OF EMPHYSEMA

Emphysema has been recognised since the 1800's, but a definition that was widely accepted did not evolve until the 1959 Ciba Guest Symposium where the participants agreed to adopt, as a working definition of emphysema, "a condition of the lung characterised by increase beyond the normal in the size of the air spaces distal to the terminal bronchiole either from dilatation or from destruction of their walls". This anatomical rather than clinical diagnosis, while being somewhat difficult to confirm during life, nevertheless led to a useful distinction from the often associated chronic bronchitis, both as regards aetiology and treatment. However, this definition

did not distinguish between overinflation such as is seen in acute asthma, the dilatation of the peripheral airspaces seen in the ageing lung, or the destruction of the lung architecture which occurs in smoking-related emphysema.

Since then the agreed definition has been modified on two occasions to emphasise the destructive process in emphysema (American Thoracic Society, 1962; Snider et al., 1985). The most recent definition states that emphysema is “A condition of the lung characterised by abnormal, permanent enlargement of the air spaces distal to the terminal bronchiole, accompanied by destruction of their walls and without obvious fibrosis” (Snider et al., 1985). The later definitions exclude many conditions which were originally included under the original definition of emphysema (Reid, 1967) and they require a tighter definition of lung normality. For example, senile lungs are characterised by a homogeneous enlargement of the alveolar airspace with some functional characteristics of emphysematous lungs (Verbeken et al., 1992), but emphysema according, to the later definitions, can only be said to be present where destruction of alveolar walls can be demonstrated.

Although tightening the definition in this way has helped promote research and understanding as to the causes of emphysema, it has brought with it additional problems in demonstrating the presence of emphysema. At present, the diagnosis of emphysema, based on the most recent definition (Snider et al., 1985), relies on the interpretation of structural changes in lung tissue. In practice, this is only available on surgically resected material, such as lung removed prior to lung transplantation, and on lungs studied at postmortem. In living subjects, chest X-rays only show a moderate correlation with macroscopic emphysema (Thurlbeck and Simon, 1978). However computed tomography (CT) scan methods correlate well with quantitative histologic assessment (Gould et al., 1988; MacNee et al., 1991) even within the range of those showing normal age change or early emphysema. Despite this, correlation of CT data with clinical assessment is poor, although it does have a better correlation with the results of pulmonary

function tests (Gould et al., 1988; MacNee et al., 1991; Heremans et al., 1992; Hruban et al., 1987).

The early stages of emphysema are virtually impossible to detect clinically and currently detection at this level can only be effectively achieved at the microscopic level. This is because normal alveolar air spaces are approximately 250 μm in diameter and are thus not visible to the naked eye. For emphysema airspaces to be visible to the naked eye, they must be greater than approximately 1 mm in diameter, at which stage three-quarters of the alveolar surface is destroyed (Weibel, 1963; Schreider and Raabe, 1957). At present, high resolution computed tomography (CT) scans are unable to detect the early stages of emphysema so that improved CT or other means will need to be developed to allow early detection of emphysema.

Emphysema is subclassified according to the distribution of abnormal airspaces within the acinus or the respiratory tissue airspaces at a single terminal bronchiole (Ciba Guest Symposium, 1959). For example, centriacinar emphysema begins at the level of respiratory bronchioles and is seen in the form of focal emphysema associated with heavy exposure to coal dust and in the more generalised centrilobular emphysema, which is the most common form seen in smokers. Panacinar emphysema involves all the acinus and is the usual pattern that occurs in homozygous α_1 -PI deficiency and in smokers (Thurlbeck, 1988). The varying distribution of the lesions in emphysema is not only dependent on the causative agent but suggests that they may differ in pathogenesis.

Emphysema may occur with or without airways obstruction. The relative contributions of emphysema and small airways abnormalities in fixed airflow obstruction in COPD remain controversial (Snider, 1992; Wright, 1992). While a number of studies have concluded that emphysema is the most important lesion causing expiratory airflow limitation, an almost equal number concluded that it did not. The most recent study concluded that the cause of the lesions responsible for small airway obstruction still needs to be identified (Gelb et al., 1996).

The destruction of the lung architecture that occurs in emphysema includes elastic fibre damage, an observation that was noted as early as 1907 (Orsos). This results in a loss of lung elasticity and elastic driving force, as reflected in reduced elastic recoil pressure measurements (Silvers et al., 1980; Petty et al., 1987) leading to expiratory airway collapse and chronic airways obstruction (Wilson et al., 1980). The loss of lung elasticity reflects the shift in the lung's pressure volume curve that is best described by an index of lung distensibility, K , an exponential function that describes the shape of the lungs' pressure-volume curve. Colebatch et al. (1979a, 1979b) originally demonstrated in patients with a clinical diagnosis of emphysema that values of K consistently fall well outside the age-related normal range. Later studies have demonstrated that K is a better predictor of emphysema than other measures of elastic recoil (e.g. Pare et al., 1982) but that it has poor sensitivity for detecting mild disease. This loss of lung elasticity together with the loss of alveolar capillaries as part of the destructive process in emphysema results in a mismatch of ventilation and perfusion and a widened arterial-alveolar difference in oxygen tension.

In conclusion, the more recent definitions of emphysema that include the destructive component of this disease are preferable, as they emphasise the damage to lung tissue that results in a loss of lung elasticity. As emphysema is difficult to detect in its early stages it is included for clinical convenience under COPD, even though emphysema may prove not to have an obstructive component.

1.1.2 PREVALENCE

Figures for incidence and prevalence of emphysema are very unreliable because, as mentioned above, it has been difficult to agree on criteria by which to make the diagnosis during life. Since the diagnosis is defined in anatomical rather than physiological terms, the best data come from post mortem surveys, where it has been found that most adult lungs contain some degree of

emphysema with the incidence increasing in the fifth to seventh decade (Thurlbeck et al., 1974). Approximately 2/3 of adult males and 1/4 of adult females will have well-defined emphysema at autopsy, although the emphysema is often limited in extent (Ingram, 1994). Therefore, it is likely that almost half of all living elderly adults have well-defined emphysema, with the remainder having some degree of emphysema with, in most cases, no apparent disability or symptoms.

The measurement of lung function with FEV₁ (forced expiratory volume in 1 sec.) has been shown to be a strong predictor of mortality from COPD (Peto et al., 1983). In a large prospective community health study (Higgins et al., 1982) airway obstruction was defined as an FEV₁/FVC (forced vital capacity) ratio < 65%. In this study airway obstruction was found in 16.3% of males aged 45-54 years. An unknown proportion of this community would be expected to have emphysema, but no figures are available in this study for the incidence of clinically significant emphysema.

The death rate attributed to COPD in different countries can vary by as much as four-fold from the lowest in Southern European countries to the highest in the United Kingdom and Eastern European countries, with the U.S.A. lying in the middle (Lamb, 1996). The incidence of emphysema varies from that of the COPD death rates, for which the lowest rates were reported in Japan and New Guinea and the highest rates were reported in the United Kingdom and the U.S.A. (Lamb, 1996). The cause of these international variations is unclear but may result in part from differences in diagnostic accuracy, as discussed in a recent review by Lamb (1996). While tobacco smoking is a major cause of COPD, international differences associated with atmospheric pollution and regions of heavy industry probably also play a role.

Table 1.1: SUMMARY OF RISK FACTORS FOR COPD
 (Adapted from a review by Silverman and Speizer (1996). Other references listed below.)

RISK FACTOR	COMMENT
Cigarette smoking	The major risk factor responsible for an estimated 95% of emphysema cases (Snider, 1986). Only 10-20% of tobacco smokers develop disease (Tetley, 1993). Other factors (Bascom, 1991; MacKenzie et al., 1994)?
Airway responsiveness	Significant correlation with decline in pulmonary function (Thurlbeck, 1995). Increased incidence in α_1 -PI deficiency (Colp et al, 1993; Eden et al, 1997). Possible common mechanism to asthma ?
Respiratory infections	Significant correlation with childhood acute respiratory infections but less so in adults (Barker et al., 1991; Johnston et al., 1998; Shaheen et al., 1998; Berglund et al., 1999)
Occupational exposures	Increased risk shown with coal and gold mining, mineral dust, cadmium fumes, and wood dust (Noertjojo et al., 1996; Hendrick, 1996). Appears additive to smoking effect.
Ambient air pollution	Increased risk in urban compared to country environments. No clear link to any specific pollutant except, perhaps, domestic wood smoke (Chen et al., 1990; Pérez-Padilla et al., 1996).
Passive smoking exposure	Increased risk in children and <i>in utero</i> for reduced pulmonary function. Uncertain in adults.
α_1-PI deficiency	Definite risk with PI types ZZ, Znull, and null-null, particularly in smokers (Seersholm et al., 1995; Piitulainen and Eriksen, 1999). Some evidence for risk with MZ and SZ (Tarjan et al., 1994; Turino et al., 1996). Variable expression – other factors (Poller et al, 1990a)? Accounts for about 1% of all emphysema (Tetley, 1993).
Other genetic factors	Association with α_1 -ACH deficiency (Eriksson et al., 1986; Lindmark et al., 1990; Poller et al., 1993). Possible association with ABO, secretor and Lewis blood group antigens (Kauffmann et al., 1996). Other inherited factors as yet unidentified.
Age	Increased risk due to normal loss of pulmonary function (Verbeken et al., 1992). Cumulative effect with smoking.

1.2 EPIDEMIOLOGY OF COPD AND EMPHYSEMA

A comprehensive list of the risk factors associated with COPD, of which emphysema is one component, is shown in Table 1.1. It can be seen from this summary, that cigarette smoking is clearly the most important in not only being responsible for the majority of COPD, but that its effect appears to be additive to many of the other risk factors. Longitudinal studies have shown an accelerated decline in FEV₁, in a dose-response relationship to cigarette smoking, associated with both the duration of smoking and the amount smoked (Burrows et al., 1977; Dockery et al., 1988). However, cigarette smokers show wide variation in their susceptibility to develop progressive COPD, with only 10-20% of tobacco smokers developing clinically evident emphysema (Bascom, 1991; Tetley, 1993). Of the factors evaluated, the pack-years of cigarette smoking were the most highly significant predictor, with a correlation coefficient of 0.38. This low correlation suggests that additional environmental factors and familial or genetic factors may contribute to the impact of smoking on the development of airflow obstruction (Pryde and Burrows, 1995). The additional risk factors that can be identified, such as occupation and environment, chest infections and α_1 -PI phenotype, do not explain the individual susceptibility of some smokers to develop COPD (Snider, 1986).

Of the genetic factors, there is the possibility that some forms of α_1 -ACH deficiency, the possession of some blood group antigen combinations and other as yet unidentified familial components, may contribute to the risk of COPD and emphysema. Nevertheless no additional clinically proven genetic factors other than α_1 -PI have, as yet, been described. In most of the studies of α_1 -PI deficiency, smoking is the overriding risk factor in that it increases the incidence of COPD over and above that due to the effect of the particular α_1 -PI phenotype. Thus cigarette smoke appears to be able to tilt the balance of the protease-antiproteases towards lung injury particularly where antiprotease function is compromised such as in α_1 -PI deficiency.

In conclusion, while the overwhelming majority of emphysema is due to smoking, it is not a simple relationship as demonstrated by the fact that not all individuals who smoke develop clinically evident emphysema. A much better understanding of the mechanisms of how emphysema develops in susceptible smokers in comparison to non-susceptible individuals is required, together with the discovery of the interplay of other, perhaps unknown, risk factors in each individual.

1.3 AETIOLOGY OF EMPHYSEMA

1.3.1 EARLY STUDIES OF EXPERIMENTAL EMPHYSEMA

There were two main theories regarding the development of emphysema during the 19th and early 20th centuries: the mechanical and the ischaemic (or atrophic) theories. The mechanical theory reasoned that upper lobe alveolar destruction and dilatation were caused by repeated coughing against obstructed airways, which trapped air distally under pressure, stretching and rupturing the alveoli (Laennec, 1835; Mendelssohn, 1845). Experimental work in animals with mechanical or surgical narrowing of the upper airways or ball valve implantation into the bronchi led to the development of alveolar distension distal to the obstruction (Loeb LM, 1930). However, it was concluded that the animals did not develop the appropriate mechanical or histological features of emphysema and that large airway obstruction alone was not sufficient to cause emphysema.

The ischaemic theory proposed that thrombosis of alveolar capillaries traversing dilated air sacs resulted in impaired nutrition and ischaemic necrosis of the alveolar septa (Waters, 1862). Cigarette smoking was thought to cause *in situ* thrombosis of these capillaries. To test this theory, glass microspheres were used to occlude small lung blood vessels in an animal model study but the resultant ischaemia failed to produce emphysema (Wright and Klierman, 1963).

In another study, chlorpromazine infusion into the bronchial arteries of horses was reported to be able to produce an emphysema-like lesion (Edwards, 1961). However, the development of emphysema was preceded by a severe arteritis of the bronchial arteries, which is not seen in natural human panlobular emphysema.

In another study a third mechanism was proposed, whereby the emphysema was caused by an immunological response to lung tissue damaged by some unknown agent. In this study, homogenised guinea pig lung tissue, which had been damaged by NO₂, was injected together with adjuvant, back into guinea pigs. However, this caused an interstitial pneumonitis to develop rather than true emphysema (Crowle, 1959).

In conclusion, none of the above theories is now in vogue, as they do not adequately explain the development of human emphysema. The most likely mechanism proposed that best describes the development of emphysema is provided by the protease-antiprotease hypothesis. The evidence for this conclusion is set out in Sections 1.4 and 1.5.

1.3.2 CADMIUM AND EMPHYSEMA

Cadmium is one of the few occupational agents where the development of COPD appears to be due solely to emphysema. While early reports were controversial due to conflicting data, a definitive study in 1988 (Davison et al.) of 101 workers and ex-workers from a cadmium alloy factory in England clearly linked cadmium fumes to the development of emphysema. However, the mechanisms involved in the development of emphysema due to the presence of cadmium are unclear. Animal studies demonstrate airspace enlargement with fibrosis after intratracheally instilled cadmium chloride (Thurlbeck, 1963) and emphysema-like lesions after multiple aerosol treatment with cadmium chloride (Snider et al., 1973). However, the lesions due to cadmium are not typical of those that are induced by elastolytic enzymes in animal studies as

suggested by the finding that elastin was not destroyed in a study with cadmium chloride using radiolabelled neonatal lung elastin in hamsters (Snider et al., 1988).

HLE, the major protease secreted by neutrophils, is now generally considered to be the elastolytic enzyme implicated in pulmonary emphysema (Janoff, 1985; Travis and Salvesen, 1983). However, HLE does not appear to be involved in cadmium-induced lung disease, as demonstrated by a study where hamsters that had been made neutropenic with anti-neutrophil globulin were still able to develop cadmium chloride-induced airspace enlargement (Hoidal et al., 1985).

Cigarettes contain significant amounts of cadmium and as a consequence smokers have been shown to have higher cadmium levels than non-smokers in their lungs and kidneys (Hirst et al., 1973). The authors report that cadmium concentration in the emphysematous lungs of smokers was in direct proportion to the severity of the emphysema. In a later study, Paakko et al. (1989) reported that the cadmium concentration in lung tissue of smokers was in direct proportion to the amount of tobacco smoked and that pulmonary cadmium levels were seen to return to the level of non-smokers in 21-22 years after cessation of smoking. While the presence of cadmium in cigarettes may not produce emphysema on its own, it may contribute to the smoking-induced disease by causing airspace enlargement.

1.3.3 EXPERIMENTAL CIGARETTE SMOKE EMPHYSEMA

As the incidence of emphysema is highest in smokers there have been a number of studies to develop an animal model of smoking-induced emphysema. However, attempts to cause emphysema in animals by prolonged exposure to cigarette smoke have met with mixed success, often producing only goblet cell metaplasia and bronchitis in early studies (Park et al., 1977; Holland et al, 1963). Some of the strongest evidence that laboratory animals develop emphysema is found in a series of studies using beagle dogs (Auerbach et al., 1967; Hammond

et al., 1970; Frasca et al., 1971; Carter et al., 1976). In one study, dogs smoked through tracheostomies for periods of over a year and developed what was thought to be emphysema, but the distribution of the lesions seen was not typical of panlobular emphysema (Auerbach et al., 1967). In a later study, also with tracheostomized dogs, air space enlargement and alveolar wall fibrosis were induced by exposure to the smoke from 2-7 cigarettes per day for 2-4 months (Frasca et al., 1983). Whilst the lungs of these dogs showed significant emphysematous changes, the data were not conclusive because of inadequate controls.

In a carefully controlled study of smoking in rats, both morphometric and pulmonary function tests indicated changes consistent with the development of emphysema in the smoke-exposed animals. However, the period of study was not long enough (6 months) to allow the emphysema-like changes to develop sufficiently so as to be significantly different from the non-smoking controls (Huber et al., 1981). One report has shown the development of significant emphysematous lesions in rats but these developed only after exposure of the rats for more than 12 months to tobacco smoke at the relatively high rate of 10 cigarettes a day, which resulted in a high mortality rate (Heckman and Dalbey, 1982).

More recent studies using the rat, guinea pig or mouse as an animal model of smoking-induced emphysema have given more promising results. When rats were exposed to cigarette smoke for 6 months in one study, there was evidence of emphysema lesions as early as 2 months based on morphometric data from histological sections of the rat lungs (Ofulue and Abboud, 1998). In this last study, the destructive component of emphysema in the smoke-exposed rats was demonstrated by the detection of lung elastin degradation, as measured by the presence of increased levels of elastin breakdown products (elastin derived peptides and desmosine). With the guinea pig as the animal model, long term exposure to cigarette smoke at the rate of 10 cigarettes a day for up to 12 months, has been reported to produce airflow obstruction associated with emphysematous air space enlargement similar to that seen in humans (Wright and Churg, 1990; Wright and Sun, 1994). Mice develop similar pathologic changes to human

emphysema after being exposed to two cigarettes a day for 6 months (Shapiro, 1995; Hautamaki et al., 1997).

In summary, it has yet to be conclusively shown that any laboratory animal has developed emphysema, as it occurs in humans, solely in response to cigarette smoke exposure. The failure of tobacco smoke definitively to produce all the characteristics of human emphysema in animal models is probably due to a combination of species difference and the short duration of the studies in comparison to smoking in humans, where it is usually a lifetime habit. For example, the mouse does not have mucus glands in the larger airways and lacks respiratory bronchioles. Besides species differences, individual susceptibility is also a factor, as demonstrated by the fact that only 10-20% of human smokers develop emphysema.

1.3.4 CONNECTIVE TISSUE DEFECTS

The connective tissue in lung has a high content of elastin in numerous elastic fibres that give the lung the elastic properties it requires for expansion and contraction functions, as part of each respiratory cycle. The stronger collagen fibres present are thought to function as the restraining framework providing overall structural stability to the organ. The extensive alterations in the lung's connective tissue framework, that are seen as air space enlargement and loss of elasticity in emphysema, are generally thought to be destructive and form one of the requirements of the more recent definitions of emphysema (See section 1.1.1). However, studies of the connective tissue framework in human emphysematous lungs have provided little information as to the specific nature of the defect. It is still not clear whether there are any changes in the whole lung content of elastin and collagen (Pierce et al., 1961; Chrzanowski et al., 1980). Although defects have been identified in the elastic fibre network of emphysematous lungs, the abnormalities appear to be localised and their impact on the overall function of the intact lung is not clear (Wright, 1961; Niewoehner and Kleinerman; 1977).

Emphysema-like lung abnormalities have been reported to be associated with some rare heritable defects in connective tissue synthesis, as reported in the Marfan syndrome (Dwyer and Troncale, 1965) and in certain extreme recessive forms of 'cutis laxa' (Turner-Stokes et al., 1983). In the case of the Marfan syndrome, it is not clearly established that the occasional bullous emphysema found in this syndrome is a consequence of the disease or some other causes (Streeten et al., 1987). Cutis laxa describes a heterogenous group of clinical and genetic conditions where pulmonary emphysema is not always found (Rybojad et al., 1999). Because of the rarity of these conditions and their diverse presentation, these conditions have provided little information on the connective tissue defects in emphysema.

Experimental animal model studies suggest that extensive tissue remodelling occurs in emphysema induced by intra-tracheal instillation of elastases, after which, rates of connective tissue degradation may be matched by synthesis. For example, exposure of the hamster lung to elastase causes a rapid loss of elastin, but collagen and elastin contents then rapidly increase to levels above baseline, during which time there is continuing airspace enlargement (Kuhn et al., 1976). Thus changes in the structural and spatial arrangements of the connective tissue components in the emphysematous lung may be more relevant than changes in the total amount of the constituents present.

The key role of elastin in maintaining the integrity of the connective tissue in the lung can be inferred from experimental animal studies. For example, blotchy mice have genetically impaired copper transport that affects lysyl oxidase, the copper dependent enzyme responsible for the lysine derived cross-linking of collagen and elastin. These mice develop progressive emphysema (Fisk and Kuhn, 1976). The tight skin mouse has a similar phenotype and has also been used in studies of inherited emphysema (Szapiel et al., 1981). Similar defects can be induced in young animals by using copper deficient diets or by a diet containing β -aminopropionitrile, an inhibitor of lysyl oxidase (Kida and Thurlbeck, 1980; O'Dell et al., 1978). However, no significant effects are apparent in normal, mature animals, given copper

deficient diets or a diet containing β -aminopropionitrile, presumably because of the low turnover rates of lung elastin and collagen in mature animals. Other agents that induce emphysema in animal models, if given with these types of diets, can induce the development of emphysema lesions of far greater severity than those produced in animals on a normal diet. Examples include the intra-tracheal installation of porcine pancreatic elastase (Kuhn and Starcher, 1980) and dietary cadmium (Niewoehner and Hoidal, 1982) when given together with β -aminopropionitrile in the diet. These animal model studies demonstrate that impaired synthesis and cross linking of elastin can lead to emphysema. There is also some evidence that cigarette smoke impairs synthesis of lung collagen and elastin under certain conditions (Laurent et al., 1983; Osman et al., 1985). Whether similar changes occur in human smokers during a lifetime of exposure to the components of tobacco smoke such as cadmium is not known.

1.3.5 OXIDANTS

Nitrogen dioxide (NO_2) is a product of most operations requiring combustion such as motor vehicle emissions (Fishbein, 1993) and is a major component of indoor pollution involving tobacco smoking and the burning of indoor cooking and heating fuels, such as natural gas and kerosene (Samet et al., 1987; Leaderer, 1986). While there are no consistent data linking NO_2 exposure to the reduction of pulmonary function in humans, emphysema-like injuries with destruction of alveoli similar to human centriacinar emphysema have been observed in a number of animal models after chronic long-term exposure to NO_2 (Freeman et al., 1972; Juhos et al., 1980; Freeman and Haydon, 1964; Glasgow et al., 1987; Chitano et al., 1995). A decrease in lung elastin and collagen has been shown in male hamsters, within 4 and 10 days respectively, after exposure to 30 ppm of NO_2 with elastin not returning to control levels until after NO_2 was stopped (Kleinerman and Ip, 1979). During NO_2 exposure, neutrophils accumulate rapidly in the lungs where there is some evidence that elastase is released as they migrate through the lung (Glasgow et al., 1987).

Airspace enlargement has also been shown with exposure of rats to hyperoxia (Riley et al., 1980) and in hamsters exposed to ozone (Lucey et al., 1990) but emphysema-like injuries have not been described. A recent review (Chitano et al., 1995), of studies of the chronic long-term exposure to ozone in experimental animals, has found that the animals mainly develop pulmonary fibrosis. One study has reported the induction of emphysematous-like lesions in dogs by using chloramine-T, a chemical oxidant that can inactivate α_1 -PI (Abrams et al., 1981).

Besides the removal of the source of the oxidants, the prevention of oxidant inactivation to the lung could theoretically be prevented or reduced by giving a drug that has antioxidant properties. However, little work has been undertaken with antioxidants, possibly because the evidence for the mechanisms involved remains controversial (Snider et al., 1992). One study using supplementation of the antioxidants, α -tocopherol and β -carotene, did not improve symptoms in patients with COPD (Rautalahti et al, 1997).

The increased risk of COPD in individuals exposed to increased NO_2 , such as in tobacco smoke, wood smoke and industrial pollution (see Table 1.1), is consistent with animal model studies mentioned in this section. Most of the evidence from animal studies supports a similar process to that occurring in human emphysema such that NO_2 induced emphysema has been used as an animal model to study the disease process of emphysema (Snider et al., 1986). The evidence indicates that exposure to increased NO_2 levels could be an important factor in the development of emphysema in humans.

1.4 THE PROTEASE-ANTIPROTEASE HYPOTHESIS IN ANIMAL MODELS

1.4.1 ENZYME INDUCED EMPHYSEMA

The association of low levels of the antiprotease α_1 -PI with emphysema, as found in the homozygous form of α_1 -PI deficiency, led to the proposal that unopposed proteolysis by enzymes released from neutrophils and macrophages was responsible for the development of emphysema. This proteolytic imbalance became known as the protease-antiprotease hypothesis. These observations led to a search for a responsible *in vivo* enzyme, although at that time no mechanism of enzymic digestion of the lung structure had been shown.

A new direction of investigation into the pathogenesis of emphysema was opened up when an experimental model imitating natural emphysema was created, almost accidentally, by Gross (Gross et al., 1964; Gross et al., 1965). He had been attempting to ameliorate the fibrotic effects of inhaled quartz dust by intra-tracheal instillation of the plant enzyme papain, and was surprised instead to note the development of a patchy airspace lesion resembling emphysema. Subsequent investigations of enzyme instillation into the lungs of animal models demonstrated that only elastolytic enzymes can induce experimental emphysema. The elastolytic enzymes obtained from cells that have access to the lung and that can cause experimental emphysema include HLE (discussed below) and more recently, another leukocyte enzyme, proteinase 3 and a macrophage enzyme, cathepsin B. Proteinase 3 (Kao et al., 1988) and cathepsin B (Lesser et al., 1992) have been shown to produce emphysema in hamsters following intratracheal installation. However, trypsin, an enzyme with no elastolytic activity, has also been shown to cause emphysema in rats after a single dose, whether given intravenously, intraperitoneally or endotracheally (Reichart et al., 1992). An explanation for this apparent contradiction, as suggested by the authors, is that the observed pulmonary leucostasis found in these rats may lead to the release of neutrophil products in the lung. These products, which include elastase,

could damage the lung in the presence of depleted antiproteases resulting from the trypsin overload.

Although early findings showed a correlation between *in vitro* elastolytic activity and *in vivo* emphysema-inducing ability (Blackwood et al., 1973; Snider et al., 1974), there are exceptions. One exception is HLE, which causes considerably less severe lesions in the lung than those induced by pancreatic elastase with comparable *in vitro* elastolytic activity. This difference is thought to be due to differences in susceptibility of various lung tissues to the two enzymes and accessibility of the two enzymes to the elastin in the lung tissue as compared to the unimpeded access of the elastases to purified elastin used *in vitro*. An example is the presence in lung tissue of sulphated proteoglycans, which are able to bind to HLE and inhibit HLE activity towards elastin (Lucey et al., 1991). On the other hand, pancreatic elastase is not inhibited by the presence of sulphated proteoglycans and thus is able to maintain its elastolytic activity *in vivo*.

1.4.2 LEUKOCYTE ELASTASE INDUCED EMPHYSEMA

Of all the elastolytic enzymes, HLE has been the most studied and is now generally considered to be the major destructive enzyme implicated in the development of pulmonary emphysema (Janoff, 1985; Travis and Salvesen, 1983). The initial evidence for HLE involvement was suggested by the development of emphysema in some individuals with an inherited deficiency of α_1 -PI, the major serum protease inhibitor of this enzyme. HLE has a broad substrate specificity, which includes the capability to degrade a wide range of extracellular matrix proteins as well as a number of serum proteins (Bieth, 1986; Hubbard et al., 1991b). Thus, HLE activity could account for much of the tissue damage observed in emphysema.

The instillation of HLE into animal lungs has been shown to cause destructive lung disease (Janoff et al., 1977; Senior et al., 1977; Snider et al., 1984). Initially, there is a reduction of lung elastin content at 24 h. followed by a rapid restoration of total lung elastin to normal levels but

with the anatomic arrangement of the new elastic fibres now being grossly disordered (Karlinsky et al., 1983). In elastase-induced emphysema there is also evidence of damage and subsequent repair to the collagen network in the lungs of experimental animals (Kuhn et al., 1976). The result is a picture of permanently increased airspace size and lung volumes, which is typical of human pulmonary emphysema.

1.4.3 EMPHYSEMA INDUCED BY PULMONARY NEUTROPHILIA

Emphysema has been produced both in rhesus monkeys (Wittels et al., 1974) and in dogs in response to repetitive intravenous injections of endotoxin, which caused sequestration of polymorphs in the lung vascular bed (Guenter et al., 1981). Similar observations were made in hamsters, in which the endotoxin was given as multiple intratracheal installations (Rudolphus et al., 1993). Repeated intravenous injections of endotoxin administered to rats over a 10 week period caused emphysema in rats made α_1 -PI deficient by the administration of D-galactosamine (Blackwood et al., 1984). Treatment with endotoxin alone produced milder changes in the rat lungs. In all these experiments it is postulated that the emphysema is induced by the release of enzymes from sequestered neutrophils; however, it has not been proven that elastin destruction occurred in these models.

1.4.4 α_1 -PROTEASE INHIBITOR (α_1 -PI) DEFICIENCY

The only animal models that have been reported to spontaneously develop air space enlargement reminiscent of human emphysema are some strains of mice (pallid, tight-skin, blotchy and beige). Of these strains, only the pallid mouse has been reported to have reduced levels of serum α_1 -PI with spontaneous emphysema occurring late in life, similar to that seen in the inherited α_1 -PI deficiency form of human emphysema. Histological examination of pallid mouse lungs has demonstrated some patchy areas of air-space enlargement with destruction of

alveolar septa, a decrease in lung elastin content and the immunological detection of an increased level of elastase bound to alveoli (Martorana et al., 1993). However some differences have been reported between the form of emphysema that is seen in the pallid mouse when compared to human form. For example, there is no increase in neutrophils in BAL (bronchoalveolar lavage) fluid (de Santi et al., 1995) as is seen in non-smoking individuals with the PI Z form of α_1 -PI deficiency (Gadek et al., 1981a; Hubbard et al., 1991c). Another difference is that, unlike humans, there does not appear to be any significant expression of α_1 -PI in the macrophages of mice (Tardiff and Krauter, 1998). Nevertheless, the histological changes reported in pallid mouse are consistent with those found in the inherited form of human emphysema and may provide new insights into the protease-antiprotease hypothesis for the development of the inherited form of human emphysema where there is an increase in protease (elastase) activity due to insufficient antiprotease (α_1 -PI).

1.5 THE PROTEASE-ANTIPROTEASE HYPOTHESIS IN HUMAN STUDIES

The protease-antiprotease or elastase-antielastase hypothesis for the pathogenesis of emphysema arose out of the original report in 1963 by Laurell and Erikssen of an association between emphysema and inherited deficiency of α_1 -antitrypsin (or α_1 -PI in this thesis). Much of the subsequent research on the pathogenesis of emphysema is based on the hypothesis that emphysema results from an imbalance in the protease-antiprotease mechanism which leads to uninhibited activity of enzymes that can hydrolyse elastin in the lung.

Other than the association of a severe form of emphysema in some individuals with rare congenital deficiencies of α_1 -PI, most of the initial support for this theory has come from intrapulmonary instillation of proteases, in particular HLE, into experimental animals leading to anatomic derangements in lung architecture which resemble human emphysema. However,

validation of this hypothesis can only ultimately come from studies of the mechanisms leading to the development of the human form of emphysema with *in vitro* and, if possible, human population studies. In this section, the evidence for an imbalance in the various proteases and antiproteases in the development of human emphysema is examined. As the protease - antiprotease imbalance is thought to occur primarily in the lung, the cells in the lung that are capable of releasing proteases, the individual proteases and the antiproteases present in the lung are examined, to establish from current literature, their normal function and possible roles in the pathogenesis of emphysema.

1.5.1 PULMONARY DEFENCE MECHANISMS

The respiratory epithelial surface is continuously exposed to microorganisms, particulates, gases, fumes and aerosols as a result of processing 6-10,000 litres of air daily in an adult. Tobacco and marijuana smoking result in an additional burden of an aerosol containing gas and tar particles. A variety of mechanical, chemical and cellular mechanisms are used to protect the lung surface. During the host defence response, inflammatory cells are attracted to the lung to facilitate the clearance of foreign agents (Harada and Repine, 1985). The three major mediators produced by inflammatory cells are oxygen metabolites, proteases and cationic proteins. The protease component forms a significant part of the defence strategy of inflammatory cells in removing foreign material. However, in excess it can also destroy the extracellular frameworks of the lung, disrupt resident cells, and stimulate further inflammation (Harada and Repine, 1985; Henson and Johnston, 1987). The protease activity is normally tightly controlled by inhibitors so that, if tissue damage has occurred, repair occurs rapidly and little if any permanent damage is seen, as for example, following pneumococcal pneumonia (Haslett, 1992). However, inadequate inhibition or control of protease activity can result in the generation of more inflammatory mediators with continued inflammation and impeded repair of tissue damage.

1.5.2 INFLAMMATORY CELLS OF THE LUNG

1.5.2.1 LEUKOCYTES

All circulating neutrophils pass through the pulmonary capillaries many times each hour and may enter the lungs in large numbers in response to chemotactic gradients (Baggiolini et al., 1992; Oppenheim et al., 1991). These cells have a short half-life of about one to two days after leaving the bone marrow (Bainton, 1992) and are removed by apoptosis, a regulated process which prevents dispersion of harmful cellular components, including proteases, into the surrounding tissue (Haslett, 1992; Haslett, 1997). The rate of apoptosis of neutrophils has been proposed to be regulated by the high concentrations of proteases, such as HLE, at inflammatory sites (Trevani et al., 1996). In the lung, neutrophils are normally present in small numbers in the alveoli, small airways, and pulmonary parenchyma and form only a small percentage of the phagocytic migratory cells in the healthy lung. However, during acute inflammatory stimuli large numbers of these cells rapidly accumulate (Wright et al., 1988). Neutrophils are also found to be increased in lung lavage samples from certain pulmonary inflammatory diseases, such as some acute forms of the adult respiratory distress syndrome (70-95% increase; Mcguire et al., 1982; Lee et al., 1981; Weiland et al., 1986; Idell et al., 1985; Fowler et al., 1982) and cystic fibrosis (50-80% increase; Davis et al., 1983; Fick et al., 1983). The presence of increased numbers of neutrophils should also be obvious in the inherited form of emphysema, if the neutrophil and its proteases are seen to play a role in the development of this long-term chronic lung disease. In non-smoking individuals with the PI Z form of α_1 -PI deficiency, more neutrophils were found in the lower respiratory tract than in normals (Gadek et al., 1981a; Hubbard et al., 1991c). The authors also refer to unpublished data suggesting that individuals with emphysema have a higher number of neutrophils than those free of the disease. They suggest that the protease-antiprotease imbalance in PI Z subjects, where α_1 -PI levels are low, is further exaggerated by an increased HLE burden from the increased numbers of neutrophils.

While the protease burden in the lung is increased with an influx of neutrophils, neutrophil enzymes other than proteases can also contribute to a protease imbalance. An example is myeloperoxidase, whose concentration in the neutrophil is at least twofold higher than that of the serine proteases. Myeloperoxidase, in the presence of H_2O_2 and Cl^- , can produce potent oxidising agents, which have the ability to inactivate α_1 -PI and secretory leukoprotease inhibitor (SLPI), resulting in a potential protease-antiprotease imbalance. The oxidants produced by myeloperoxidase are normally regulated by the presence of antioxidants such as superoxide dismutase, glutathione, and catalase thus protecting the lung against both possible proteolytic and oxidant injury (Snider, 1989).

It has long been recognised that in smokers significantly increased numbers of circulating neutrophils are attracted to the lung to facilitate the clearance of irritants in the cigarette smoke (Cone et al., 1971). While in the lungs of smokers there can be an approximately 10 fold increase of absolute neutrophil numbers, the percentage of neutrophils remains unchanged relative to an overall increase in the total number of cells recovered in bronchoalveolar fluid (McGowan et al., 1983; Hunninghake and Crystal, 1983; Hoidal and Niewoehner, 1983a). Thus, in smokers, the lung has not only an increased burden of neutrophils with the accompanying inflammatory products but also a much larger content of another inflammatory cell, the macrophage, as discussed in the following section.

1.5.2.2 ALVEOLAR MACROPHAGES

The most prominent phagocytic cell in normal lung is the alveolar macrophage (almost 90% of total phagocytes), a local cell responsible for the normal function of removing foreign material deposited in the peripheral lung. In contrast to neutrophils, macrophages are long lived; their half-life varies from several days to weeks, depending on the tissue involved. Within the lung, they live about 10 days (Van Furth, 1992). Macrophages, like neutrophils, are attracted to sites of inflammation under the influence of locally produced chemotactic factors, some of which are

selective for macrophages (Baggiolini et al., 1992; Oppenheim et al., 1991). Alveolar macrophages are capable, when activated by surface stimuli, of releasing chemotactic mediators which are likely to account for the increase in neutrophils at the site of inflammation (Baggiolini et al., 1992; Oppenheim et al., 1991). Macrophages also may regulate unwanted neutrophil enzyme activity during resolution of inflammation via apoptosis (Haslett, 1992; Savill et al., 1992). Besides chemotactic factors, macrophages also secrete a neutrophil degranulating agent and can ingest, and later release, HLE.

Cigarette smoking is associated with an approximately 5 to 10 fold increase in cells recovered by BAL. Of these cells, macrophages comprise over 98 %, while leukocytes represent only 1% (Merchant et al., 1992). Macrophages are also prominent in the respiratory bronchioles of cigarette smokers where emphysematous changes are first apparent (Niewoehner et al., 1974). Whilst macrophages appear to be involved in the control of the number of neutrophils in the lung, the extent of their direct involvement in the degradation of connective tissue, including elastin, remains unknown.

1.5.3 PROTEASES

1.5.3.1 LEUKOCYTE PROTEASES

The azurophil (or primary) and specific (or secondary) granules of neutrophils contain compounds responsible for the hydrolytic, proteolytic and oxidative breakdown of phagocytosed material. HLE is the major protease stored in the azurophil granules and can potentially be secreted in millimolar amounts in response to various inflammatory stimuli (Janoff, 1985, Campbell, 1986). HLE is a neutral serine proteinase with a broad substrate specificity which includes the capability of degrading a wide range of extracellular matrix proteins of the lung including elastin, collagen, proteoglycan, fibronectin, and laminin (Hubbard et al., 1991b) as well as lung surfactant (Pison et al., 1989, Lewis et al., 1992), a number of serum proteins such as fibrinolytic and coagulation factors, complement, and immunoglobulins

G and M (Hubbard et al., 1991b). Thus uncontrolled HLE activity on its own could account for much of the tissue damage observed in emphysema and in other acute and chronic inflammatory processes (Sanborg and Smolen, 1988; Travis, 1988).

In addition to HLE, azurophil granules also store other neutral serine proteinases (cathepsin G, azurocidin, and proteinase 3) as well as other components such as acid hydrolases, myeloperoxidase, and the defensins. All these components of the azurophil granules are probably released alongside, and work together with, HLE. For example, cathepsin G has been shown to work synergistically with HLE in the degradation of extracellular matrix (Boudier et al., 1991). Like HLE, proteinase 3 (Rao et al., 1991) and, to a lesser degree, cathepsin G (Hubbard et al., 1991b) both have the capacity to degrade extracellular matrix components including elastic fibers. Azurocidin appears to have no enzymatic activity but, like cathepsin G, acts as an antimicrobial agent (Gabay et al., 1989).

Stored within the specific granules of neutrophils are two metalloproteinases; gelatinase B and neutrophil collagenase. Metalloproteinases comprise a family of structurally related zinc dependent enzymes that are able to degrade components of the extracellular matrix (Birkedal-Hansen et al., 1993). These neutrophil proteinases are stored in a proenzyme form, which require activation after secretion. This is usually effected by other neutrophil products such as oxidants generated by stimulated neutrophils (Weiss, 1989). Collagenase can also be activated by plasmin (Murphy and Docherty, 1992) and by limited HLE and cathepsin G activity (Hubbard et al., 1991b). Metalloproteinase activity is specifically inhibited by the tissue inhibitors of metalloproteinases (TIMPS 1 and 2) and by α_2 -macroglobulin, which is found in small amounts in the lung. In combination, activated neutrophil collagenase and gelatinase B can degrade fibronectin and elastin and a wide range of collagens (Murphy and Docherty, 1992). Singularly, however, they are less effective, as each enzyme has a restricted range of extracellular matrix components as substrate. For example, of the metalloproteinases, only gelatinase B can degrade elastin (Senior et al., 1991).

Phagocytosis by neutrophils involves direct recognition or receptor-mediated binding of compounds, particles, or microbes on the neutrophil cell surface, and their uptake into a phagolysosome that has been built by fusion of the intracellular granules with neutrophil cell surface membrane (Sandborg and Smolen, 1988). The phagocytosed compound is then digested in the phagocytic vacuole filled with lysosomal enzymes and oxygen radicals. Neutrophils have also been found to form protected extracellular compartments between their plasma membranes and proteins such as fibrinogen, fibronectin or elastin-coated surfaces. This extracellular compartment is inaccessible to plasma protease inhibitors and allows unrestrained proteolytic activity to occur (Loike et al., 1992). However, neutrophil enzymes and oxygen radicals also appear to escape or be actively exocytosed in regulated amounts to the extracellular space as part of their normal function. For example, the release of extracellular enzymes and oxidants from neutrophils may occur in the following situations:

- In the process of neutrophil migration, to assist the neutrophil's passage through barriers of cells and extracellular matrix in response to chemotactic signals (Glasgow et al., 1987).
- During successful phagocytosis, where the phagolysosome is not completely closed, allowing granule contents to be released (Klebanoff and Clark, 1978).
- After disintegration of the neutrophil following cell death (Goldstein, 1976).
- During so called "frustrated phagocytosis" where the neutrophil is unable to ingest large foreign bodies (e.g. cigarette smoke tars, dust particles, etc.) or kill and destroy foreign organisms (eg., resistant bacteria) (Klebanoff and Clark, 1978).

In emphysema, there is a continual influx of neutrophils into the lungs in response to the inhalation of cigarette smoke and other likely causative agents, such as environmental and occupational dust and smoke particles. This is thought to result in the release of an excess of extracellular neutrophil proteases and oxidants with the most likely mechanism being "frustrated phagocytosis". In the protease-antiprotease hypothesis, the lungs of emphysematous individuals are in a chronic inflammatory state in which the excess extracellular neutrophil

proteases and oxidants cause damage to lung connective tissue leading to the characteristic irreversible changes seen in emphysema.

1.5.3.2 MACROPHAGE PROTEASES

Human mononuclear phagocytes synthesise and secrete a range of proteinases capable of degrading extracellular matrix. However, the type of proteinase that the phagocyte is capable of producing changes on differentiating from a monocyte to a macrophage. Whereas monocytes produce serine proteinases of the same type that are found in neutrophils, macrophages produce mainly metalloproteinases (Shapiro, 1994). Currently nine macrophage metalloproteinases have been identified, including collagenases, stromelysins, gelatinases, matrilysin and macrophage elastase. Like leukocyte metalloproteinases, the macrophage enzyme activity is zinc dependent and is specifically inhibited by the tissue inhibitors of metalloproteinases (TIMPs 1 and 2). Presumably, such a wide range of metalloproteinases is required by the macrophage for complete tissue degradation as discussed further below.

Macrophage proteinases are thought to be involved in certain chronic inflammatory conditions such as rheumatoid diseases (eg. there is increased collagenase activity in rheumatoid synovial fluids; Birkedal-Hansen et al., 1993) and could play a role in the development of other chronic inflammatory diseases such as emphysema. Cathepsin B, D, L and S are synthesised and secreted by macrophages and may also play a role in elastin degradation (Chapman and Stone, 1984). Macrophages may be involved in proteolytic mechanisms of lung injury since they synthesise and secrete enzymes which, when working together, degrade a spectrum of extracellular matrix components similar to that degraded by neutrophil proteinases (Murphy and Docherty, 1992; Shapiro, 1994; Senior et al., 1991; Murphy et al., 1991).

The finding, that several metalloproteinases have elastolytic activity when working together, and the fact that freshly harvested blood monocytes contain the serine proteinases HLE and

cathepsin G (Welgus et al., 1990) suggests that macrophages and monocytes can degrade elastin, and hence contribute to the development of emphysema. It has been found that a subpopulation (20-30%) of circulating monocytes is capable of being recruited rapidly by chemoattractants to sites of inflammation. This subpopulation of monocytes has neutrophil-like pro-inflammatory properties which includes avid adherence to the extracellular matrix, the ability to produce reactive oxygen species, high proteinase content, and proteolytic activity against elastin and fibronectin (Owen et al., 1994a; Owen et al., 1994b). This subpopulation of monocytes may have a role in the lung injury that occurs in emphysema.

Two recent studies by Shapiro and co-workers (Zheng et al., 2000; Wang et al., 2000) in mice lend support to the proposal that macrophage metalloproteinases may have a more significant role in the development of human emphysema. In the first study (Zheng et al., 2000), IL-13, a critical cytokine in asthma, caused emphysema in mice with enhanced lung volumes and compliance, mucus metaplasia and inflammation. The mouse metalloproteinases, MMP-2, -9, -12, -13, and -14 and cathepsins B, S, L, H, and K were all found to be induced by IL-13. In addition, treatment with MMP or cysteine proteinase antagonists significantly decreased the emphysema and inflammation. In the second study (Wang et al., 2000), interferon gamma, a product of CD8(+) lymphocytes, was found to cause emphysema in mice where there was induction and activation of MMP-12 and cathepsins B, H, D, S, and L. Despite there being significant differences in physiology between mice and humans (Shapiro, 1997; Tardiff and Krauter, 1998), these studies suggest that the investigation of macrophage metalloproteinase function in human emphysema should be more closely examined.

1.5.3.3 OTHER CELL PROTEASES

Other inflammatory cells, such as lymphocytes, eosinophils and mast cells, contain proteolytic enzymes, although it is unclear whether they have a role in extracellular matrix turnover. Fibroblasts and other similar mesenchymal cells synthesise metalloproteinases which are

required for pulmonary morphogenesis, and remodelling and repair of damaged or regenerating lung tissue (Murphy and Docherty, 1992). Resident alveolar cells of the lung may also be induced to secrete proteinases in inflammatory situations, as has been demonstrated with gelatinase B expression from unidentified cells within the alveolar wall of lungs of cigarette smokers (Shapiro, 1995).

1.5.4 DIRECT EVIDENCE FOR ELASTASE INVOLVEMENT IN EMPHYSEMA

1.5.4.1 PRESENCE OF ELASTASE IN EMPHYSEMATOUS LUNG

An excess of elastase activity in the lung is proposed to lead to emphysema as part of the protease-antiprotease or elastase-antielastase hypothesis. Thus the presence of HLE, the most likely candidate, should be able to be demonstrated in the lungs of emphysematous patients and in smokers, at least in the 10-20% of smokers who develop clinically significant emphysema. However, there are conflicting reports in the literature, not only concerning the role of HLE in the pathogenesis of emphysema, but also the detection of whether there are increased levels of HLE in the emphysematous lung. Most free HLE released into the lung would be rapidly bound by antiproteases with any excess likely to be found attached to the connective tissue of the lung surface. Damiano et al. (1986) has demonstrated the immuno-localisation of HLE on elastic fibres in surgically resected tissue from the lungs of smokers and showed that the amount of HLE bound was proportional to the amount of emphysema that was present. These findings were supported by Ge et al. (1990) but in similar study Fox et al. (1988) reported no increase in HLE in association with elastin in emphysematous lung tissue. It is important to note, in interpreting the data, that the immunological presence of HLE does not necessarily indicate that the HLE is functionally active.

As most free HLE in the lung is rapidly bound by antiproteases, such as α_1 -PI, the simplest way of detecting HLE excess is the immunological measurement of the complexed form of HLE in BAL fluid. A number of studies have used this type of assay. One study has demonstrated a

rapid increase in elastase values in BAL from smokers, minutes after cigarette exposure (Fera et al., 1986). Another study demonstrated a relationship between elevated HLE and reduced antielastase levels in BAL fluid and emphysema (Fujita et al., 1990). Increased HLE - α_1 -PI complexes were also found in BAL from both patients with emphysema (Trefz et al., 1992) and subjects with subclinical emphysema (Yoshioka et al., 1995). The condition of subclinical emphysema was defined as those individuals who showed low attenuation areas on CT scans, consistent with the early changes seen in emphysema but who did not demonstrate clinical signs of emphysema (Yoshioka et al., 1995).

Unbound leukocyte proteolytic enzymes, including HLE, are capable of being taken up or “internalised” by macrophages via receptors on the macrophage surface (Campbell et al., 1979; Campbell, 1982; McGowan et al., 1983). The macrophage can subsequently release these “internalised” enzymes in their active form; a process which may contribute to an elastase burden in the lungs of emphysematous individuals. This proposal is supported by the finding that higher elastase activity was found in the alveolar macrophages isolated from individuals with CT scans showing low attenuation areas when compared to alveolar macrophages from individuals with normal CT scans (Betsuyaku et al., 1994).

While some attempts to show increased elastase activity in cigarette smokers have often been inconclusive (Gadek et al., 1979; Carp et al., 1982; Stone et al., 1983), other studies have found small amounts of elastase-like esterase activity in the BAL fluid of smokers compared to non-smokers (Stockley and Ohlsson, 1982; Janoff et al., 1983a; Niederman et al., 1984). However, in most of these studies the type and source of the elastase activity has not been clearly defined. For example, Janoff et al. (1983a) found that the elastase-like activity was suppressed partially and equally by inhibitors of both metalloproteinases and serine proteases, but surprisingly, not by α_1 -PI. In another study, where elastin was used as substrate, the elastase activity was inhibited more effectively by inhibitors of metalloproteinases than by serine proteases inhibitors

(Niederman et al., 1984). The above reports indicate that free elastase activity could be due either to serine proteases or metalloproteinases and that the source might be the leukocyte or the alveolar macrophage.

Some support for macrophage activity in the development of emphysema is suggested by reports, which demonstrate increased levels of cathepsin enzymes in response to tobacco smoke. Cathepsin B levels were found to be approximately three times higher in the macrophages of healthy smokers and approximately ten times higher in the BAL fluid of smokers when compared to non-smokers (Chang et al., 1986). Cathepsin D levels were found to be elevated in the macrophages and BAL fluid of smokers (Chang et al., 1989). Cathepsin L activity is also increased in rat macrophages in response to tobacco smoke (Lesser et al., 1989). While the above reports suggest that the cathepsins may play a role in the inflammatory response of macrophages to tobacco smoke it is not known whether cathepsins have a role in the development of emphysema.

The prevailing evidence suggests that HLE is the prime candidate responsible for the degradation of lung tissue in emphysema. However, because of the chronic nature of the disease, any or more than one of the elastolytic enzymes, that can potentially be released in the lung over a long period of time, may have a role in the development of emphysema (Table 1.2; adapted from Shapiro, 1995; Chapman et al., 1994). The enzyme(s) involved in the degradation of the extracellular matrix of the lung in emphysema may be dependent on the conditions created in the tight junction or "microenvironment", between inflammatory cells such as neutrophils or macrophages and extracellular matrix surfaces. In this "microenvironment", inhibitors such as α_1 -PI may be at reduced levels or excluded and normal physiological conditions, such as pH and oxidant levels, may be altered (Campbell and Campbell, 1988; Weiss and Regiani, 1984). In the unique conditions created in this "microenvironment", certain elastolytic enzyme activity may be favoured which would not be seen under more normal physiological conditions. For example, the neutrophil, which generates oxygen free radicals

when activated, can therefore, at the same time as releasing granule proteinases, “switch off” α_1 -PI and SLPI function by oxidative inactivation of these inhibitors to allow unrestricted HLE activity in this “microenvironment” (Kramps et al., 1991).

1.5.4.2 DEGRADATION PRODUCTS FROM ELASTASE ACTIVITY

Because emphysema is a destructive process, quantitation of degradation products from alveolar wall components has been studied as a measure of elastase activity within the lungs. As elastin is a prominent component of the alveolar wall, most of the assays have been developed to measure either elastin-derived degradation products such as desmosine, a cross-linking amino acid unique to elastin, or elastin-derived peptides. A third approach has been to measure the presence of specific fibrinopeptides, which are generated only by the unique action of HLE on fibrinogen.

When elastin is degraded, desmosine is excreted in the urine as one of the components of the peptides produced by elastin degradation. A radioimmunoassay for desmosine in urine reported increased levels in experimental animals treated with intratracheal elastase (Goldstein and Starcher, 1978; Janoff et al., 1983b). However, the same assay for urinary desmosine in human subjects with suspected elastolytic degradation of the lung has given conflicting results. One study showed increased 24 hour urinary excretion of desmosine in 11 subjects with COPD as compared with 23 healthy non-smokers (Harel et al., 1980) but increased levels were not found in two later studies using this assay. In the first of these, on a group of 157 subjects, elevated desmosine levels were not found in the urine of smokers with COPD when compared with normal smokers or non-smokers (Davies et al., 1983). In the second study, no difference in urinary desmosine levels was found between normal individuals, PI Z subjects with emphysema and subjects with interstitial lung diseases; nor were elevated levels found in PI Z children when compared with age-matched control children (Pelham et al., 1985). In a re-evaluation of the radioimmunoassay method, by including preliminary purification steps as part of the assay,

Starcher and Scott (1992) showed that the original radioimmunoassay overestimated urinary desmosine due to the presence in urine of cross-reacting material, whose presence could mask significant differences between subject groups.

At about the same time, Stone et al. (1991) reported an improved method for the assay of urinary desmosine, based on HPLC with preparative chromatography and isotope dilution steps. This procedure was claimed to avoid the measurement of interfering substances detected in the earlier radioimmunoassay method. It gave values that were 10-20% of the previously reported radioimmunoassay values (Stone et al., 1991). Using this HPLC assay, Stone et al. (1995a) reported that urinary desmosine excretion in apparently healthy smokers was 50% higher than in a control group of healthy subjects of similar age who had never smoked. In a later study using this improved assay, together with lung function data from a 12 year longitudinal study, it was shown that smokers with a rapid decline in lung function excreted significantly more desmosine in their urine than did smokers experiencing normal rates of decline (Gottlieb et al., 1996). The results from the improved assay suggest that elevated urinary excretion of desmosine may be an early biologic marker of the development of COPD and lend support to the claim that elastase degradation of elastin is important in the pathogenesis of emphysema.

Besides measurement of desmosine content, elastin peptides have been measured directly with ELISA methods using specific antibodies for elastin peptides. Some of the reported assays have been more successful than others in differentiating COPD subjects from other groups. Patients with COPD have been shown, in several studies, to have elevated plasma elastin peptide levels compared with levels in normal non-smokers (Darnule et al., 1982; Kucich et al., 1985; Dillon et al., 1992; Schriver et al, 1992; Akers et al., 1992). In some of these studies smokers were shown to have higher levels than non-smokers (Kucich et al., 1985; Schriver et al, 1992; Akers et al., 1992). Dillon and co-workers (1992) have reported that plasma elastin peptide levels correlate with the lung function parameter, K, but no correlation was shown in this and another study with the lung function parameter, FEV₁ (Dillon et al., 1992; Frette et al., 1992). Schriver

et al (1992) found significant differences in elastin peptides levels (both in plasma and urine) between subjects with COPD, smokers and non-smokers. Subjects with COPD had the highest levels and smokers had intermediate levels compared to non-smokers. Elastin peptides have also been measured in BAL, where levels were significantly elevated in current smokers compared with age-matched current non-smokers (Betsuyaku et al, 1996).

In contrast to the above reports, one assay for elastin peptides was unable to differentiate between any of the patient and control groups studied which included non-smokers, smokers and individuals with α_1 -PI deficiency (Frette et al., 1992) and patients with emphysema (Frette et al., 1996). In addition, no relationship was found with FEV₁ in non-smokers, smokers and individuals with α_1 -PI deficiency (Frette et al., 1992) and in coal miners (Frette et al., 1997). Although an explanation for these discordant results is not known, notable differences with this assay are that aortic kappa elastin (ethanolic KOH generated elastin peptides) was used to prepare the antisera and that the serum elastin peptide levels found were detected in ug/ml range compared to ng/ml quantities as found in most of the other major reported assays (Kucich et al., 1985; Dillon et al., 1992; Schriver et al, 1992). One possible explanation is that the different antigen used could result in an antibody preparation with significantly different specificities to that used in the other assays.

As neither desmosine nor elastin peptide assays are tissue specific, the site of the excess elastin degradation in smokers and individuals with COPD has not been established. However, in view of the known inflammatory response in the lungs of cigarette smokers, and as the lung is rich in elastic tissue, it must be a favoured site of origin for elastin peptides in smokers. This is supported by the observation that significant increases in elastin degradation products have only been reported in smoking and COPD subjects, and in patients with another degenerative lung disease, cystic fibrosis (Stone et al., 1995b). Elastin peptide levels were reported to be significantly higher in subjects with COPD than in the other lung disease groups studied (pneumonia, asthma, tracheobronchitis). They were also significantly higher than in disease

groups with the potential for non-pulmonary elastin degradation, such as, inflammatory arthritis and atherosclerotic vascular disease (Akers et al., 1992). Further support for the concept that increased elastin degradation products are derived from the lungs in emphysema comes from preliminary evidence that there is a decrease in urinary desmosine excretion in PiZ patients treated with α_1 -PI (Snider et al., 1993; Stone et al., 1995c). All these studies suggest that elastin degradation products are of lung origin and thus would appear to be useful markers of degradative lung disease.

The current working hypothesis for the pathogenesis of emphysema has HLE as the most likely elastolytic enzyme responsible for the degradation of lung tissue in emphysema (Janoff, 1985). To demonstrate that there is increased HLE activity in the pathogenesis of emphysema, Weitz et al. (1986) have developed an assay based on the measurement of fibrinopeptide A α 1-21 and its degradation products which result from the unique capacity of HLE to cleave the A α 21(Val)- A α 22(Glu) bond of fibrinogen. Using this assay as an index of *in vivo* HLE activity, a five fold elevation of plasma elastase-specific fibrinopeptides was found in a group of smokers compared with non-smokers (Weitz et al., 1987). Further, individuals with α_1 -PI deficiency had plasma elastase-specific fibrinopeptides markedly higher than those in smokers (Weitz et al., 1986; Weitz et al., 1987). In a later study, Weitz et al. (1992) reported that fibrinopeptide levels significantly correlated with the degree of α_1 -PI deficiency, with PiZ individuals having the highest levels and PiMZ individuals having intermediate levels compared to normal PiM subjects. In addition, this study suggests that α_1 -PI deficiency and cigarette smoking have additive effects on plasma elastase-specific fibrinopeptides levels, which would explain why PiZ and some PiMZ subjects are at greater risk for the development of emphysema if they smoke (Weitz et al., 1992). Elastase-specific fibrinopeptides levels in non-smoking PiZ subjects were also found to be inversely related to FEV₁ (Weitz et al., 1992). The assay data from this group provide some of the best direct evidence of *in vivo* elastolysis,

due to the activity of HLE and is consistent with the concept that unregulated HLE activity causes lung destruction leading to emphysema.

1.5.5 ANTI-PROTEASES

While there is good evidence for the role of α_1 -PI in the inherited form of emphysema, there is still controversy over the relative contribution of other anti-proteases to the more common type of emphysema where α_1 -PI levels are normal. In this section, a number of the candidate anti-proteases, including α_1 -PI, are discussed with a view to their potential role in the pathogenesis of emphysema.

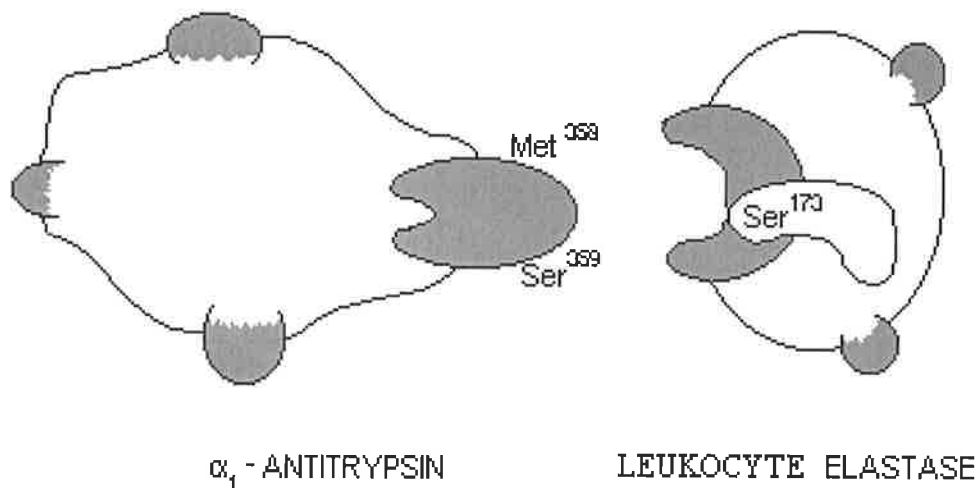
1.5.5.1 ALPHA-1-ANTIPROTEASE (α_1 -PI)

α_1 -PI is a small 52 kDa glycoprotein which is predominantly produced by the liver, but lesser amounts are produced by a variety of other tissues. The primary function of α_1 -PI is to protect body tissue, particularly that of lung, against the action of HLE. The importance of α_1 -PI is emphasized by the fact that it is one of the few antiproteases that can inhibit HLE, the main protease candidate in this hypothesis.

α_1 -PI reaches most tissues and organs, including the lower respiratory tract, by simple diffusion from the circulation, although there is evidence that locally produced α_1 -PI may have an important protective function at the tissue site of production (Carlson et al., 1988; Koopman et al., 1989). An example, relevant to the pathogenesis of emphysema, is the expression of α_1 -PI by protease containing inflammatory cells (such as neutrophils, monocytes and macrophages) found in the lung (du Bois et al., 1991; Perlmutter, 1991; Perlmutter and Pierce, 1989). Neutrophils can also store the antiprotease and release it together with its target protease, HLE, when activated (Paakko et al., 1996). The most likely reason for the secretion by the neutrophil

of α_1 -PI together with its target enzyme is that it may be part of a mechanism for modulating local HLE activity (Paakko et al., 1996). Thus α_1 -PI provides not only a systemic protective function against HLE activity in the lower respiratory tract, through diffusion of α_1 -PI synthesised by the liver, but also a local protective function at the cellular level where it may regulate HLE activity through its co-secretion with HLE.

In addition to its inhibitory activity towards HLE, α_1 -PI exhibits some activity against most other serine proteases, including trypsin, chymotrypsin, proteinase 3, pancreatic elastase, cathepsin G, plasmin and thrombin. However, its primary physiological function is thought to be the inhibition of HLE (Travis and Salvesen, 1983) as demonstrated, for example, by the fact that α_1 -PI inhibits HLE at least 10 times more rapidly than any other protease tested (Beatty et al., 1980). α_1 -PI functions as a pseudosubstrate by forming a tight equimolar complex with specific target proteases, such as HLE. The reactive site of α_1 -PI, the so called PI residue, is centred on the aminoacid residues Met³⁵⁸ - Ser³⁵⁹ and is part of an external loop protruding from the molecule (Figure 1.1). This Met³⁵⁸ - Ser³⁵⁹ tip fits closely into the reactive pocket of HLE (Figure 1.1). A disadvantage of exposure of the reactive centre on the stressed loop is that the α_1 -PI molecule is liable to inactivation, either by cleavage of the loop or by oxidation of the reactive Met³⁵⁸ to methionine sulphoxide. The latter derivative of the oxidised α_1 -PI molecule is now too large to fit the reactive centre of HLE (Crystal et al., 1989; Carrell, 1986) resulting in a significant reduction of its inhibitory function. For example, the binding association constant (k_{ass}) which describes the binding affinity of α_1 -PI for HLE is reduced after oxidation from $10^7 \text{ M}^{-1} \text{ sec}^{-1}$ to $10^4 \text{ M}^{-1} \text{ sec}^{-1}$ (Beatty et al., 1980).



**Figure 1.1 A diagrammatic representation of the reactive site of α_1 -PI centred at the stressed loop containing Met³⁵⁸ - Ser³⁵⁹ which is susceptible to cleavage on interaction with the reactive pocket of HLE.
(α_1 -PI can be inactivated by oxidation of the Met³⁵⁸ to methionine sulphoxide.)**

As of 1988 (Brantly et al.), more than 75 different PI alleles had been identified, of which the common M allele (frequency greater than 95%) is associated with normal α_1 -PI levels. Only a few alleles result in decreased serum levels of α_1 -PI. The S and Z alleles are associated with slightly and markedly reduced α_1 -PI levels, respectively, while the null alleles are associated with the complete absence of α_1 -PI (Brantly et al., 1988; Crystal et al., 1989). The most studied is the PI Z mutation, which is responsible for more than 95% of all α_1 -PI deficiencies. The PI Z form is also slightly less effective as an inhibitor due to a slower association rate with human leukocyte elastase (HLE) than the normal PI M form of α_1 -PI (Lomas et al., 1993).

α_1 -PI is the most abundant of the antiproteases in human serum (Carrell, 1986), as well as in most tissues and organs, including the lung. Sampling of the fluid on the epithelial surface of

the lower respiratory tract of normal persons has demonstrated average α_1 -PI levels of 3 to 4 μM , which represents about 10% of normal serum levels (Wewers et al., 1987). However, even at this concentration, α_1 -PI has been reported to constitute the majority of the anti-elastase activity in the normal lower respiratory tract. For example, more than 80% of the anti-elastase activity in BAL has been attributed to α_1 -PI in some studies (Wewers et al., 1987; Wewers, 1989) and 50% or more in some other studies (Afford et al., 1988; Walsh et al., 1992).

It has been estimated that only about 1% of all emphysema is due to the inherited homozygous α_1 -PI deficiency while the majority of cases (95%) occur in smokers with normal α_1 -PI levels (Tetley, 1993). To include subjects with this more common form of emphysema in the protease-antiprotease hypothesis for emphysema, a “two hit concept” has been proposed in which smoking leads to emphysema by inducing both an increase in elastase activity (see Section 1.5.2) and by decreasing α_1 -PI activity (Wewers, 1989; Hunninghake and Crystal, 1983). It has been proposed that α_1 -PI activity is reduced, along with other susceptible proteases in the lung of smokers, as a result of inactivation by oxygen radicals present in the inhaled tobacco smoke and by the release of oxygen radicals from macrophages and leukocytes (Johnson and Travis, 1979; Carp and Janoff, 1978; Carp et al., 1982).

However the evidence supporting the oxidative inactivation of α_1 -PI in smokers is mixed, with not all reports being able to demonstrate the presence of functionally inactivate or oxidised α_1 -PI. As this is one of areas investigated in this thesis, the evidence will be reviewed briefly. In support of the oxidative proposal is a report that BAL from smokers contained α_1 -PI that was 40% less effective as an inhibitor of HLE than α_1 -PI from non-smokers when compared to the total amount of α_1 -PI present (Gadek et al., 1979). Similar findings were seen in rats after short term exposure to cigarette smoke (Janoff et al., 1979). It has also been shown that the rate of inhibition of HLE by α_1 -PI in BAL fluid from smokers is reduced, as demonstrated by an approximately two fold reduction in the k_{ass} constant of α_1 -PI for HLE in cigarette smokers

compared to non-smokers (Wewers et al., 1989). More direct evidence for compromised α_1 -PI function in the lungs of smokers is seen where the α_1 -PI purified from BAL fluid of smokers took twice as long to inhibit HLE as did α_1 -PI purified from BAL fluid of non-smokers (Ogushi et al., 1991). Oxidative damage to the α_1 -PI molecule as a result of smoking has also been demonstrated by the isolation of α_1 -PI containing methionine sulphoxide from lung secretions of cigarette smokers but not from the lung secretions of non smokers (Carp et al., 1982). While oxidative inactivation of α_1 -PI is most likely to be detected in the lung lining fluid which comes into contact with tobacco smoke, some workers have been able to detect small amounts of α_1 -PI inactive towards elastase in serum or plasma from smokers (Beatty et al., 1982; Chowdhury et al., 1982; Cox et al., 1984; Bridges et al., 1985).

While the above reports support the proposition that α_1 -PI activity in smokers is functionally compromised, other studies using similar methods, have failed to find any noticeable differences in the elastase inhibitory capacity of α_1 -PI obtained by BAL from smokers and non-smokers (Stone et al., 1983; Boudier et al., 1983; Stockley and Afford, 1984; Abboud et al., 1985; Walsh et al., 1992). Even the use of specific monoclonal antibodies to differentiate the native and oxidised forms of α_1 -PI in BAL fluid, failed to find any differences between smokers or non-smokers in one study (Campbell et al., 1987). Two other reports have failed to detect the presence of inactivated α_1 -PI in serum or plasma from smokers (Lellouch et al., 1985; Walsh et al., 1992).

While the evidence for increased or free HLE activity in the pathogenesis of emphysema is strong (section 1.5.4), the evidence, as described above, for compromised α_1 -PI activity in smoking associated emphysema is inconsistent. The variable results from these studies may be explained by differences in sensitivity and specificity in being able to detect the small amounts of inactivated α_1 -PI present in BAL fluid and serum or plasma from smokers. In comparison, the degree of inactivation of α_1 -PI is much greater and more easily detected in an acute disease,

such as ARDS, than the more subtle inactivation which is proposed to occur in a life-long chronic lung disease, such as smoking associated emphysema. In addition, inactivation of α_1 -PI is likely to occur only in those parts of the lung exposed to tobacco smoke, as suggested by the pattern of emphysema that is seen in the lungs of smokers (Thurlbeck, 1988) and, as proposed elsewhere (Campbell and Campbell, 1988; Weiss and Regiani, 1984), inactivation of α_1 -PI may be localised to the pericellular junction between the inflammatory cell and the extracellular matrix surface of the lung. In conclusion, the possibility that there are only small amounts of inactivated α_1 -PI localised to sites of inflammatory cell degranulation in the emphysematous lung may explain, when combined with methodological differences, the difficulty in detecting the presence of inactivated α_1 -PI in BAL and serum from smokers.

1.5.5.2 ALPHA-2-MACROGLOBULIN (α_2 -M)

α_2 -M is unique in its ability to inhibit all four classes of proteases (serine, cysteine, aspartate and metallo- proteinases) suspected of having a role in emphysema (Travis, 1988). While α_2 -M appears to be a non-specific protease inhibitor it may have an important regulatory role towards proteases, as suggested by the fact that it binds preferentially and more rapidly to collagenase than the specific tissue inhibitor, TIMPS (Cawston and Mercer, 1986). While α_2 -M function is primarily restricted to the vascular system and interstitial fluid (Tollefsen and Saltvedt, 1980) because of its size (M_r 725,000), it is also found in small amounts in the lung due to diffusion from the vascular system and local synthesis by macrophages (Stockley et al., 1979; White et al., 1981). Thus, the presence of α_2 -M in the lung suggests that it may have a role as part of the lung antiprotease system.

α_2 -M functions as an antiprotease by unwinding its strands to open a cavity for entrance of the protease and then retwisting the strands to encapsulate the protease (Qazi et al, 1998). However, while having a broad specificity, α_2 -M is not a very effective antiprotease as it does not

completely inhibit most protease activity. For example, when complexed to α_2 -M, proteases typically retain 80-100% of their hydrolytic activity against low molecular weight substrates and 10% or less against large proteins (Barrett, 1981). This has led to the proposal that α_2 -M-HLE complexes may play a role in the development of pulmonary emphysema by allowing the encapsulated HLE limited access to lung tissue (Galdston et al., 1979; Stone et al., 1979). This proposal is supported by the detection of α_2 -M-PPE complexes in BAL from the lungs of hamsters with PPE-induced emphysema (Stone et al., 1982).

Because of its broad specificity as a protease inhibitor, α_2 -M is thought to act as an important backup inhibitor to the more specific inhibitors, such as α_1 -PI and TIMPS. Some support for this role is suggested by the fact that α_2 -M inhibitory function appears to compensate for compromised α_1 -PI function, as suggested by the finding of significantly increased levels of α_2 -M in all types of α_1 -PI deficient individuals (Fryksmark et al., 1983; Brissenden and Cox, 1983). Another report has also described an apparent increased elastase binding capacity of α_2 -M in individuals with COPD and normal α_1 -PI levels (Kilroe-Smith et al, 1989). The above reports would suggest that α_2 -M has a role as a high-affinity broad spectrum proteinase inhibitor but there is little evidence, even from subjects with α_2 -M deficiency (Poller et al, 1989; Kruger, 1993), to support α_2 -M having a major role in the development of emphysema.

1.5.5.3 ALPHA-1-ANTICHYMOTRYPSIN (α_1 -ACH)

α_1 -ACH is a major plasma acute phase protein that can increase 5-7 fold in concentration after an inflammatory insult (Aronsen et al., 1972) suggesting that it has an important role in controlling certain enzyme(s) associated with inflammation. It is synthesised and secreted by hepatocytes and by alveolar macrophages and is detectable in lung lavage fluid. α_1 -ACH has been shown to inhibit chymotrypsin-like proteases (Beatty et al., 1980; Travis et al., 1978) and

mast cell chymases (Travis et al., 1978; Reilly et al., 1982). The most rapid inhibition is seen towards cathepsin G (Beatty et al., 1980; Travis et al., 1978) which suggests that its function is to regulate cathepsin G activity. Although cathepsin G is a relatively poor protease, it can degrade some components of connective tissue and is much more effective in this capacity when it can work synergistically with HLE (Boudier et al., 1991). As both enzymes are co-released from leukocytes *in vivo*, this is a likely mode of action in an inflammatory response. However, the presence of HLE specifically inactivates α_1 -ACH allowing cathepsin G unrestricted activity (Potempa et al., 1991), which suggests that the regulation of cathepsin G activity may not be the prime function of α_1 -ACH as earlier studies have suggested.

However, a role for α_1 -ACH in defending the lung against proteolytic attack is suggested by reports of an association between α_1 -ACH deficiency and disturbances of lung (Eriksson et al., 1986; Weidinger et al., 1992; Poller et al., 1993). At this stage, the mechanism and the extent of involvement of α_1 -ACH deficiency with the development of COPD is uncertain. Nevertheless, it is possible that a deficiency in α_1 -ACH function may be one of the familial factors that could explain why only some individuals with an α_1 -PI deficiency and only some smokers develop emphysema.

1.5.5.4 SECRETORY LEUKOPROTEASE INHIBITOR

SLPI (secretory leukoprotease inhibitor; also called antileukoprotease or bronchial mucus proteinase inhibitor) is a low molecular weight boomerang-shaped protein (M_r 11,700) (Hochstrasser et al., 1972) responsible for more than 80% of antiprotease activity in bronchitic sputum (Stockley et al., 1986). SLPI is a reversible inhibitor of trypsin, cathepsin G and chymotrypsin (Schiessler et al., 1978) as well as a fast acting inhibitor of HLE (Boudier and Bieth, 1989). It is also an effective inhibitor of mast cell chymase in the presence of heparin, a glycosaminoglycan which is found as part of the lung tissue surface (Walter, 1996).

Early studies indicated that SLPI appeared to have two reactive sites, but a later study has shown that all enzymes bind to a single site on the inhibitor (Eisenberg et al., 1990). The binding site on SLPI is susceptible to oxidation, with a resultant loss of inhibitory activity against HLE (Kramps et al., 1989). This oxidative inactivation of SLPI is similar to that which occurs with α_1 -PI suggesting a methionine residue at the reactive site as found in α_1 -PI (see previous section 1.5.5.1). SLPI is localised in secretory granules of serous cells in the upper respiratory tract (Kramps et al., 1981), is also found in Clara and goblet cells of the small peripheral airways of the lung (DeWater et al., 1986) and is synthesised by epithelial cells (Abe et al., 1991). SLPI has also been reported to be the major inhibitor that is found in the neutrophil cytosol, where it has been proposed that it may act as a protective screen against proteinases spilling from the azurophilic granules into the cytosol (Sallenave et al., 1997a). Based on the recovery of SLPI and α_1 -PI in lavage samples from different parts of the lung, SLPI has been suggested to be the main inhibitor in the larger airways, whereas α_1 -PI is predominant in the peripheral airways (Kramps et al., 1988).

No inherited deficiency of SLPI or a role in the development in emphysema, similar to that seen for α_1 -PI, has been reported as yet. However some of its functions are similar to that of α_1 -PI. For example, both SLPI and α_1 -PI concentrations are increased in BAL from emphysema patients, presumably to balance the increased elastolytic load in the lungs, although the mechanism is unknown (Willems et al., 1989; Trefz et al., 1992). Like α_1 -PI, SLPI is inactivated by oxidants *in vivo*, as has been demonstrated in a patient with cystic fibrosis (Vogelmeier et al., 1991), although it has not been reported in emphysema. As is the case for α_1 -PI inhibition of induced emphysema in animal studies, SLPI is also able to significantly diminish the development of Escherichia coli lipopolysaccharide - mediated pulmonary emphysema (Rudolphus et al., 1993) and to prevent the development of HLE induced emphysema in hamsters (Rudolphus et al., 1994).

What is unique about SLPI is that it is able to inhibit the contact-dependent proteolysis that occurs between HLE and a substrate surface; a property not possessed by any other known endogenous inhibitor. Campbell et al. (1982, 1988) have shown that large inhibitors such as α_1 -PI and α_2 -M are partially excluded from the leukocyte-substrate interface and are thus ineffective as proteolytic inhibitors once the leukocyte is bound to the substrate. However, the low molecular weight inhibitor, SLPI, is able to inhibit HLE after it is bound to elastin (Morrison et al., 1990) and inhibits ongoing proteolysis by leukocytes already bound to substrate (Rice and Weiss, 1990).

In conclusion, SLPI has been shown to be one of the major serine proteinase inhibitors in the lung and the fact that it has been found to be associated with elastic fibres (Willems et al., 1986; Rudolph et al., 1994) suggests that this antiprotease may have a role in protecting the lung against elastolytic proteinases.

1.5.5.5 *ELAFIN*

More recently, another low molecular weight inhibitor, elafin, has been found to be secreted by Clara cell and type II pneumocyte cell lines. It had been found previously only in skin (Sallenave et al., 1993). Elafin has also been shown to be present in expectorated sputum (Sallenave et al., 1993), in BAL fluid from normal subjects and in elevated amounts in BAL fluid from individuals with farmer's lung and lymphocytic alveolitis (Tremblay et al., 1996). Elafin appears to be synthesised as a 12-14,000 M_r precursor and then processed to the secreted 6,000 M_r protein. The importance of elafin as an antiprotease in the lung and determination as to whether it has a role in the development of emphysema awaits further investigation.

1.5.5.6 MONOCYTE/NEUTROPHIL ELASTASE INHIBITOR

Monocyte/neutrophil elastase inhibitor (M/NEI) is a protease inhibitor that has been found in high levels in human neutrophils, monocytes and macrophages (Remold-O'Donnel, 1989). This inhibitor can regulate the activity of the neutrophil proteases: HLE, cathepsin G and proteinase 3. M/NEI is a nonglycosylated single polypeptide of M, approximately 42,000 with an essential cysteine at the reactive centre of the molecule (Renold-O'Donnel, 1992). Initial studies suggest that it is an oxidation-sensitive molecule that would only be able to inhibit serine proteases in the immediate vicinity of the carrier cells. Its function in the regulation of proteolytic activity from neutrophils, monocytes and macrophages is at present unknown. Further studies may determine whether it has any role in protecting the lung against elastolytic proteinases.

1.5.5.7 OTHER ENDOGENOUS INHIBITORS

A number of endogenous compounds, other than the known proteases, have been reported to inhibit HLE activity to varying degrees. The most potent of these compounds are the sulphated GAGs, such as heparin, heparan sulphate, chondroitin sulphate and dermatan sulphate (Redini et al., 1988a; Walsh et al., 1991a; Drag and Petersen, 1994). However it has not been determined whether endogenous sulphated GAGs have an anti-elastase role *in vivo*. Although the occurrence and location of sulphated GAGs *in vivo* on cell surfaces and as components of proteoglycans in cell and basement membranes (Höök et al., 1984; Marcum and Rosenberg, 1984) lends some support for this role. The finding that sulphated GAG's, such as heparan sulphate, chondroitin sulphate and dermatan sulphate, are synthesised and secreted by neutrophils (Bartold et al, 1989) would suggest that sulphated GAG's may have a role in regulating the activity of HLE secreted by neutrophils.

Elastin peptides have also been reported to be potent inhibitors of HLE (Bonnaure-Mallet et al., 1995) with the formation of an unusually stable complex between HLE and elastin peptides rich

in the alanine/glycine and hydrophobic domains of elastin (Tyagi and Simon, 1993). Tyagi and Simon (1993) have suggested that the elastin peptides, released during elastinolysis, have a regulatory role towards HLE activity at local sites of proteolysis.

Other compounds in the lung may also be able to influence HLE activity. One such compound is pulmonary surfactant, which is found as a thin layer on the alveolar surface where it may be able to partially protect the alveolar surface against HLE activity. This proposal is suggested by the report that the administration of pulmonary surfactant resulted in a significant inhibition of elastase-induced emphysema in mice (Otto-Verberne et al., 1992). The above examples demonstrate that HLE activity may also be regulated by other components *in vivo* other than the specific anti-proteases.

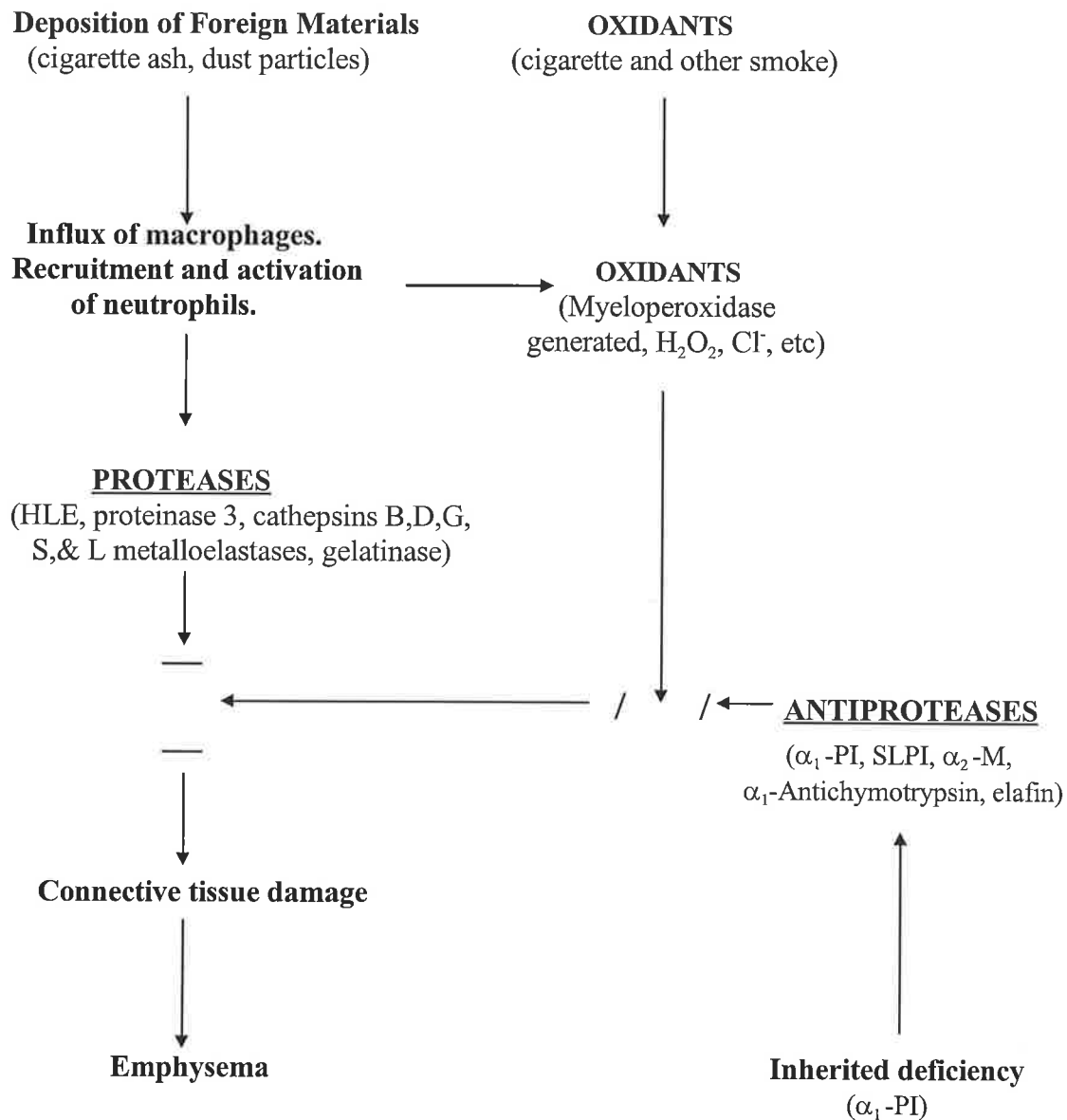


Figure 1.2: Diagram outlining the protease-antiprotease hypothesis of emphysema.

Note that not all the proteases have elastolytic activity but may have a role, by working in parallel or synergistically with the major elastases such as HLE, in damaging the connective tissue of the lung. α₁-PI and SLPI appear to be the only antiproteases that are inactivated by oxidants. Oxidant activity is normally regulated by the presence of antioxidants such as superoxide dismutase, glutathione, and catalase (not shown).

1.6 THE PROTEASE-ANTIPROTEASE HYPOTHESIS: REDRESSING THE IMBALANCE

Despite the fact that the protease-antiprotease hypothesis is only supported by indirect evidence, it has stimulated much study of the proposed mechanisms in the development of emphysema (see Figure 1.2), in the course of which a variety of new methods for preventing emphysema have been tested. Most of the procedures have involved reducing the protease levels or increasing the antiprotease levels within the lungs.

1.6.1 REDUCING THE PROTEASE LEVELS

If the protease-antiprotease hypothesis is accepted as the basis of the pathogenesis of emphysema, one would predict that the development of emphysema would be prevented or reduced by lowering the protease load, particularly of elastolytic enzymes in the lungs of susceptible individuals. One way of achieving this would be to prevent exposure to the agent responsible for the influx of inflammatory cells into the lungs, thereby preventing the release of proteases and oxidants responsible, in this model, for the development of emphysema.

As discussed previously, there is a wide range of indirect evidence from population and animal model studies to support the view that smoking is the major risk factor in the pathogenesis of emphysema. The influence of smoking is such that it overrides every other risk factor, including the only clinically proven genetic factor, α_1 -PI deficiency. Thus smoking appears to be able to alter the balance of the protease-antiproteases in the lung resulting in elastolytic lung damage. One likely mechanism for the protease excess is the release of elastases from macrophages and neutrophils attracted to the cigarette smoke particles together with reduced antiproteases due to inactivation by smoking and phagocyte derived oxidants.

Numerous population studies have shown that mortality and incidence rates increase with increasing exposure to cigarette smoke and that there is an improvement in mortality and

morbidity rates in smokers who quit compared with smokers who continue to smoke. Smokers who are able to stop smoking reduce their rate of decline of FEV₁ from about 70 ml a year to about 30 ml a year (Cohen et al., 1991). Despite quitting, ex-smokers still have an excessive age-adjusted overall mortality when compared to lifetime non-smokers, which is most likely to be due to the fact that the lungs and some other organs have already been permanently damaged (Cohen et al., 1991). However most smokers cannot give up the habit, as it is so highly addictive (U.S. Surgeon General, 1988) that even the most intensive smoking cessation programs have not been very successful. In one program, for example, about 75% of individuals were still smoking one year after completing the program (Ockene et al., 1990). One strategy to reduce the development of COPD in smokers, who cannot give up the habit, has been to promote the use of low tar, low nicotine cigarettes. However, results with this type of cigarette have been disappointing or absent especially in COPD, although there appears to be a reduction in the prevalence of cough and the risk of lung cancer (U.S. Department of Health and Human Services, 1989).

Another strategy for influencing the balance of protease-antiprotease activity in smokers is to prevent neutrophils from degranulating and releasing enzymes and oxidants into the lung. One such drug that has this potential is colchicine, which has been shown to reduce HLE secretion *in vitro*. However, when smokers with COPD were treated with colchicine in a controlled study, there was no change in the HLE load or elastase degradation products (desmosine and elastin peptide levels) in the smokers' BAL (Cohen et al., 1990). A later controlled study from the same group showed that colchicine was partially effective in that it was able to reduce HLE levels by 51.5% in BAL fluid from ex-cigarette smokers with COPD (Cohen et al., 1991). The authors attempt to explain this difference by suggesting that the difference between the two studies is most likely to be due to the fact that colchicine is too mild a drug to overcome the ongoing stimulation of leukocytes in current smokers, but is able to modify degranulation in the less stimulated leukocytes of ex-smokers. However, on the above evidence colchicine is ineffective in smokers. Nevertheless, the use of a drug, with a similar mode of action, that could

significantly alter the protease-antiprotease balance in the lung, holds much promise, particularly where it could be included in cigarettes of smokers who are unable to give up smoking.

1.6.2 INCREASING THE ANTIPROTEASE LEVELS

Most research into the effects on emphysema of altering the protease-antiprotease balance has been directed at supplementing the antiprotease system using either purified or recombinant α_1 -PI, other endogenous inhibitors or one of the many synthetic inhibitors that have been developed. Replacement therapy with α_1 -PI, purified from plasma, has been used in clinical trials in α_1 -PI deficient patients (PiZ) and has been shown to maintain adequate plasma and lung concentrations without inducing appreciable side effects when given either parentally or by inhalation (Gadek et al., 1981(a); Hubbard et al., 1988; Hubbard et al., 1989). This therapy has been used in most of the 1000 PiZ patients enrolled since 1989 in the US National Heart, Lung, and Blood Institute α_1 -PI deficiency registry (Luisetti and Travis, 1996). So far, preliminary reports of replacement therapy show little or no decline in lung function over time, but in most subjects the period of therapy is still short. For example, in a recent study, the authors have concluded from the first year of a program of weekly infusions of α_1 -PI to PiZ individuals that there may be a slowing in the rate of decline in FEV1 after 1 year (Seersholm et al., 1997). To date, the most comprehensive study has monitored 14 patients for periods of from 12 months to four years, where it was concluded that replacement therapy 'may be associated with decreased hospitalisation and possibly with stabilisation of lung function' (Barker et al., 1994). At this stage of the program, it is unproven that replacement therapy with purified α_1 -PI can prevent the development of emphysema in α_1 -PI deficient patients.

To prove definitively that replacement therapy can prevent the development of emphysema in α_1 -PI deficient patients requires the use of large amounts of α_1 -PI in large population studies

over many years. A weakness of this type of study is to obtain sufficient quantities of purified α_1 -PI. An alternate source of α_1 -PI is to use that produced by recombinant technology where it may be possible to synthesise sufficient amounts for large scale replacement studies. However studies have shown that the non-glycosylated form produced by recombinant technology is more rapidly excreted in urine when compared to the glycosylated native form of α_1 -PI (Casalero et al., 1987; Schnebli, 1991). The importance of the carbohydrate component is suggested by the fact that it constitutes nearly 20% of the molecular mass of native α_1 -PI (Schnebli, 1991). At this stage, the recombinant form of glycosylated α_1 -PI has only been produced in small amounts (Garver et al., 1987) but it is obvious from the preliminary studies that for it to be as effective as the purified form, the recombinant form may require the carbohydrate component of the molecule to be equally as effective.

A number of other recombinant forms of α_1 -PI have been produced in which there are alterations in the P₁ residue, which is thought to be responsible for the specificity of the inhibitor. Two examples are the recombinant α_1 -PI MET³⁵⁸→VAL (Travis et al., 1985) and MET³⁵⁸→LEU (Jallat et al., 1986), both of which are better inhibitors of HLE than the native α_1 -PI as well as being oxidation resistant, unlike the native form. The improved recombinant forms of α_1 -PI offer the potential to be more effective and longer lasting inhibitors of HLE, particularly for use, in the short term in acute conditions such as ARDS. However, the long term administration of oxidation-resistant forms of α_1 -PI for the prevention of the development of emphysema would need to be carefully evaluated so that it did not interfere with the normal physiological role that HLE has in inflammatory reactions, such as chemotaxis, phagocytosis, and acute phase protein synthesis (Travis and Fritz, 1991). In addition, there is also the question as to whether the presence of oxidation-resistant forms of α_1 -PI would interfere in the regulation of the function of the oxidation susceptible native form of α_1 -PI by oxidants released by macrophages and neutrophils. So far none of these modified forms of α_1 -PI has been clinically evaluated.

As α_1 -PI is not the only inhibitor found in BAL, it has been proposed that replacement therapy with a combination of α_1 -PI and other inhibitors found in BAL could provide a more balanced and effective form of therapy for the prevention of the development of emphysema. To date, the only inhibitor investigated to any extent for this role has been SLPI. Initial studies have demonstrated that recombinant SLPI can be delivered to the lung successfully as an aerosol, where it has been shown to move from the epithelial surface to the lung interstitium, providing not only a potential increase in the anti-HLE screen but also a potential increase in antioxidant levels by increasing glutathione levels (Gillissen et al, 1993; Stolk et al, 1995; Vogelmeier et al, 1996).

Because of the difficulties in obtaining sufficient amounts of α_1 -PI for large scale clinical replacement studies, many synthetic protease inhibitors have now been evaluated as inhibitors of HLE *in vitro* (Powers and Harper, 1986; Groutas, 1987). Some of the more promising candidates have also been investigated for their ability to prevent elastase-induced emphysema in laboratory animals. These include:- suc-ala-ala-pro-val-chloromethylketone in hamsters (Stone et al., 1981; Fletcher et al., 1990); a peptide boronic inhibitor in hamsters (Soskel et al., 1986; Stone et al.; 1990); a peptide aldehyde reversible inhibitor in hamsters (Kennedy et al., 1987); specific β -lactam inhibitors in hamsters (Bonney et al., 1989; Fletcher et al., 1990); SR 26831, a tetrahydrothieno pyridine derivative in rats and rabbits (Herbert et al., 1991); ICI 186756, a peptide aldehyde inhibitor in hamsters (Williams et al., 1991); ICI 200355, a peptide trifluoromethyl ketone inhibitor in hamsters (Williams et al., 1991); ONO-5046, a low molecular weight inhibitor in guinea pigs (Sakamaki et al., 1996). In general, all the above inhibitors are very effective in preventing the development of HLE-induced emphysema in laboratory animals but because of the extensive evaluation studies and clinical trials that are required to detect possible clinical side effects none has, as yet, been evaluated for use in humans.

As well as synthetic inhibitors, a number of natural compounds, such as polypeptidic anti-elastases and sulphated GAG's and their derivatives have been evaluated as anti-elastase compounds. The more promising candidates have also been investigated for their ability to prevent elastase-induced emphysema in laboratory animals. These include:- CY222, a low molecular weight heparin fraction of mean M_r 4,500 in hamsters and mice (Redini et al., 1988b; Lafuma et al., 1991); Arteparon, an oversulphated derivative of chondroitin sulphate in hamsters (Rao et al., 1990); sheep lung surfactant in mice (Otto-Verberne et al., 1992); and an elastin derived peptide preparation of mean M_r 57,000 in rats (Bonnaure-Mallet et al., 1995). None of these preparations has been evaluated in clinical studies. However, they are attractive as therapeutic agents because, being derived from endogenous compounds, they are likely to produce less clinical side effects and so more rapidly gain approval for clinical use.

In general, inhibitors of HLE are effective in preventing or reducing the HLE-induced acute tissue damage that initially occurs prior to the development of the emphysematous lesions that are seen in experimental animals. The evidence that HLE inhibitors are as effective in preventing the more chronic lesions found in human emphysema is at present not available, primarily because of the difficulty in meeting ethical requirements for the use of most of these compounds in human studies.

Emphysema develops slowly over a number of years, even in α_1 -PI deficient patients, so that a long time period and many patients will be needed in trials to assess the benefits and side effects of any type of replacement therapy. A native α_1 -PI preparation (Prolastin[®]), which is already being used in clinical studies, and recombinant α_1 -PI and SLPI, and perhaps some derivatives of other endogenous compounds (eg. heparin derivatives) appear likely to offer the highest degree of safety for long term clinical use. Of these agents, the low molecular weight, synthetic inhibitors, will require more development initially, but seem likely to be cheaper and

technically easier to apply by an aerosol than are protein preparations. Some of the low molecular weight, synthetic inhibitors may even be active when given orally. The impetus to develop synthetic inhibitors is also driven by the fact that they are likely to have wider applications for the treatment of other conditions in which HLE has been implicated in the pathogenesis. Examples range from acute conditions such as septic shock and ARDS, to chronic conditions such as cystic fibrosis and rheumatoid arthritis, and include infectious diseases like AIDS.

Gene therapy is the newest approach to the treatment of lung disease. The lung is an attractive target organ for gene therapy because of its accessibility through the airways or the vasculature. Therapeutic gene delivery has largely focused on introducing corrective genes in lung diseases arising from single gene defects, such as cystic fibrosis. More recently interest has been centred on gene therapy as a tool in the treatment of more complex pulmonary conditions, such as emphysema (Sallenave et al., 1997b). It has been proposed that modification of critical components of the inflammatory process, so as to increase the expression of anti-elastase genes, could circumvent elastase mediated lung damage in emphysema. In the case of α_1 -PI deficiency, one approach would be to replace the PiZ gene with the PiM gene so as to restore normal α_1 -PI levels in the lung of deficient individuals and to prevent the development of the inherited deficiency form of emphysema.

1.6 SUMMARY

From this review of the literature, it can be seen that the pathogenesis of emphysema is complex and that the critical mechanisms still remain to be elucidated. The protease-antiprotease hypothesis has been very useful in that it has provided a framework for many directions in emphysema research, the results of which, in general, are held to support the hypothesis. However, it is important to note that almost all of the evidence that supports this theory is still

indirect. In the following paragraphs the major evidence supporting the theory is noted with comment on what further research is required to prove, modify or disprove the theory.

Evidence for HLE being the major protease part of the hypothesis

HLE has been shown in animal studies, to induce lesions typical of emphysema, including increased airspace size and lung volumes. These have been induced in the lungs of the animal by sequestering leukocytes into the lung or more directly by introducing HLE, or other elastases, intratracheally.

HLE has been shown to have a broad substrate specificity, so that it is capable of degrading a wide range of extracellular matrix proteins, including elastin. It can potentially be secreted in millimolar amounts, in response to various inflammatory stimuli, from the azurophil granules of leukocytes and also to some extent from monocytes and macrophages. No other elastolytic enzyme has been found in the lung in such large amounts, nor does any other elastase have such broad substrate specificity. There appears to be sufficient evidence to conclude that this enzyme, on its own, could account for the bulk of the tissue damage observed histologically in emphysema.

The finding of elevated elastin degradation products (such as elastin derived peptides in plasma, urine and BAL and in recent studies, desmosine in urine) supports the presence of increased elastase activity, most likely HLE, in the lungs of emphysema patients. More specific evidence for a role of HLE in the pathogenesis of emphysema can be inferred from two reports that HLE has not only been found to accumulate on interstitial elastin in areas of the human lung affected by emphysema, but also that the amount of HLE present strongly correlated with the local severity of emphysema. Markedly elevated plasma elastase specific fibrinopeptides, which are only derived from HLE activity on fibrinogen, have been reported in individuals with α_1 -PI deficiency where the fibrinopeptide level correlates with the degree of α_1 -PI deficiency. As

those individuals with α_1 -PI deficiency have a greater risk of developing emphysema, the presence of increased HLE activity supports a role for HLE in the pathogenesis of the inherited form of emphysema. Thus, the overall evidence strongly supports a role for HLE in the pathogenesis of emphysema, if not the primary causal agent. Nevertheless, further research into the development of, and use of more specific markers of lung destruction would be desirable, as it would allow the identification of those individuals with a high rate of lung degradation and the monitoring of the effectiveness of treatment with anti-HLE inhibitor therapy. These studies would also further clarify the extent of the involvement of HLE in the pathogenesis of emphysema.

The variable pathogenesis and different forms of emphysema suggests that there may be other factors causing an imbalance in the protease - antiprotease balance allowing increased HLE activity in the lung. Such factors could include a local variation in the lungs of concentrations of other elastolytic enzymes (eg. metalloproteinases, cathepsin G, proteinase 3), proteinase inhibitors (eg. α_1 -PI, SLPI, elafin) and oxidants (eg. neutrophil oxidants, external oxidants such as tobacco smoke and other occupational and environmental agents). However, at this stage, this is only speculation as there is no evidence in the literature to support the possible role of other factors in human studies.

Despite an abundance of information on the activity of HLE in the lung, definitive proof will require prospective studies with specific HLE inhibitors or control of HLE expression at the gene level. The elimination of free HLE from the lungs of patients with the inherited PiZ form of α_1 -PI deficiency and in smokers with augmentation therapy (using α_1 -PI, SLPI and the new range of specific HLE inhibitors) should give more definitive proof that HLE is the primary causative agent in the pathogenesis of human emphysema and whether the protease - antiprotease hypothesis is still valid.

Evidence for α_1 -PI being the major antiprotease part of the hypothesis

α_1 -PI inhibits HLE at least 10 times more rapidly than any other protease tested (Beatty et al., 1980) and therefore it is claimed that the primary function of α_1 -PI is to inhibit HLE. As discussed previously, most of the evidence in the literature supports the view that HLE is the prime candidate responsible for the degradation of lung tissue in emphysema. If the evidence is accepted that the primary inhibitory role of α_1 -PI is towards HLE, together with the evidence that reduced α_1 -PI levels are linked with the development of the inherited form of emphysema, then α_1 -PI must be strongly considered to be the major antiprotease in the protease - antiprotease hypothesis.

If α_1 -PI is the critical protease in the pathogenesis of emphysema, one would expect it to be the major anti-elastase in the lower respiratory tract where the emphysematous lesions occur. This is indeed the case where a number of studies have reported that α_1 -PI constitutes the majority of the anti-elastase activity in the normal lower respiratory tract, unlike many of the other major protease inhibitors, which are only present in minor amounts (eg. SLPI, α_2 -M).

While adequate levels of α_1 -PI have been shown to prevent emphysema, the absence of α_1 -PI does not necessarily lead to the development of emphysema. This is found in individuals with the homozygous form of α_1 -PI deficiency (PI Z), in which a recent study has estimated that only 10-15% of such individuals develop emphysema (Knight et al., 1997). This suggests that factors in addition to α_1 -PI deficiency play a role in the development of emphysema. One of the other factors could be a deficiency in one of a number of other HLE inhibitors, such as SLPI, elafin, α_2 -M, α_1 -ACH or M/NEI. However, only a deficiency in either α_1 -ACH or α_2 -M has been reported to be associated with the development of emphysema in some individuals, but neither have been linked with α_1 -PI deficiency. While there is no reported association between an inherited deficiency of α_1 -PI and the other known HLE inhibitors, there is more of a

likelihood of association between α_1 -PI and certain inhibitors, which are subject to oxidative inactivation in the more common smoking induced form of emphysema. For example, SLPI, elafin and M/NEI are inactivated by oxidants similar to that reported for α_1 -PI. It is possible that variable inactivation of these other inhibitors by smoking and/or oxygen free radicals together with oxidative inactivation that has been reported for α_1 -PI could explain the different susceptibilities of individual smokers to develop emphysema. In conclusion, the contribution, if any, of these other inhibitors and their relative oxidative status in smokers to the development of emphysema remains to be determined.

Another factor in the variability of the development of emphysema between individuals could be explained by differences in the susceptibility of the lung matrix to HLE activity due to subtle genetic differences in the composition of the lung matrix. For example, variations in the sulphated GAGs of the extracellular matrix of the lung could conceivably contribute to variable HLE digestion of the lung as has been reported *in vitro* where sulphated GAGs have shown variable inhibitory activity towards HLE. Similarly, a change of sequence or structure of some of the proteins in the lung extracellular matrix, such as has been shown with the different isoforms of elastin, collagen and fibronectin (Boyd et al, 1993), may also contribute to an alteration in the susceptibility of the lung matrix to HLE activity. At present the extent and the significance of variations in the composition of the extracellular matrix of the lung and the effect that this variation has on HLE digestion of the lung is not known.

Role of cigarette smoking in emphysema

Although smoking is the major risk factor for the development of emphysema, with a direct relationship between the amount of cigarettes smoked and respiratory damage, there is a wide variation in the susceptibility to develop progressive emphysema, as demonstrated by the fact that only 10-20% of tobacco smokers develop clinically evident emphysema. One genetic factor

that has been reported to explain some of this variability is the finding that an α_1 -PI deficiency (PI Z) in a smoker results in the much earlier development of emphysema (approximately 20 years), which probably reflects the severely limited antiprotease reserves (less than 20% of normal) in the smoker's lungs (Tetley, 1993). However, no other host or familial factors that make individual smokers more susceptible to emphysema have, at present, been identified. Until these other variables are identified, it is likely that mechanisms involved in the development of emphysema will not be fully understood.

Understanding the mechanism of emphysema generated by smoking has been hampered by the failure to cause true emphysema lesions in animals by prolonged exposure to cigarette smoke. While the obvious species difference is the major factor, there is also the problem that most of these studies have been of a short duration in comparison to the lifetime habit in humans. Another factor is that, as in human studies, there is likely to be variable susceptibility of individual animals to the effects of tobacco smoke which is exacerbated by the small number of animals used in some of the reported studies. In human studies, the most likely hypothesis to explain the development of smoking induced emphysema is that increased numbers of neutrophils are attracted into the lungs by the tobacco smoke and tar. The neutrophils are activated at the site of smoke particles on the lung surface, releasing HLE and oxidants, which combined with the oxidants in tobacco smoke, inactivate α_1 -PI and possibly other inhibitors of HLE. The result is an imbalance between HLE and its inhibitors localised at the site of the smoke particles on the lung surface resulting in conditions favourable for HLE activity. However, there are large gaps in the available evidence to support this hypothesis. In particular, the evidence for the inactivation of α_1 -PI by tobacco smoke and leukocyte generated oxidants and free radicals in the lungs of smokers is inconclusive and awaits further research.

Conclusion

Despite the weakness in a lot of the available evidence, together with the complexity of the problem and, in particular, the difficulty in getting data from human studies, the protease-antiprotease hypothesis still provides a useful framework for the investigation of the pathogenesis of emphysema. This is despite the fact that the identities of the candidate protease(s) and antiprotease(s) are still unclear. Considerable evidence implicates HLE as the main protease candidate in the pathogenesis of injury to elastic fibres in the lung parenchyma that leads to the subsequent development of pulmonary emphysema. Other elastolytic enzymes have been shown to be released from leukocytes (eg. proteinase 3, cathepsin G, metalloproteinases) or secreted by monocytes (leukocyte type enzymes) and alveolar macrophages (metalloproteinases, ingested leukocyte enzymes) and possibly by other resident cells in the lung. It is possible that they may play a role in the pathogenesis of emphysema. After examining the literature, I consider that the above elastolytic enzymes play a secondary role to HLE as the primary elastolytic enzyme in the development of emphysema. For example the different subclasses of emphysema, such as centrilobular, panacinar, etc., is due to HLE activity but that the relative protease concentration imbalance in that part of the lung may be initially contributed to by other elastolytic enzyme (eg. Cathepsin G, which works synergistically with HLE). I consider that HLE is still the main protease part of the protease-antiprotease hypothesis and for this reason a study of its activity forms the major part of this thesis.

The increased incidence of emphysema in individuals with an inherited α_1 -PI deficiency (e.g. PI Z) can be partially explained by an inadequate level of functional α_1 -PI, as the main antiprotease, although the fact that emphysema does not occur in all individuals suggests that other, as yet unexplained factors, are involved. However, in the more common smoking induced form of emphysema there is even less evidence to explain how the disease arises. It is still unclear as to whether the disease arises from either the smoke components and the

inflammatory cell products inactivating α_1 -PI and other inhibitors (eg. SLPI) or simply from the increased elastolytic load on the lung overwhelming the antiproteases or a combination of both.

From the above literature review it is concluded that HLE is the best candidate for the protease and α_1 -PI is the most favoured or key antiprotease in the protease-antiprotease hypothesis. For this reason, the primary aim of the work in this thesis was to examine *in vitro* the protease-antiprotease hypothesis with HLE as the main protease component and α_1 -PI as the main antiprotease component. Initial studies examined the functional activity of α_1 -PI in smokers and in patients with emphysema so as to detect the presence of inactivated α_1 -PI. If the protease-antiprotease theory holds true, a reduction in antiprotease levels in smokers and patients with emphysema, as a result of inactivation of α_1 -PI, should lead to a protease imbalance which should be detected by sensitive assays developed as part of this thesis project.

During the course of measuring enzymatic activity of HLE, it was noted that a number of components in the assay systems influenced the outcome of the assays. A detailed study using the HLE assay has shown that these components included proteins (eg. elastin peptides), surfactants (eg. pulmonary surfactant), GAGs (eg. heparin), and a number of endogenous and exogenous inhibitors. As HLE is known to have a broad substrate specificity that allows it to bind and interact with numerous structural protein components and metabolites in the lung, it was not surprising that a number of lung components were found to bind to HLE. What was surprising was the finding that a number of lung components have an inhibitory effect towards HLE which supports the proposal that components, in addition to the defined lung antiproteases, may play a significant regulatory function on HLE activity in the lung and could partly explain the variable development of emphysema in α_1 -PI deficient individuals and smokers. In particular, it has been shown that sulphated GAGs, particularly heparin, are potent inhibitors of HLE. A detailed study of the binding of, and modifying effects of heparin on, HLE

has been completed, resulting in the finding that there are high-affinity binding sites for HLE along the heparin chain. This finding raises the possibility that GAGs, such as heparin, may have an important regulatory influence on HLE activity in the lungs. It is postulated that they function as a component of the antiprotease screen on the lung surface.

Hypothesis and Aims of this study.

1. To demonstrate that the protease - antiprotease hypothesis holds true under conditions in which HLE is the principal protease and α_1 -PI is the principal antiprotease.
2. To determine whether other specific and non-specific inhibitors have a functional role in the protease - antiprotease hypothesis.
3. To examine the relative specificity and effectiveness of a variety of other specific and non-specific inhibitors towards HLE.
4. To apply these data to the proposal that HLE is the major candidate in the protease - antiprotease hypothesis.

CHAPTER TWO

MATERIALS AND METHODS

2.1 INTRODUCTION

The studies reported in the following chapters describe a number of the aspects of the interaction of HLE with a range of endogenous and synthetic inhibitors so as to further investigate the central role of this enzyme in the elastase - anti-elastase model of a number of inflammatory diseases such as pulmonary emphysema. A large part of this study relied on assays for HLE activity under a variety of assay conditions. Variations of assays for this enzyme together with other methods and the materials used are described in this chapter.

All chemicals and biochemicals described throughout this thesis were of analytical grade or better if available.

2.2 MATERIALS

2.2.1 ENZYMES

HLE (EC 3.4.21.37; 875 units/mg of protein) was from Elastin Products Co., USA. HLE was demonstrated to be at least 90% active using previously published kinetic constants with Suc-A-A-A-NA as substrate (Nakajima et al., 1979) as described in Section 4.2.

Other human leukocyte enzymes used were cathepsin G (EC3.4.21.20; Elastin Products) and myeloperoxidase (EC 1.11.1.7; Calbiochem Corp, CA. USA).

Pancreatic enzymes used were PPE (EC 3.4.21.36; Calbiochem), trypsin (EC 3.4.21.4; porcine type IX; Sigma Chemical., USA) and chymotrypsin (EC 3.4.21.1; bovine type 11; Sigma).

Heparin lyase I (heparinase, EC 4.2.2.7 and heparin lyase II (heparitinase II, no assigned EC number) were from Seikagaku Kogyo, Tokyo, Japan.

2.2.2 NON-ENZYME PROTEINS AND PEPTIDES

α_1 -PI (Cat# A9024)	Sigma, USA
α_2 -M	Sigma.
α -Benzoyl-DL-arginine-p-nitroanilide (trypsin substrate)	Sigma.
Aprotinin (Trasylol, 10,000 KIU/mL)	Bayer AG, Germany.
Elastin peptides (α -elastin) preparation	“In house” preparation. (Section 2.3.6)
Human albumin	Behringwerke AG, Germany.
Human antithrombin III	Calbiochem, USA
Human lung elastin	Elastin Products, USA
Lung surfactant protein	Gift from Dr. J. Powers Flinders University, Australia.
MeO-Suc-A-A-P-V-NA (elastase substrate)	Sigma.
n-Acetyl-L-alanyl-L-alanyl-L-azalanorvaline p-nitrophenyl ester	Auspep, Australia.
n-Succinyl-(L-alanine) ₂ -proline-L-phenylalanine-p-nitroanilide (chymotrypsin and cathepsin G substrate)	Sigma.
Suc-A-A-A-NA (elastase substrate)	Calbiochem.
VPGVG & VGVAPG (custom synthesised)	Auspep.

2.2.3 GLYCOSAMINOGLYCANS

Chondroitin sulphate A (bovine trachea)	Sigma.
Chondroitin sulphate B (dermatan sulphate, pig skin)	Seikagaku Kogyo, Japan.
Chondroitin sulphate C (shark cartilage)	Seikagaku Kogyo.
Heparan sulphate (bovine kidney)	Sigma.
Heparan sulphate (bovine intestinal)	Sigma

Heparan sulphate (fast form, bovine intestinal)	Sigma
Heparin (porcine)	David Bull Laboratories, Australia.
Heparin (porcine)	Fisons, U.K.
Heparin (ovine)	David Bull Laboratories.
Heparin (bovine, Cat# H-5765)	Sigma.
Heparin (source not stated)	Commonwealth Serum Laboratories, Australia.
Heparin (OP 381/2, M_r 850-1000)	Opocrin S.P.A., Italy
Heparin (OP 381/1, M_r 2000)	Opocrin S.P.A.
Heparin (OP 2123, M_r 4500)	Opocrin S.P.A.
Heparin (Kabi 2165, M_r 4000-6000)	Kabi Vitrum AB, Sweden
Heparin tetra-, hexa-, and octa-saccharide gel marker	Gift from Dr. Turnbull, University of Manchester, U.K.
[³ H]-heparin & [³ H]-heparan sulphate	“In house” preparation. (Section 2.3.3)
Hyaluronic acid (rooster combs)	Pharmacia, AB, Sweden.

2.2.4 OTHER REAGENTS

Alcian Blue	Sigma.
Antiserum to human α_1 -PI (Cat. No. 003-11)	Atlantic Antibodies, USA.
2, 2'-Azinobis(3-ethylbenzthiazoline)-6-sulphonic acid	Sigma.
Biogel P2	Bio-Rad, USA
Brij 35 (polyoxyethylene monolauryl ethers)	Sigma
Bromphenol Blue	Sigma.
Chlorophenol Red	Aldrich, USA.

CNBr-activated Sepharose 4B	Pharmacia.
Complete and incomplete Freund's adjuvant.	Commonwealth Serum Labs
D-Glucuronic acid lactone	Sigma
1,9-Dimethylmethylen blue	Koch-Light, U.K.
Dimethylsulphoxide	Sigma.
EAH-Sepharose 4B & ECH-Sepharose 4B	Pharmacia.
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride	Calbiochem.
ICI 186756 (low molecular weight aldehyde inhibitor of HLE)	ICI Americas, Wilmington, USA.
ICI 200355 (a peptide trifluoromethyl ketone inhibitor of HLE)	ICI Americas
Lung surfactant	"In house" preparation. (Section 2.3.7).
Meta-hydroxydiphenyl	K & K Laboratories, USA
NaB ³ H ₄	Amersham, Australia.
Peroxidase-conjugated swine antibody to rabbit immunoglobulin	Dako A/S, Glostrup, Denmark.
Phenylmethanesulphonyl fluoride	Sigma.
Protein Standard Plasma (Human, Cat. # OTF1-03, stated α_1 -PI value 4.2 g/l)	Calbiochem.
Rabbit antiserum to elastin peptides (α -elastin) preparation	"In house" preparation. (Section 2.3.8)
Sodium dodecyl sulphate (SDS)	BDH Chemical, Poole, U.K.
Triton X-100 (octylphenol-polyethyleneglycolether-formaldehyde polymer)	BDH
Tween 80 (polyoxyethylene sorbitan mono-oleate)	BDH

2.3 METHODS

2.3.1 BRONCHOALVEOLAR LAVAGE

BAL was performed as described previously (Bell et al., 1981; Walsh et al., 1992). Briefly, subjects were premedicated with atropine 0.6 mg intramuscularly. A 0.5% (w/v) solution of lignocaine was used for topical anaesthesia of the airways. An Olympus IT bronchoscope (Olympus, Japan) was wedged into a subsegment of the right middle lobe. Three 50 mL aliquots of sterile 140 mM physiological saline were instilled and recovered by gentle aspiration into separate containers. A portion of the second recovered specimen was used for differential and total cell counts. Aliquots 2 and 3 were then pooled and centrifuged at 2000 x g for 10 min to remove cells and debris. Half the supernatant was concentrated to one-fiftieth of the original volume for normal BAL and one-fifth for ARDS BAL (see Assay Sample Handling, Section 2.4.1.4) in a Minicon B15 concentrator (Amicon Corp., USA) and frozen with the unconcentrated supernatant and a matched serum (collected within 20 min after the lavage) at - 70 °C until analysed.

2.3.2 PREPARATION OF HUMAN LEUKOCYTE ELASTASE-SEPHAROSE GELS

HLE-Sepharose gels were prepared by adding 2 mg of HLE to a 3 ml suspension of EAH-Sepharose 4B or ECH-Sepharose 4B gel with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride as condensation agent according to a standard procedure provided by Pharmacia. All solutions were adjusted to pH 4.5 prior to mixing. The mixture was mixed end-over-end for 24h at room temperature. Approximately 50 – 70% of HLE was estimated to be bound to the gels (approx. range of 0.3 to 0.4 mg of HLE / ml of gel) by estimation of residual HLE activity using the assay described in Section 2.4.2.2.

2.3.3 PREPARATION OF [³H]-HEPARIN AND [³H]-HEPARAN SULPHATE.

[³H]-heparin and [³H]-heparan sulphate were prepared by reduction of the reducing-end anhydromannose of heparin (porcine, David Bull) or heparan sulphate (bovine kidney, Sigma) chains by adding 0.1 ml of NaB³H₄ (10 mCi) in 0.1 M NaOH to 4 mg of Heparin (porcine, David Bull) in 0.9 ml of 0.05 M sodium phosphate buffer (pH 7.4) and adjusting the pH to 7.4 with 0.1 M HCl. The solution was allowed to stand for 2 hr. in ice and then 18 hr. at 4°C. Excess NaB³H₄ was destroyed by acidification in a fume hood followed by dialysis against the phosphate buffer. Specific activity of [³H]-heparin and [³H]-heparan sulphate was typically in the range of 4 – 6 x 10⁶ cpm / mg. The specific activity of the end-labelled heparin was typical for the procedure used in this study, although higher activity could have been achieved with changes to the procedure. Nevertheless the activity was satisfactory for this study. CPM was used as the units throughout this thesis as all values were comparisons between data from the same β-counter using the same assay conditions and instrument settings

2.3.4 PREPARATION OF ANTITHROMBIN –SEPHAROSE.

Human antithrombin III (2 mg) was coupled via amino groups to approximately 5 ml of CNBr-activated Sepharose 4B with the recommended procedure provided by Pharmacia with the activated Sepharose except that the reaction was performed in the presence of excess amounts of acetylated heparin as described by Hook et al (1976). The acetylation of any free amino groups present on heparin should prevent any coupling of heparin to the Sepharose beads. Acetylated heparin was prepared by treating heparin with acetic anhydride (Danishefsky and Steiner, 1965). Excess acetylated heparin was added to the antithrombin III to saturate the heparin binding site on the antithrombin molecules and thus prevent this site from binding to the Sepharose beads.

2.3.5 PREPARATION OF 12-22 % ACRYLAMIDE GELS.

Discontinuous PAGE gels with a linear acrylamide and cross-linker gradient (12-22% total acrylamide, pH 8.3) were prepared as previously described (Rice et al., 1987) but with a number of modifications which are described as follows. The gels (20x40x0.15 cm) were prepared by adding 60 mL of 12% gel into the reservoir and 60 ml of 22% gel into the mixing chamber of the linear gradient maker (Pharmacia). The acrylamide solutions were then pumped at 10 ml/min with a peristaltic pump (model CH4A-302, Amicon, USA) into the top of the gel casting unit (essentially two offset glass plates and spacers sealed with tape and held with clamps; Owl Scientific, U.S.A) forming a linear gradient from the bottom to the top. The top of the gel was layered with approximately 0.5 ml of distilled water, sealed and allowed to stand overnight before use.

2.3.6 ELASTIN PEPTIDES (α -ELASTIN) PREPARATION.

The soluble elastin peptides (α -elastin, approx. 70,000 molecular weight as determined by SDS PAGE) were prepared from insoluble human lung elastin by hydrolytic cleavage with 0.25 M oxalic acid for 1 h at 100 °C according to the method of Partridge et al (1985). This hydrolysis of the elastin sample was repeated five times. The protein concentration of the dialysed soluble elastin peptides was determined by the Folin-Lowry method (Lowry et al., 1952) using human albumin as standard.

2.3.7 ISOLATION OF PULMONARY SURFACTANT.

Surfactant was isolated from washings of sheep lung according to the procedure described by Hawgood et al (1985). Briefly, lungs were lavaged with 5 mM Tris/HCl, 100 mM NaCl, pH 7.4. The lavage was centrifuged at low speed (150 g) to remove cellular material and then at high speed (20,000 g) to pellet the surfactant which was then washed and stored at -70° C.

All procedures, including the lavage, were performed at 4° C. Phospholipid content was calculated according to the method used by Bartlett (1959).

2.3.8 RABBIT ANTISERUM TO ELASTIN PEPTIDES (α -ELASTIN) PREPARATION.

New Zealand white rabbits were purchased from the South Australian Government Department of Agriculture (Gilles Plains, South Australia). Antisera of high titre that was successfully used in the ELISA for elastin peptides (see Section 2.4.4) were raised in two rabbits as reported previously (Dillon et al., 1992) and is described briefly as follows. The primary inoculation was administered subcutaneously with multisite injections. Each rabbit received 0.5 mg of an elastin peptides (α -elastin) preparation (see Section 2.3.6) in a total volume of 0.5 ml of saline emulsified in an equal volume of complete Freund's adjuvant. Subsequent immunisations at weekly intervals were given as 0.25 mg of elastin peptides in 0.5 ml of saline with an equal volume of incomplete Freund's adjuvant for at least 3 months. When the antiserum was of sufficient titre, the rabbits were bled by venipuncture and the antiserum was stored at 4°C with 0.02 % sodium azide.

2.3.9 AFFINITY CHROMATOGRAPHY OF HEPARIN ON HUMAN LEUKOCYTE ELASTASE-SEPHAROSE

Heparin (4 mg; porcine, David Bull) with [³H]-heparin (approx. 3 x 10⁶ cpm, see Section 2.3.3) as tracer in a volume of 0.75 ml was loaded onto a column of either HLE-EAH- or HLE-ECH-Sepharose gel (see Section 2.3.2) in 50 mM/l Tris/HCl, 50 mM/l NaCl, 0.01 % Brij 35, 0.02% NaN₃, pH 7.5 buffer and washed with 10 ml of the same buffer with a flow rate of 0.3 ml/min. Bound heparin fractions were eluted with a 70 ml linear 0.05 – 2 M/l NaCl gradient in the same buffer. Fractions of 1 ml were collected and radioactivity in each was determined. Fractions containing bound heparin peaks were pooled, dialysed (Spectra/por dialysis tubing,

MWCO:3500, Spectrum, USA) against distilled water, lyophilised and reconstituted with distilled water to original column load volume. Heparan sulphate (bovine kidney, Sigma) was also fractionated on HLE-Sepharose by essentially the same procedure.

2.3.10 AFFINITY CHROMATOGRAPHY OF HEPARIN ON ANTITHROMBIN-SEPHAROSE.

The affinity gel was prepared (see Section 2.3.4) and heparin was separated on the gel into high and low affinity fractions following the principles of a previously described method (Hook et al., 1976). Affinity chromatography was performed with 100 μ g of heparin with [3 H]-heparin as tracer with approximately 70% (low affinity fraction) not being adsorbed in the presence of 150 mM NaCl. The remaining bound heparin (high affinity fraction) was eluted with 3M NaCl. The low affinity fraction was recycled on the column to remove any remaining high affinity heparin. The high and low affinity pools were then dialysed (Spectra/por, MWCO:3500) against distilled water, lyophilised and reconstituted with distilled water to original column load volume.

2.3.11 DIGESTION OF HEPARIN WITH HEPARIN LYASES.

2.3.11.1 HEPARIN BOUND TO HUMAN LEUKOCYTE ELASTASE-SEPHAROSE.

Heparin (1 mg; porcine, David Bull) with [3 H]-heparin as tracer (approx. 0.7×10^6 cpm) was bound to HLE-EAH-Sepharose in 5 mM phosphate, 100 mM NaCl, pH 7.15 buffer and then incubated with heparin lyases (Seikagaku Kogyo) for 30 hours at 30 $^{\circ}$ C. Heparin lyase I (heparinase, EC 4.2.2.7); 5 miu and heparin lyase II (heparitinase II, no assigned EC number); 5 miu were added at the start of the incubation with further two lots of 5 miu of heparin lyase I added at approximately 10 and 20 hours because of the instability of this enzyme (see Lohse and Linhardt, 1992). Digested heparin and heparinases are then eluted off the gel with the same buffer. The digestion procedure was then repeated to ensure complete digestion of unbound or loosely bound heparin chains. The remaining tightly bound fraction was then eluted off the gel

with 2 M NaCl, desalted on a 360 x 20 cm Biogel P2 column (Bio-Rad) and concentrated by lyophilisation. All fractions were boiled for 1 min at 100°C to inactivate any residual enzyme. Unbound heparin was also digested as a control.

2.3.11.2 HEPARIN BOUND TO HUMAN LEUKOCYTE ELASTASE

The digestion procedure is as described above in 2.3.11.1 except that free HLE was used in place of HLE-EAH-Sepharose and that the digestion procedure was not repeated. At the end of the 30 hr. digestion period, the heparin/HLE/heparinase mixture was dialysed against 50 mM Tris/HCl, 100 mM NaCl, pH 8.0 using Spectrapor dialysis tubing (MWCO:3500, Spectrum) to remove digested and unbound heparin fragments. To isolate the remaining bound heparin fragments from HLE and the heparinases, an apoprotin-Sepharose affinity column was used in a procedure reported by Martodam et al., 1979. The apoprotin-Sepharose was prepared by coupling aprotinin (20 mg) via amino groups to approximately 10 ml of CNBr-activated Sepharose 4B with the recommended procedure provided by Pharmacia. Prior to chromatography, NaCl was added to the dialysed heparin/HLE/heparinase mixture to bring the concentration to 1M so as to disassociate the undigested heparin from HLE. The mixture was added to the affinity column and then washed with 1M NaCl in the above Tris buffer to elute the heparin fractions which were desalted on a 360 x 20 cm Biogel P2 column (Bio-Rad) and concentrated by lyophilisation. The HLE was eluted from the apoprotin-Sepharose column with 50 mM sodium acetate, 1M NaCl, pH 8.0.

2.3.12 ELECTROPHORETIC SEPARATION OF HEPARIN FRACTIONS WITH 12-22 % POLYACRYLAMIDE GEL ELECTROPHORESIS

Electrophoresis was performed with two resolving gel buffers as previously described (Rice et al., 1987) but with a number of modifications described, as follows. A comb of 20 teeth of 20 x 5 x 1.5 mm was used as a well former in the stacking gel which allowed 40 µl samples added

together with 40 μ l of 50% (w/v) sucrose. An oligosaccharide marker consisting of a tetrasaccharide, a hexasaccharide and an octasaccharide fraction prepared from porcine heparin was used to estimate the size of the heparin fragments. Electrophoresis was run on an adjustable vertical electrophoresis system (Owl Scientific) at 600 V in a 4 °C room to provide cooling for 16 hours or until the gel markers Bromphenol Blue (1 mg/ml) and Chlorophenol Red (1 mg /ml) had moved approximately 35 cm and 38 cm respectively into the resolving gel. The gel was removed from the plate and stained and fixed in 0.5% (w/v) Alcian Blue in 2% (v/v) acetic acid for 30 min. followed by destaining with several washes of 5% acetic acid. The bands were made visible with silver staining using a method described previously (Al-Hakim and Linhardt, 1991).

2.3.13 HIGH PRESSURE LIQUID CHROMATOGRAPHY ESTIMATION OF THE SIZE OF ELASTIN PEPTIDES IN THE PRESENCE OF HEPARIN.

Human lung elastin (10 mg) in 1 ml of 50 mM Tris/HCl, 140 mM NaCl and 0.02% sodium azide pH 7.8 containing 100 μ g of HLE was incubated at 37°C for 60 h. An identical preparation, but with 8 mg of heparin added, was also used. HLE activity was stopped with 50 μ l of 5 mg of phenylmethanesulphonyl fluoride per ml of dimethylsulphoxide. Samples were centrifuged and the protein concentration was determined in the supernatants. The weight of residual elastin was determined by weighing the dried pellets. The supernatants were freeze-dried and reconstituted in 200 μ l of distilled H₂O. Samples (10, 20 or 50 μ l) were loaded on to a Bio-Sil gel filtration column (SEC-400; 300 mm x 7.5 mm; Bio-Rad Laboratories, Richmond, USA) with a guard column (75 mm x 7.5 mm, Bio-Rad). The HPLC instrument (Varian 5000; Varian Associates Inc., Palo Alto, USA) was used with an on-line ultraviolet detector and a Varian 4270 integrator. Sodium phosphate buffer (100 mM, pH 6.8) was used with a flow rate of 1.0 ml/min. The column was calibrated with a mixture of proteins of known Mr (Bio-Rad gel filtration standard).

2.3.14 HIGH PRESSURE LIQUID CHROMATOGRAPHY OF HEPARIN FRACTIONS

Size exclusion chromatography was used to estimate size differences between heparin fractions isolated by HLE-Sepharose chromatography (see Section 2.3.9). The HPLC Bio-Sil gel filtration column and procedure are the same as described in Section 2.3.13 except that the column was fitted to a Bio-Rad HPLC 7000 system with a 1706 UV monitor set at 205 nm (Bio-Rad).

2.4 ASSAYS

2.4.1 α_1 -PI AND ELASTASE INHIBITORY FUNCTION OF PLASMA, SERUM, AND BRONCHOALVEOLAR LAVAGE.

2.4.1.1 *INSTRUMENTATION.*

A Cobas Bio centrifugal analyser with a DENS (Data Evaluation of Nonlinear Standards) curve option from Hoffman-LaRoche, Basle, Switzerland. Analyser settings used are listed in Table 1 and have been previously reported (Walsh et al., 1992).

2.4.1.2 *ASSAY STANDARDS.*

Protein Standard Plasma (human, stated α_1 -PI value 4.2 g/l) was our routine laboratory standard which had previously been used to determine serum reference intervals. The use of this commercial standard allowed a comparison of the assay values obtained for the volunteer and patient groups in this study with the previously derived reference range (1.6-3.5 g/l). The α_1 -PI in this standard was shown to be fully active in the functional assays by reducing the standard with 2-mercaptoethanol (Johnson and Travis, 1978). No inhibition was seen in the functional assay if the α_1 -PI was inactivated by oxidising the standard with n-

chlorosuccinimide (Jori et al., 1968), demonstrating that in plasma the inhibition is due to α_1 -PI alone. This standard was used as the assay calibrator in the assays described below in this section.

Purified α_1 -PI was functionally titrated against an active site-titrated preparation of PPE. The azapeptide, N-acetyl-L-alanyl-L-alanyl-L-azavanorvaline p-nitrophenyl ester was used as the active site titrant as described by Powers et al (1984). The purified α_1 -PI demonstrated that the commercial standard had a value 20-30% too high, which is consistent with other commercial preparations as discussed by Wewers et al (1987). However, since our routine laboratory assay and reference intervals are derived using this commercial standard value, we have for convenience used the stated commercial concentration in this study.

2.4.1.3 *SUBJECTS*

Serum samples were collected from 17 normal children (age <15 years). Normal adult serum samples (35 non-smokers, 20 smokers) were from a voluntary blood donor service. Serum samples were also collected from patients with established ARDS (n = 5) admitted to the Intensive Care Unit of the Royal Adelaide Hospital, and from pulmonary emphysema patients. Patients with emphysema (n = 19) were defined clinically by the presence of bullous disease on chest x-ray, and abnormal results for lung function tests. The Mann-Whitney U-test was used to test the significance of differences between subject groups.

BAL was performed in the following subjects. One group consisted of eight volunteers with no history of pulmonary disease, normal clinical examination and normal pulmonary function as demonstrated by spirometry, single breath diffusion capacity for carbon monoxide, and pulmonary elastic recoil using exponential analysis of the pressure volume curve (Colebatch et al., 1979b). Four subjects were life-long non-smokers, and the other four

were current smokers with a history of at least 30 pack-years. The age range was 30-50 years. The smokers were allowed to smoke up to the time of the pre-medication injection, this being approximately 30 minutes prior to the BAL. The second group consisted of 5 ARDS patients who were clinically defined by a partial pressure of oxygen of less than 75 mm Hg with an inspired oxygen of 0.5 or greater, new diffuse bilateral infiltrates on x-ray, and a pulmonary artery wedge pressure of less than 18 mm Hg. Patients with clinical evidence of primary bacterial pneumonia or bronchoscopic evidence of bronchitis were excluded.

All subjects who participated in this study gave informed consent.

2.4.1.4 *ASSAY SAMPLE HANDLING.*

A preliminary study to establish whether anticoagulants interfered in the assays was performed on blood samples from 7 normal volunteers collected into heparin (12.5 International units/ml), sodium citrate (12 mM), dipotassium EDTA (5 mM) and sodium fluoride/potassium oxalate (23 mM/15 mM). All assays were optimised for linearity between 5 and 50 mg/l of α_1 -PI. To bring readings within this range all plasma and serum samples were diluted 1 in 100 with saline prior to assay. Because of the wide range of α_1 -PI concentrations found in normal volunteer BAL ($n = 8$, 0.22-3.75 mg/l) and particularly in ARDS patients BAL ($n = 5$, 9.0-400 mg/l), both unconcentrated and concentrated BAL samples were used to prepare serial dilutions to obtain 3 assay readings where possible within the linear portion of the assay standard plots (5-50 mg/l).

2.4.1.5 *ESTIMATION OF α_1 -PROTEASE INHIBITOR WITH PORCINE PANCREATIC ELASTASE INHIBITION ASSAY.*

This assay has been previously reported (Walsh et al., 1992). The assay buffer was 50 mM Tris HCl, 50 mM NaCl, 0.01% (v/v) Brij 35, pH 8.0. PPE was prepared in the assay buffer as a stock 1 g/l reagent and stored at -70° C in 50 μ l aliquots. The stock HLE was diluted 1 in 250 in the assay buffer to give the working enzyme concentration of 4 mg / l such that 1.0 μ g is added in the “reagent volume” (Table 2.1). Suc-A-A-A-NA was used as the elastase substrate. It was prepared as a 0.2 mM stock solution in dimethyl sulfoxide and was diluted in the assay buffer 1 in 50 to give a concentration of 4 mM/l for the “start reagent”.

2.4.1.6 *ESTIMATION OF α_1 -PROTEASE INHIBITOR WITH HUMAN LEUKOCYTE ELASTASE INHIBITION ASSAY.*

This assay has been previously reported (Walsh et al., 1992). HLE was prepared in the assay buffer (see in Section 2.4.1.5) as a stock 1 g/l reagent and stored at -70° C in 50 μ l aliquots. The stock HLE was diluted 1 in 500 in the assay buffer to give the working enzyme concentration of 2 mg/l such that 0.5 μ g is added in the “reagent volume” (Table 2.1). MeO-Suc-A-A-P-V-NA was used as the elastase substrate. It was prepared as a 0.2 M stock solution in dimethyl sulfoxide and was diluted in the assay buffer 1 in 50 to give a concentration of 4 mM for the “start reagent”.

2.4.1.7 *IMMUNO-TURBIDIMETRIC α_1 -PROTEASE INHIBITOR ASSAY.*

This assay has been previously reported (Walsh et al., 1992). The assay buffer was 15 mM phosphate, 140 mM NaCl, 15 mM NaN_3 pH 7.4 containing 4% (w/v) polyethylene glycol (PEG) Mr 6000. Antiserum to human α_1 -PI was diluted 1 in 50 in the buffer/ PEG solution to give the working reagent that is added as the “reagent volume” (Table 2.1).

2.4.2 MEASUREMENT OF ELASTASE INHIBITION.

2.4.2.1 *HUMAN LEUKOCYTE ELASTASE (2.5 µg) WITH SUC-A-A-A-NA AS SUBSTRATE.*

This assay has been previously reported (Walsh et al., 1991a) and was performed on a Cobas-Bio centrifugal analyser as follows. Eighty µl of sample (e.g. a HLE inhibitor) was mixed with 250 µl of the assay buffer (see in Section 2.4.1.5), containing 2.5 µg of HLE. After incubation at 25°C for 60 s, the residual HLE activity was measured by the addition of 20 µl of 5 mM of Suc-A-A-A-NA in the same buffer. The rate of hydrolysis was monitored at 405 nm for 30 min. Residual HLE activity in the presence of an inhibitor was expressed as a percentage of the activity found in the absence of the inhibitor.

2.4.2.2 *HUMAN LEUKOCYTE ELASTASE (0.5 µg) WITH MeO-SUC-A-A-P-V-NA AS SUBSTRATE (15 µl sample)*

This assay was performed on the Cobas-Bio centrifugal analyser with as follows. Briefly, 15 µl of sample (eg, a HLE inhibitor) was mixed with 250 µl of the assay buffer (see in Section 2.4.1.5) containing 0.5 µg of HLE. After incubation at 25°C for 60 s, 20 µL of 5 mM of MeO-Suc-A-A-P-V-NA in the same buffer was added. The rate of hydrolysis was monitored at 405 nm for 5 min. Residual HLE activity in the presence of an inhibitor was expressed as a percentage of the activity found in the absence of the inhibitor.

2.4.2.3 *HUMAN LEUKOCYTE ELASTASE (0.5 µg) WITH MeO-SUC-A-A-P-V-NA AS SUBSTRATE (75 µl sample).*

This assay is a modification of that described in 2.4.2.2 but with sample, enzyme and substrate volumes being of equal volume to allow variations in the order of addition of reagents. Briefly the assay used 75 µl of sample (eg, a HLE inhibitor), 75 µL of assay buffer

(see in Section 2.4.1.5) containing 0.5 μg of HLE and 75 μl of 0.75 mM of MeO-Suc-A-A-P-V-NA in the same buffer.

2.4.2.4 HUMAN LEUKOCYTE ELASTASE (150 ng) WITH MeO-SUC-A-A-P-V-NA AS SUBSTRATE

This assay is a more sensitive variant of the above assay (Section 2.4.2.2) and was used for monitoring the inhibitory activity of heparin fractions from affinity column separations (Section 2.3.9) and heparinase digests of heparin (Section 2.3.11). Briefly, 80 μl of a suitably diluted heparin fraction was mixed with 150 μl of the assay buffer (see in Section 2.4.1.5), containing 150 ng of HLE and then the residual HLE activity was measured with 20 μl of 2.5 mM of MeO-Suc-A-A-P-V-NA in the same buffer. The rate of hydrolysis was monitored at 405 nm for 15 min.

2.4.2.5 HUMAN LEUKOCYTE ELASTASE WITH HUMAN LUNG ELASTIN AS SUBSTRATE.

This assay has been previously reported (Walsh et al., 1991a). Elastin was added in triplicate to a microtitre tray as a 5 μg suspension (particle size <250 μm diameter) in 100 μL of 50 mM Tris, 140 mM NaCl and 0.02% NaN_3 , pH 8.0. HLE was added as 5 ng in 100 μl of the Tris buffer. Assay samples (eg, HLE inhibitors) were added in triplicate in 100 μl of the Tris buffer. After 24 h at 25°C in a humid chamber, 100 μl of the supernatant was removed and soluble elastin peptides were measured with an ELISA method (Section 2.4.4).

2.4.2.6 *PORCINE PANCREATIC ELASTASE WITH SUC-A-A-A-NA AS SUBSTRATE.*

This assay has been previously reported (Walsh et al., 1991a) and was performed as described for the HLE assay in Section 2.4.2.1 except that PPE was used in place of HLE and that the assay was monitored for 10 min rather than 30 min.

2.4.3 MEASUREMENT OF CATHEPSIN G, CHYMOTRYPSIN AND TRYPSIN INHIBITION.

These assays have been previously reported (Walsh et al., 1991a) and are described briefly below.

2.4.3.1 *CATHEPSIN G AND CHYMOTRYPSIN ASSAY.*

This assay was performed as described for the HLE assay in Section 2.4.2.1 except that cathepsin G (0.1 μg) or chymotrypsin was used in place of HLE with n-Succinyl-(L-alanine)₂-proline-L-phenylalanine-p-nitroanilide replacing the elastase substrate. The assay was monitored for 15 min. Assay variations included performing the assay at 37°C with a preincubation step of 30 min also at 37°C to detect any inhibition of this enzyme by heparin at this temperature.

2.4.3.2 *TRYPSIN ASSAY.*

This assay was performed as described for the HLE assay in Section 2.4.2.1 except that trypsin was used in place of HLE with α -Benzoyl-DL-arginine-p-nitroanilide replacing the elastase substrate. The assay was monitored for 15 min.

2.4.4 ELISA FOR ELASTIN PEPTIDES.

This assay has been reported previously (Dillon et al., 1990; Walsh et al., 1991a; Dillon et al., 1992). Briefly, an elastin peptide (α -elastin) preparation (see Section 2.3.6) was used to prepare standards. These standards together with unknowns were added to U-bottom microtitre plates in triplicate with a known amount of antisera (see Section 2.3.8) present in excess; the plates were then incubated overnight at 4°C. The assay mixture was then transferred to ELISA plates, which had been coated previously with 0.01 μ g of elastin peptides. The ELISA plates were incubated for 1 h at room temperature.

The presence of bound rabbit antibody on the ELISA plates was detected by the addition of peroxidase-conjugated goat antibody to rabbit immunoglobulin and subsequent colour development with 0.08% hydrogen peroxide and 2, 2'-azinobis(3-ethylbenzthiazoline)-6-sulphonic acid/mL. After sufficient colour development (usually 60 min.) the absorbance was read at 405 nm.

The antigen concentration of the unknowns was related to the standard curve (assay range 0.5-50 ng/ml), which was included on each plate to compensate for plate-to-plate variation. The inter-batch coefficient of variation was 5.2 % and the intra-batch coefficient of variation was 4.3 %, both at 0.4 ng/mL (n= 10).

2.4.5 URONIC ACID CONTENT OF GLYCOSAMINOGLYCANS.

This assay is a modification of that reported by Blumenkrantz and Asboe-Hansen, 1973. Briefly, 50 μ l of standards (0-200 μ g/ml of uronic acid prepared from D-glucuronic acid lactone) and unknown samples in duplicate were heated in a boiling water bath for 5 min in sealed glass digestion tubes together with 300 μ L of the sulphuric acid/tetraborate reagent. The samples were cooled on ice and each assay sample was split into two 140 μ l aliquots to which was then added 2 μ l of meta-hydroxydiphenyl. All samples were read within 5 min. at 520 nm on the Cobas-Bio centrifugal analyser. Thus four readings were taken on each assay sample.

The Cobas-Bio cuvette rotor and sample tip were immediately discarded after the assay so that the sulphuric acid/tetraborate reagent did not damage the analyser.

2.4.6 TOTAL SULPHATE CONTENT OF GLYCOSAMINOGLYCANS.

Heparin fractions and heparin standards in duplicate were boiled in 0.75 M HCl for 1 hr. in sealed glass digestion tubes and then lyophilised in a Speedvac Concentrator (Savant Instruments, USA). The lyophilised samples and standards were reconstituted in distilled water and the sulphate content was determined with a turbidimetric method (Lundquist et al., 1980) adapted to the Cobas-Bio analyser. The method is described briefly as follows:- 8 μ L samples of hydrolysed heparin fractions and heparin standards together with sulphate standards (0-50 mM/l sulphate in the form of sulphuric acid) were diluted with 80 μ L of distilled water, then acidified with 215 μ L of 0.2 mM/l HCl followed by 75 μ L of BaCl₂/polyethylene glycol/Na₂SO₄ reagent (as in Lundquist et al., 1980). The increase in turbidity due to BaSO₄ was determined at 600 nm after 5 min.

2.4.7 QUANTITATION OF SULPHATED GLYCOSAMINOGLYCANS.

This dye binding method was primarily used to monitor eluted heparin and heparan sulphate fractions from affinity column separations (Section 2.3.9). The assay used is a modification of the 1,9-dimethylmethylene blue method reported by Farndale et al (1986) which, in this study, was adapted to the Cobas-Bio analyser. The method is described briefly as follows:- 16 mg of DMB dye was dissolved in 50 ml of 20% ethanol (AR grade) and stored as 5 ml aliquots at -20°C. Before assay a thawed 5 ml aliquot of the dye was added to 100 ml of 0.2 M glycine/HCl buffer and the pH adjusted to pH 3.0 to make the working dye reagent. The assay uses 25 μ l of sample or heparin standards (0-50 μ g/ml) to which was added 150 μ l of the working dye reagent and the absorbance was read at 525 nm after 1 minute.

2.4.8 ASSAY FOR THE DETERMINATION OF THE KINETIC CONSTANTS OF HLE.

The assay used by Nakajima et al (1979) to determine the kinetic constants for HLE, with Suc-A-A-A-NA as substrate was adapted to the Cobas-Bio analyser. Briefly, 25 μ l of a variable amount of substrate (0.0156-8 mmol/L of Suc-A-A-A-NA) and 2.5 μ g of HLE in 250 μ l, both in 0.1M HEPES buffer (pH 7.5) containing 0.5 M NaCl and 10% dimethylsulfoxide, were incubated at 25^o C for 120 s. The rate of hydrolysis of the substrate was monitored at 405 nm for 30 min. after the substrate and enzyme were mixed. Assays were also run in the presence of 0.01% Brij 35 in the assay buffer.

Table 2.1: Cobas Bio settings

Parameter Listing	Immunoturbidimetric α_1 -PI Assay	PPE Inhibition Assay	HLE Inhibition Assay
1 Units	mg/l	mg/l	mg/l
2 Calculation factor	1000	1000	1000
3 Standard 1 conc	5	5	5
3 Standard 2 conc	10	10	10
3 Standard 3 conc	20	20	20
3 Standard 4 conc	30	30	30
3 Standard 5 conc	40	40	40
3 Standard 6 conc	50	50	50
6 Limit	0	0	0
7 Temperature ($^{\circ}$ C)	25.0	25.0	25.0
8 Type of analysis	7.5	7.5	7.5
9 Wavelength (nm)	340	405	405
10 Sample volume (μ l)	50	25	10
11 Diluent volume (μ l)	00	00	00
12 Reagent volume (μ l)	250	250	250
13 Incubation time (sec)	0	600	10
14 Start reagent volume (μ l)	0	20	20
15 Time of first reading (sec)	1.0	30.0	30.0
16 Time interval (sec)	20	30	10
17 Number of readings	20	11	15
18 Blanking mode	1	1	1

2.5 DATA INTERPRETATION AND STATISTICAL ANALYSIS

Before any assay data were accepted for interpretation, the assay was performed in at least triplicate, within assay, and in quadruplicate between assays. This assessed the reliability of the assay and allowed the data to be scrutinised for outliers before being subjected to statistical analysis. The paired *t* test was used to determine whether there was a significant difference between two sets of assay data. The Mann-Whitney U-test was used to test the significance of differences between subject groups. This non-parametric test was chosen as it makes no assumptions about the underlying distribution and is particularly suited to small data sets, as used in this thesis. A significant difference for both tests was considered where $p < 0.05$, with $p < 0.01$ being highly significant.

CHAPTER THREE

ANTI-ELASTASE ACTIVITY OF PLASMA, SERUM AND BAL.

3.1 INTRODUCTION

Although the cause of emphysema remains uncertain, it is currently thought that it results from an imbalance of the protease-anti-protease systems in the lung with HLE being implicated as the main elastolytic enzyme as discussed in chapter one of this thesis. This imbalance leads to digestion of elastin, with structural changes and dilatation of air spaces that are the hallmarks of this disease. The main protease inhibitor that regulates excessive HLE activity is α_1 -PI. As α_1 -PI is responsible for 80-90% of the anti-elastase activity towards HLE in serum (Ohlsson, 1978) and in the peripheral airways (Gadek et al., 1981b, Kramps et al., 1988), a reduction in α_1 -PI levels or function should, according to the theory, lead to the development of emphysema. A lower concentration of α_1 -PI with a reduced inhibitory function towards HLE has been reported in the severe genetic PiZ form of α_1 -PI deficiency which is frequently associated with pulmonary emphysema in adults (Eriksson, 1965, Sharp et al., 1969). However the question as to whether there are reduced levels of α_1 -PI and increased amounts of functionally inactive α_1 -PI present in the majority of emphysema cases, which are due to smoking, remains unresolved. It is known that α_1 -PI can be inactivated by oxidising agents resulting in the loss of binding of α_1 -PI towards HLE. This oxidative inactivation of α_1 -PI can occur during acute pulmonary inflammation due to the release of various oxidants from activated neutrophils (Zaslow et al., 1983), an example being the acute inflammatory lung injury that is seen in ARDS (Lee et al., 1981; McGuire et al., 1982; Cochrane et al., 1983). In this chapter, the total HLE inhibitory capacity and the inhibitory capacity due to α_1 -PI alone was determined in serum and BAL from non-smokers, smokers and patients with emphysema and ARDS. The aim being to determine whether there are any changes in reduced levels of α_1 -PI and increased amounts of functionally inactive α_1 -PI in individuals with emphysema and in smokers compared to non-smokers. The

ARDS group was used as a positive control because of the increased amount of functionally inactive α_1 -PI that has been reported in this acute inflammatory disease of the lung.

The development of the assays to measure total HLE inhibitory capacity and the inhibitory capacity due to α_1 -PI alone is described as follows. As HLE is the protease that is generally accepted to be involved in the lung destruction seen in emphysema, and is reported to bind with α_1 -PI many times faster than any other protease (Beatty et al., 1980; Travis and Salvesen, 1983), it was used to develop a rapid assay for functional α_1 -PI. The rapid binding of α_1 -PI to HLE (10 sec in this assay) was combined with rate-analysis capability of the Cobas Bio centrifugal analyser, the sensitive substrate MeO-Suc-A-A-P-V-NA, and the HLE activator Brij 35 so as to allow a fast assay (180 sec) suitable for rapid processing of a large number of samples. This assay (see Section 2.4.1.6) was used to measure the inhibitory activity of α_1 -PI in plasma and the HLE inhibitory capacity of BAL fluid from normal subjects and individuals with emphysema and ARDS. This assay was compared to two other α_1 -PI assays on the Cobas Bio analyser. One was an immuno-turbidimetric assay which uses a specific antibody towards α_1 -PI (see Section 2.4.1.7) to allow the measurement of all immuno-reactive α_1 -PI present, whether it is functional or not. The other was a PPE inhibitory assay that measures the inhibition due to functional α_1 -PI alone (see Section 2.4.1.5). The assay parameters used on the Cobas Bio centrifugal analyser for the three assays are compared in Table 2.1 (Chapter 2).

3.2 ASSAY EVALUATION

MeO-Suc-A-A-P-V-NA was used as substrate for the HLE inhibition assay on the basis of data reported previously (Nakijima et al., 1979) that it was 200 times more sensitive than the commonly used elastase substrate Suc-A-A-A-NA. This substrate allowed the development of an automated rapid assay (3 min assay time) on the Cobas Bio analyser for the detection of HLE. α_1 -PI has a high association rate with HLE ($t_{1/2 \text{ ass}} = 0.61$ msec; Travis and Salvesen, 1983) which allowed the use of the minimum incubation time on the analyser of 10 seconds. No

significant difference was observed when either a 10 sec or a 15 min incubation step was used prior to substrate addition for the assay of plasma or BAL (see Section 3.5). While a difference was noted for purified α_1 -PI where 86% inhibition was seen after a 10 sec. compared to a 15 min. incubation period (Figure 3.1), no significant difference was seen with the standard used in the assay, Protein Standard Plasma (Figure 3.2). The use of this sensitive substrate on the Cobas Bio analyser, together with Brij 35, allowed for a rapid assay of 3 min with 10 μ l of a 1 in 100 dilution of plasma or serum (Table 2.1). Brij 35 was added to the assay buffer on the basis of reports that surfactants activate HLE (Starkey and Barrett, 1976; Cook and Ternai, 1988a; Wenzel et al., 1990; Walsh et al., 1991b). In this assay system final concentrations of Brij 35 greater than 0.005% increased sensitivity by increasing HLE activity by as much as 40% (see Section 5.2.1 and Figure 5.1). A Brij 35 concentration of 0.01% was used routinely in the assay. The increased activity of HLE in the presence of Brij 35 allowed the use of a smaller amount of HLE (0.5 μ g) in the assay so that low concentrations of HLE inhibitors can be detected, as in BAL fluid.

Suc-A-A-A-NA is the most effective (Nakijima et al., 1979) and commonly used substrate for PPE and has been widely used in a number of studies to demonstrate the presence of inactive α_1 -PI in plasma and BAL on the basis that significant inhibition of PPE is only seen with functional α_1 -PI (eg. Klumpp and Bieth, 1979; Boudier et al., 1983; Morrison et al., 1987). Assay conditions and instrument parameters were optimised to obtain the same rate of absorbance change as found for the HLE inhibition assay (Figure 3.3; Table 2.1).

All α_1 -PI inhibition values were obtained from the linear part of the calibration plots (5-50 mg/l, Figure 3.3) using the DENS (Data Evaluation of Nonlinear Standards) curve option on the analyser to allow for any non-linearity. The inhibition assays were compared with an immunoturbidimetric assay for α_1 -PI on the Cobas Bio Analyser. The settings for this assay are shown in Table 2.1.

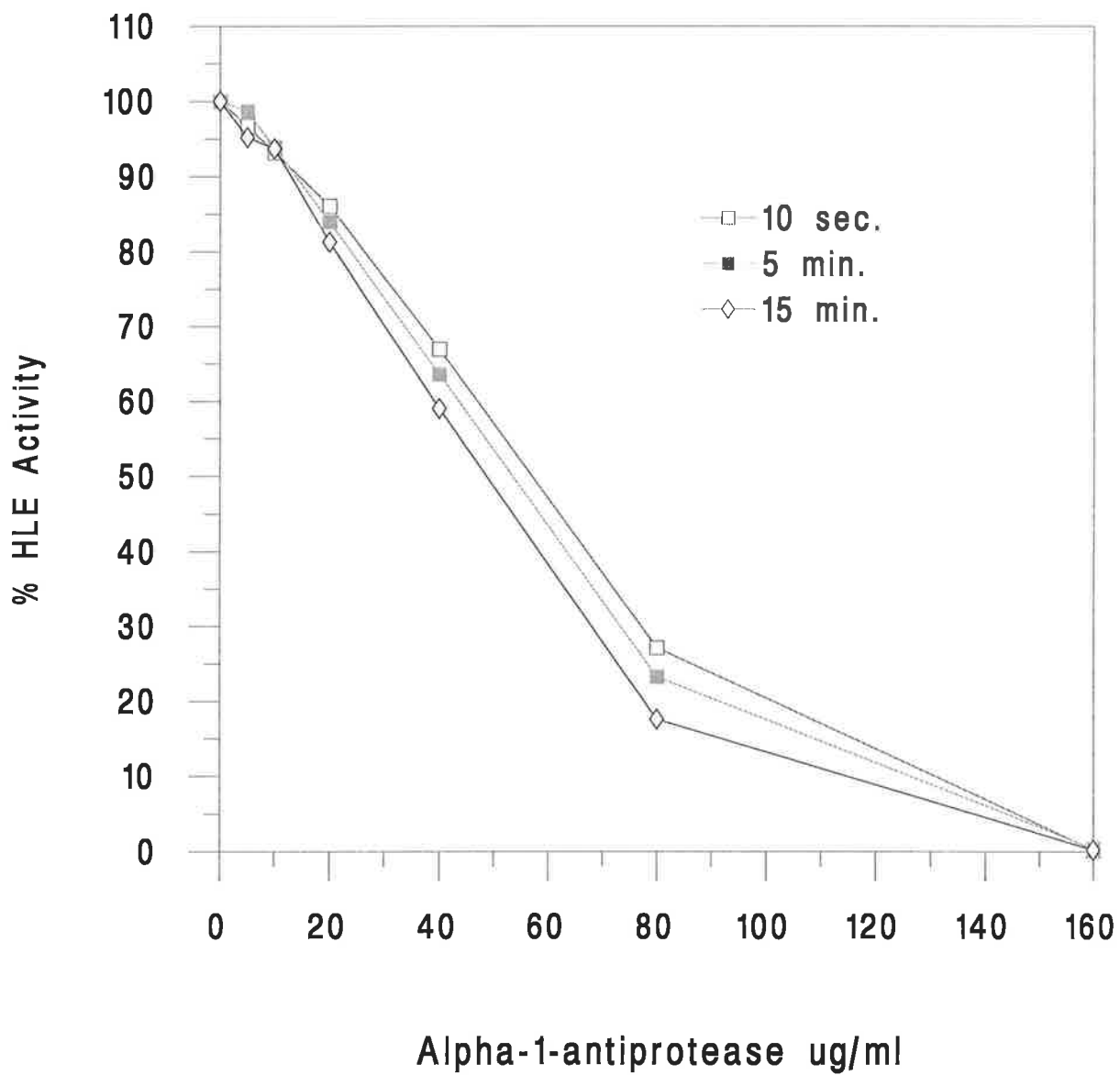


Figure 3.1: Effect of incubation time on alpha-1-antiprotease inhibition of HLE with MeO-Suc-A-A-P-V-NA as substrate. Assay as described in Section 2.4.16

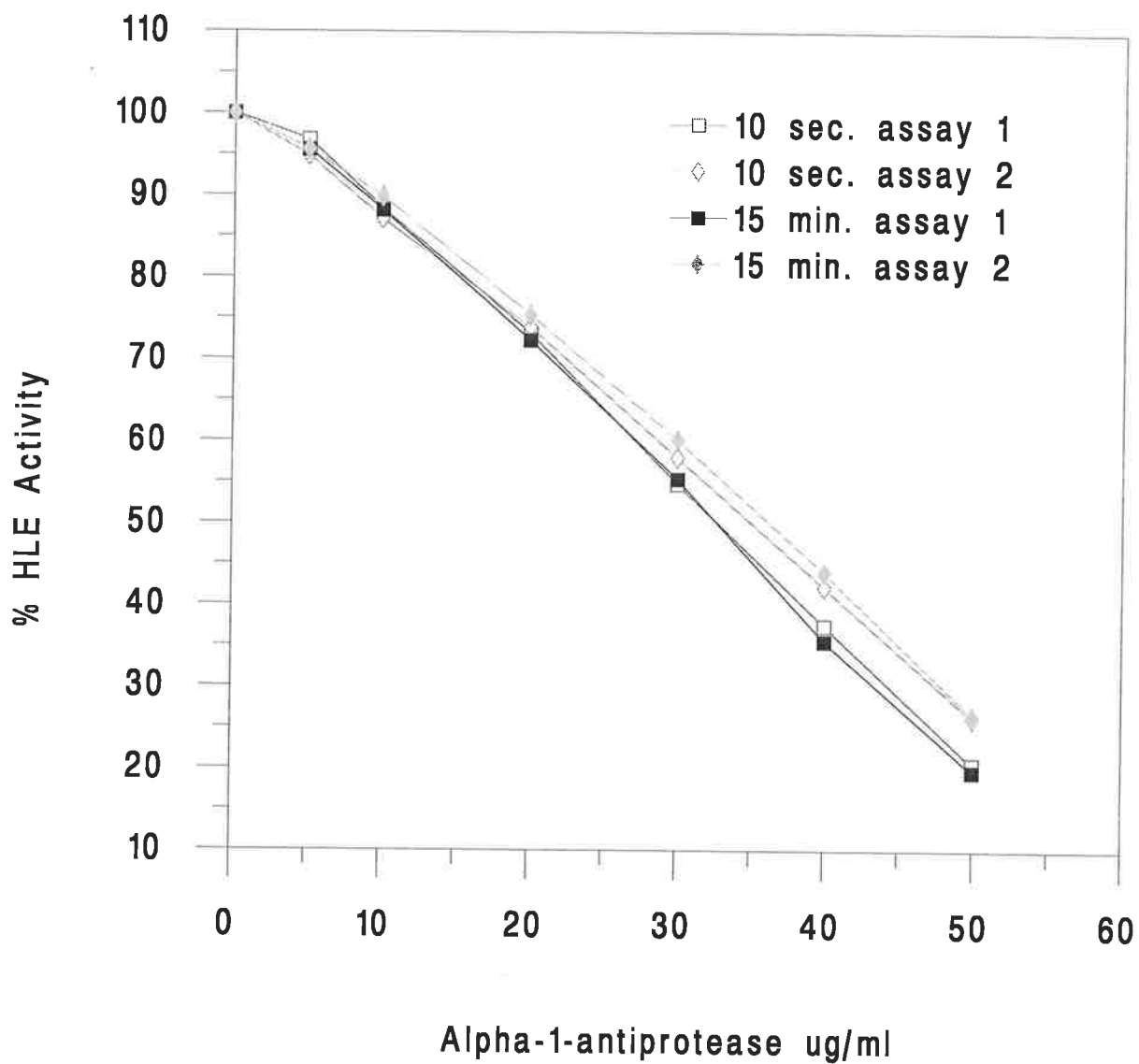


Figure 3.2: Effect of incubation time on plasma (Behring standard plasma) inhibition of HLE with MeO-Suc-A-A-P-V-NA as substrate. Assay as described in Section 2.4.1.6

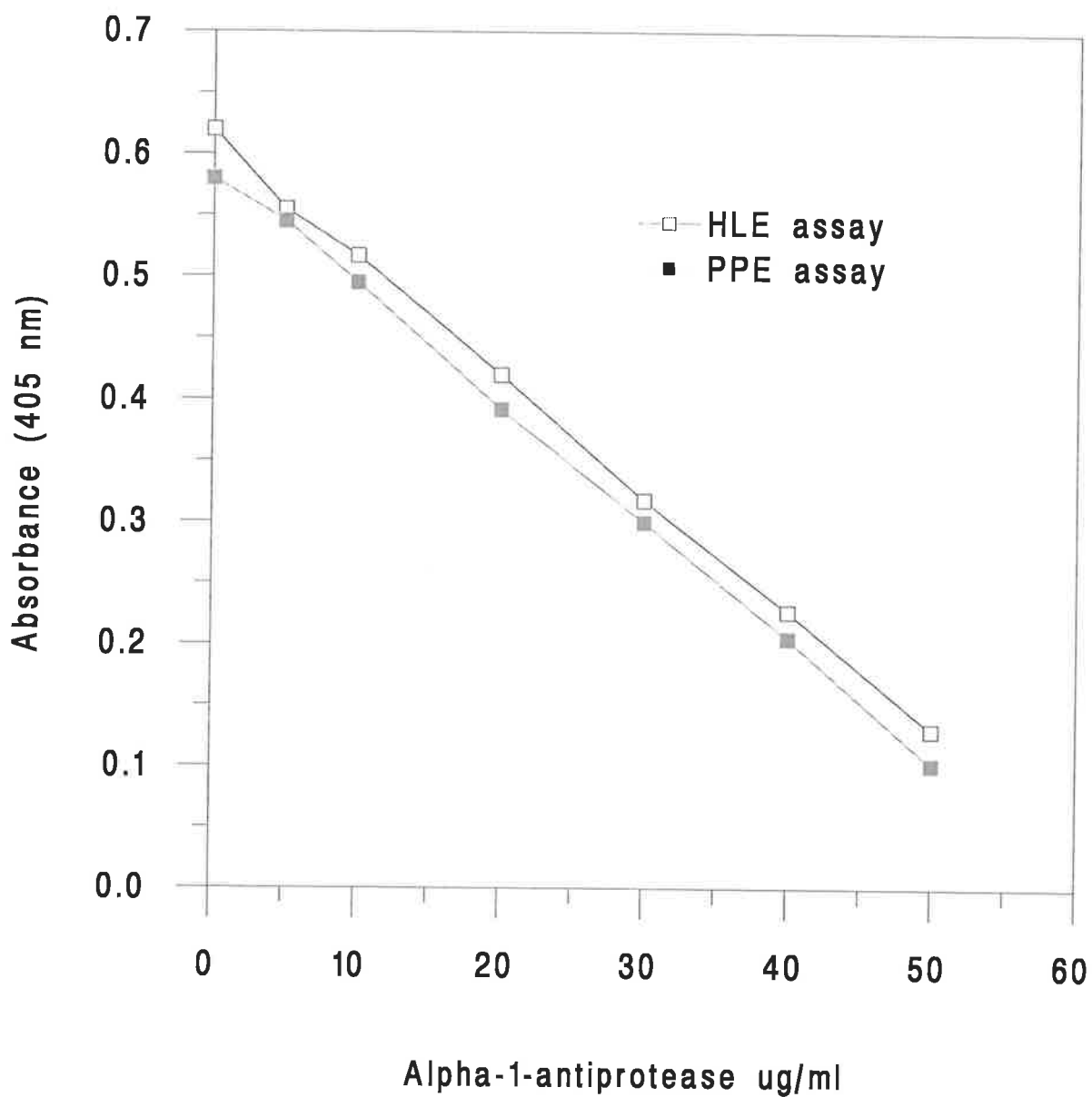


Figure 3.3: Calibration plots for HLE and PPE inhibition assays with Behring protein standard plasma as calibrator. MeO-Suc-A-A-P-V-NA and Suc-A-A-A-NA as substrates for HLE (Section 2.4.1.6) and PPE (Section 2.4.1.5) respectively.

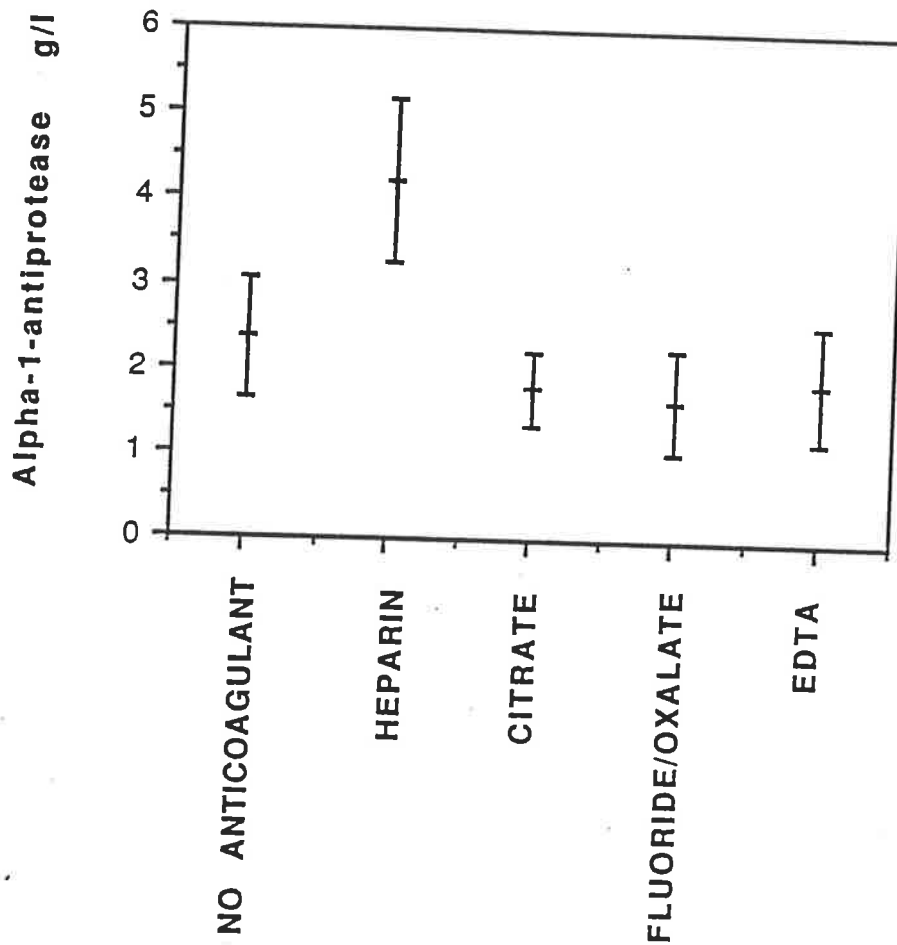


Figure 3.4: Effect of anticoagulants on HLE inhibition assay. Serum and plasma samples from normal volunteers (n=7) were collected into the anticoagulants described in Section 2.4.1.4. HLE inhibition assay is as described in Section 2.4.1.6.

3.3 EFFECT OF ANTI-COAGULANTS

Initial studies with heparinised plasma in the HLE inhibition assay gave apparent α_1 -PI values greater than twice that measured with the PPE inhibition and the immuno-turbidimetric assays. This apparent elevation in α_1 -PI activity was found to be due to the presence of heparin which inhibited HLE activity (see Chapter 4). An initial study was then undertaken to determine what anti-coagulants could be used in the three α_1 -PI methods. It was found that plasma containing commonly used anti-coagulants and serum could be employed in these assays except plasma containing heparin, which gave results significantly higher in the HLE inhibition assay (Figure 3.4). Thus, heparinised samples must be avoided if the HLE inhibition assay is used.

Table 3.1: Imprecision of assay

	Sample	Mean \pm SD (g/L)	CV (%)
Intra-Assay (n = 20)			
Immuno-turbidimetric	A	1.25 \pm 0.02	1.8
	B	2.58 \pm 0.0	0.9
HLE Inhibition	A	1.16 \pm 0.04	3.5
	B	2.46 \pm 0.04	1.5
PPE Inhibition	A	1.26 \pm 0.06	4.7
	B	2.42 \pm 0.06	2.4
Inter-Assay (n = 15)			
Immuno-turbidimetric	B	3.10 \pm 0.11	3.4
HLE Inhibition	B	2.80 \pm 0.15	5.3
PPE Inhibition	B	2.75 \pm 0.18	6.6

3.4 SERUM α_1 -PROTEASE INHIBITOR CONCENTRATIONS

It is important in comparison studies, where different assays are used to measure the same parameter, that reproducibility of the assays be known so that results can be correctly interpreted allowing for this assay variability. Within run and between run imprecision were

determined with one sample below the reference range (Sample A) and one in the middle of the reference range (Sample B). The intra-batch and inter-batch imprecision for both samples (A and B) were satisfactory for the HLE inhibition assay, and fell between the figures found for the immuno-turbidimetric and PPE inhibition assays (Table 3.1).

The immuno-turbidimetric and HLE inhibition assays were used to determine α_1 -PI concentrations in children, normal adults and adults with emphysema or ARDS (Figure 3.5). The reference intervals for normal homozygotes (α_1 -PI phenotype MM) were previously determined in our routine laboratory on blood donor serum samples with the immuno-turbidimetric α_1 -PI assay to be 1.6-3.5 g/l. In the present study the reference intervals were found to be lower. The range (mean \pm 2 SD) for the combined adult non-smokers, and smokers (n = 55) were 1.0-3.3 g/l and 1.0-2.8 g/l for the immuno-turbidimetric and HLE inhibition assays respectively. Total (immuno-turbidimetric) and functional (inhibition) α_1 -PI were significantly increased in children (both $p < 0.01$) and in adults with emphysema ($p < 0.05$ and $p < 0.01$ respectively) when compared to those for normal adults (Figure 3.5). The mean values in smokers did not differ significantly from those in non-smokers. Values for ARDS were significantly higher than in any other group (both $p < 0.05$).

When the functional α_1 -PI concentration (HLE assay) was expressed as a percentage of the immuno-turbidimetric α_1 -PI concentration (Figure 3.6) the only difference noted was that for the ARDS group where the percentage was reduced ($p < 0.05$). The PPE inhibition assay on the above groups correlated well with the HLE inhibition assay (see also comparison data in Section 3.5).

3.5 COMPARISON OF α_1 -PROTEASE INHIBITOR CONCENTRATION IN SERUM WITH BAL FLUID

Both HLE and PPE inhibition assay values were expressed as a percentage of the immuno-turbidimetric α_1 -PI concentrations in a group of normal volunteer smokers (n = 4) and non-

smokers (n = 4) as shown in Figures 3.7 and 3.8. Serum α_1 -PI was 91% active in non-smokers and 97% active in smokers for the PPE assay (Figure 3.7), and 94% active in non-smokers and 96% active in smokers in the HLE assay (Figure 3.8a).

In BAL fluid, using the PPE inhibition assay, the α_1 -PI was 70% active in non-smokers and 85% active in smokers (Figure 3.7). However, in the HLE assay the figures were 138% and 153% respectively (Figure 3.8a), demonstrating the presence of HLE inhibitors other than α_1 -PI in normal BAL fluid. Nevertheless, there was no significant difference in values for BAL fluid between smokers and non-smokers. There was also no significant difference between smokers and non-smokers immuno-turbidimetric α_1 -PI concentrations in serum or BAL samples (Figures 3.7, legend).

In patients with ARDS there was an approximately two-fold increase for immuno-turbidimetric α_1 -PI concentrations in serum ($p < 0.05$) (Figure 3.9: mean \pm SD, 4.50 ± 2.45 g/l) when compared to normal volunteers (Figure 3.7: non-smokers, 2.08 ± 0.39 ; smokers 2.03 ± 0.30). However, the α_1 -PI was only 76% active in both the PPE and HLE inhibition α_1 -PI assays ($p < 0.01$) as shown in Figures 3.9 and 3.10. In BAL fluid from ARDS patients there was a greater than 40-fold increase in the immunoturbidimetric α_1 -PI ($p < 0.01$) (Figure 3.9: 0.101 ± 0.167 g/L) when compared to normal volunteers (Figure 3.7: non-smokers, 0.00112 ± 0.00094 , smokers, 0.00235 ± 0.00150). However, the α_1 -PI in BAL fluid from ARDS patients was only 37% active as measured in the PPE inhibition α_1 -PI assay ($p < 0.05$) (Figure 3.9). As is the case for normal BAL fluid inhibition of HLE (Figure 3.8a), the inhibition of HLE by ARDS BAL fluid was elevated (118%; Figure 3.10a) also indicating the presence of other HLE inhibitors in this fluid.

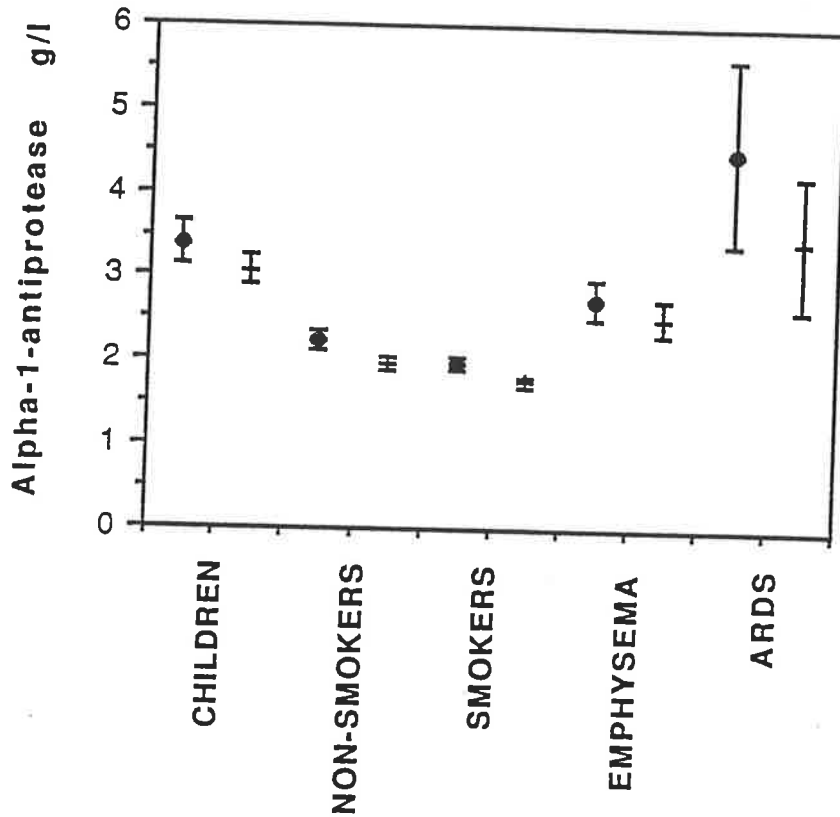


Figure 3.5: Comparison of serum immuno-turbidimetric (●) and HLE inhibition assays (+) for the estimation of α_1 -PI concentration in children (<15 years, $n = 17$), normal adults (non-smokers, $n = 35$; smokers, $n = 20$) and adults with emphysema ($n = 19$) and ARDS ($n = 5$). Each group is shown as mean \pm standard error. Assay procedures are as described in Sections 2.4.1.6 and 2.4.1.7.

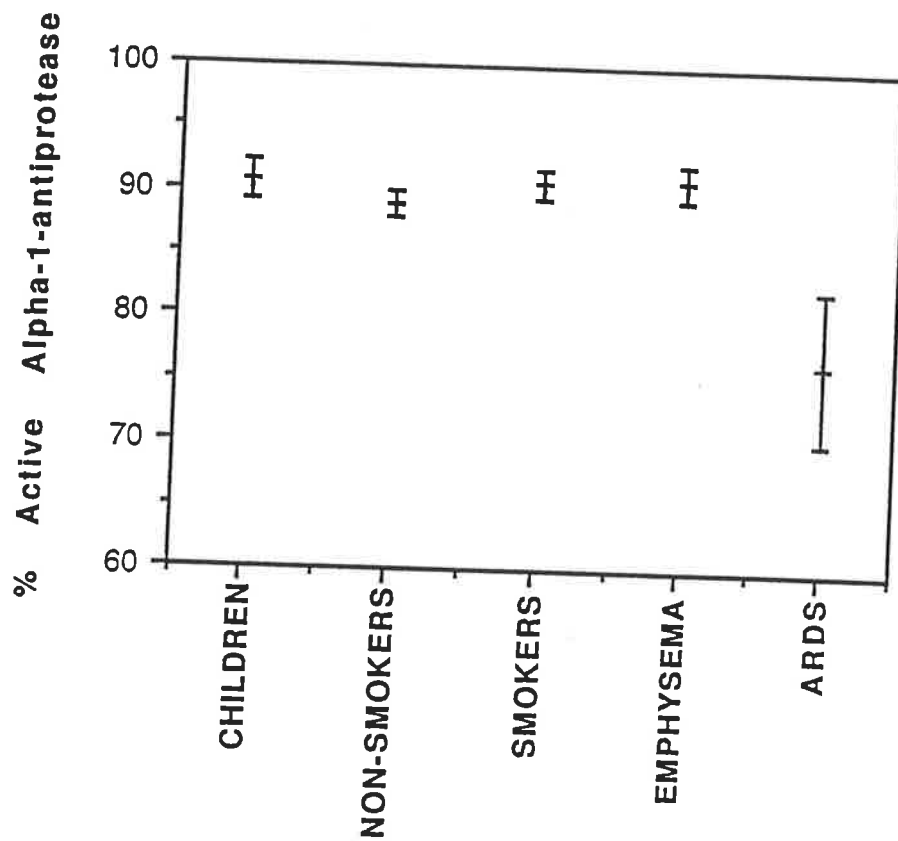


Figure 3.6: Comparison of % active α_1 -PI concentration in children (<15 years, $n = 17$), normal adults (non-smokers, $n = 35$; smokers, $n = 20$) and adults with emphysema ($n = 19$) and ARDS ($n = 5$). Active α_1 -PI concentration is determined as a ratio of serum HLE inhibition and immuno-turbidimetric assays. Each group is shown as mean \pm standard error. Assay procedures are as described in Sections 2.4.1.6 and 2.4.1.7.

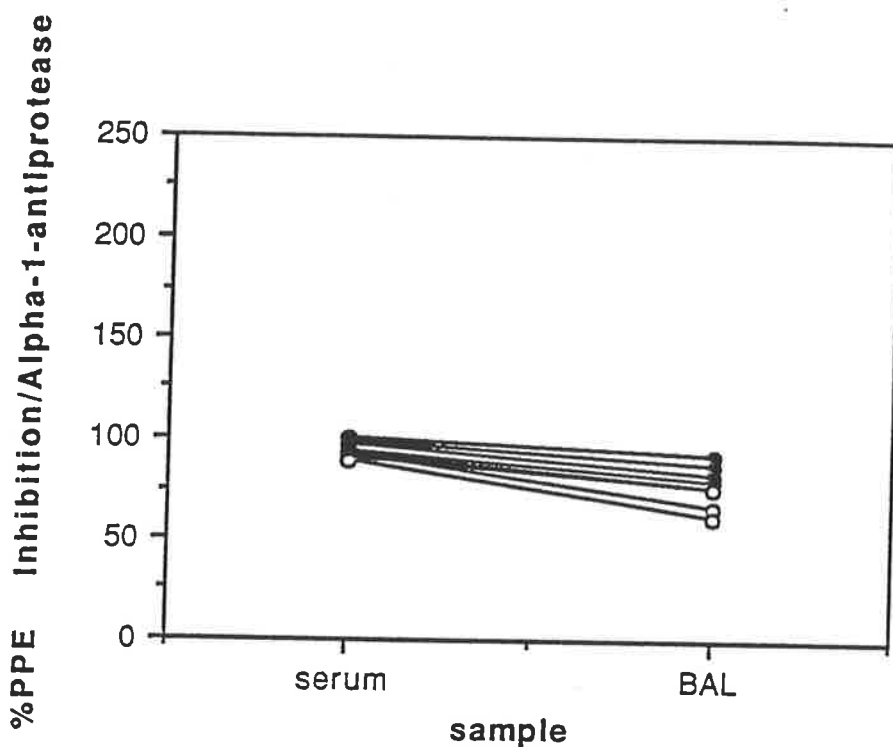


Figure 3.7: Comparison of % active α_1 -PI concentration in serum and BAL from normal volunteers - 4 non-smokers (o), 4 smokers (●) using the PPE inhibition assay expressed as a ratio of the immuno-turbidimetric assay. The immuno-turbidimetric α_1 -PI concentration (g/l, mean \pm SD) for non-smokers and smokers respectively were: Serum, 2.08 ± 0.40 , 2.03 ± 0.31 ; BAL, 0.00112 ± 0.00094 , 0.00235 ± 0.00150 . The PPE inhibitory capacity values for non-smokers and smokers respectively were: Serum, 1.89 ± 0.38 , 1.97 ± 0.27 ; BAL, 0.00072 ± 0.00079 , 0.00202 ± 0.00130 . Assay procedures are as described in Sections 2.4.1.5 and 2.4.1.7.

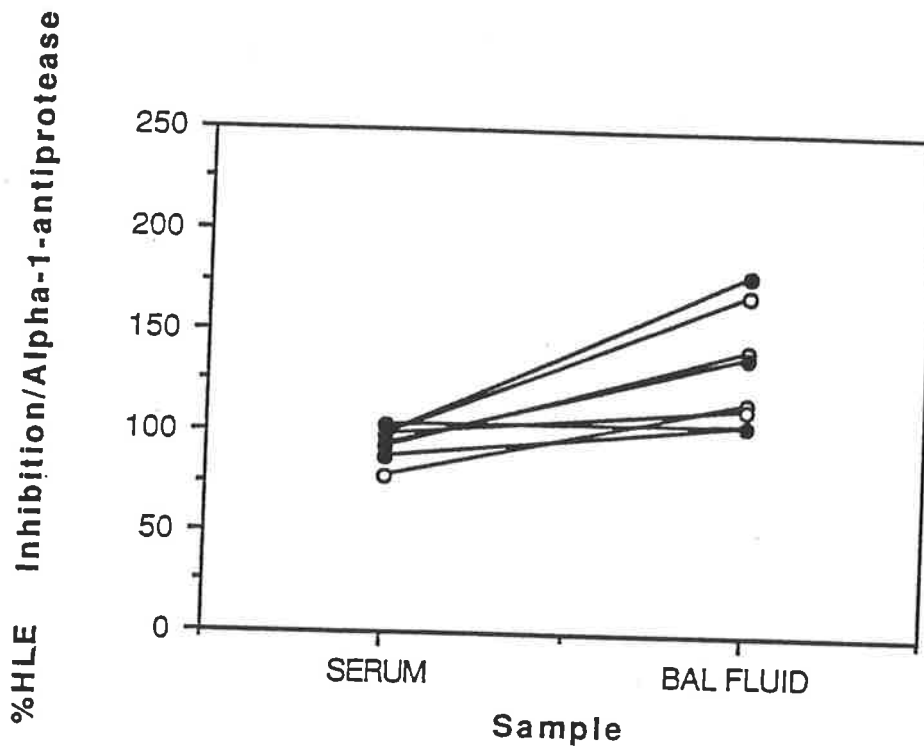
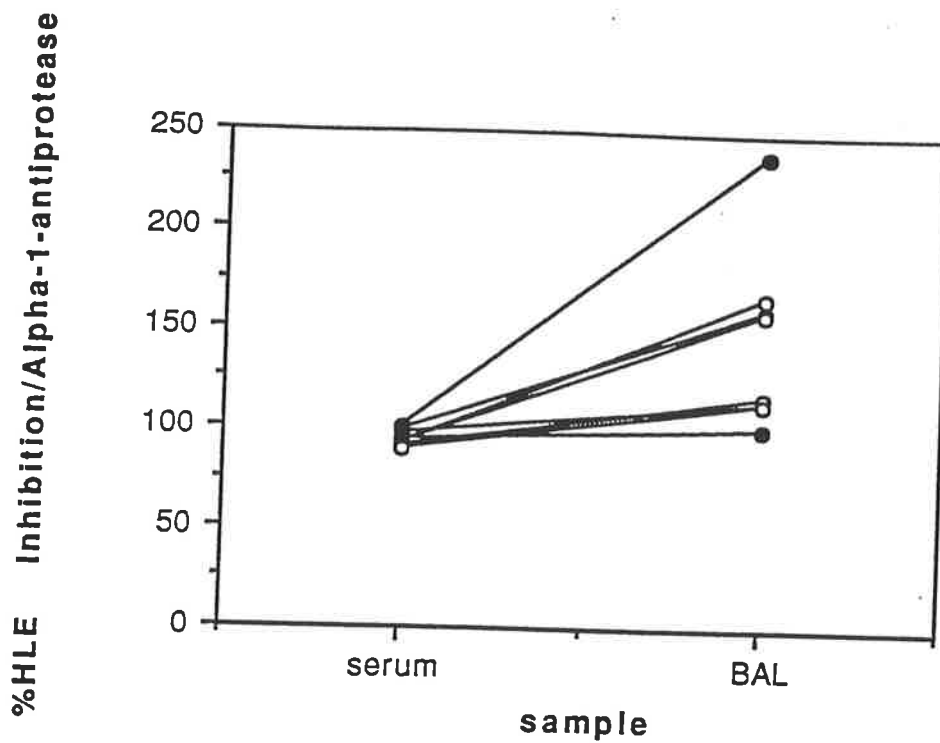


Figure 3.8 a & b: Comparison of % active α_1 -PI concentration in serum and BAL from normal volunteers - 4 non-smokers (o), 4 smokers (●) using the HLE inhibition assay with 10 sec (a) or 15 min (b) incubation expressed as a ratio of the immuno-turbidimetric assay. The immuno-turbidimetric α_1 -PI concentrations are as for Figure 3.7. The HLE inhibitory capacity values for non-smokers and smokers respectively were: Serum, 2.15 ± 0.33 , 1.95 ± 0.29 ; BAL, 0.00141 ± 0.00101 , 0.00383 ± 0.00286 . Assay procedures are as described in Sections 2.4.1.6 and 2.4.1.7.

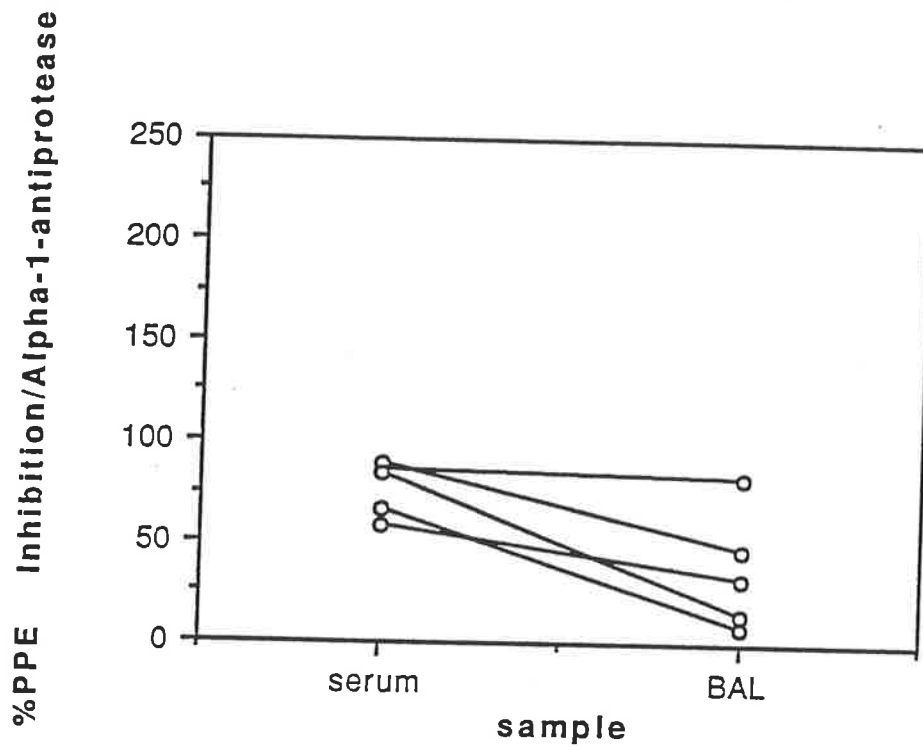


Figure 3.9: Comparison of % active α_1 -PI concentration in serum and BAL from ARDS patients ($n = 5$) using the PPE inhibition assay expressed as a ratio of the immuno-turbidimetric assay. The immuno-turbidimetric α_1 -PI concentration (g/l, mean \pm SD) for ARDS patients in serum and BAL respectively were: 4.50 ± 2.45 and 0.101 ± 0.167 . The PPE inhibitory capacity values for ARDS patients in serum and BAL respectively were: 3.44 ± 1.76 , 0.072 ± 0.144 . Assay procedures are as described in Sections 2.4.1.5 and 2.4.1.7.

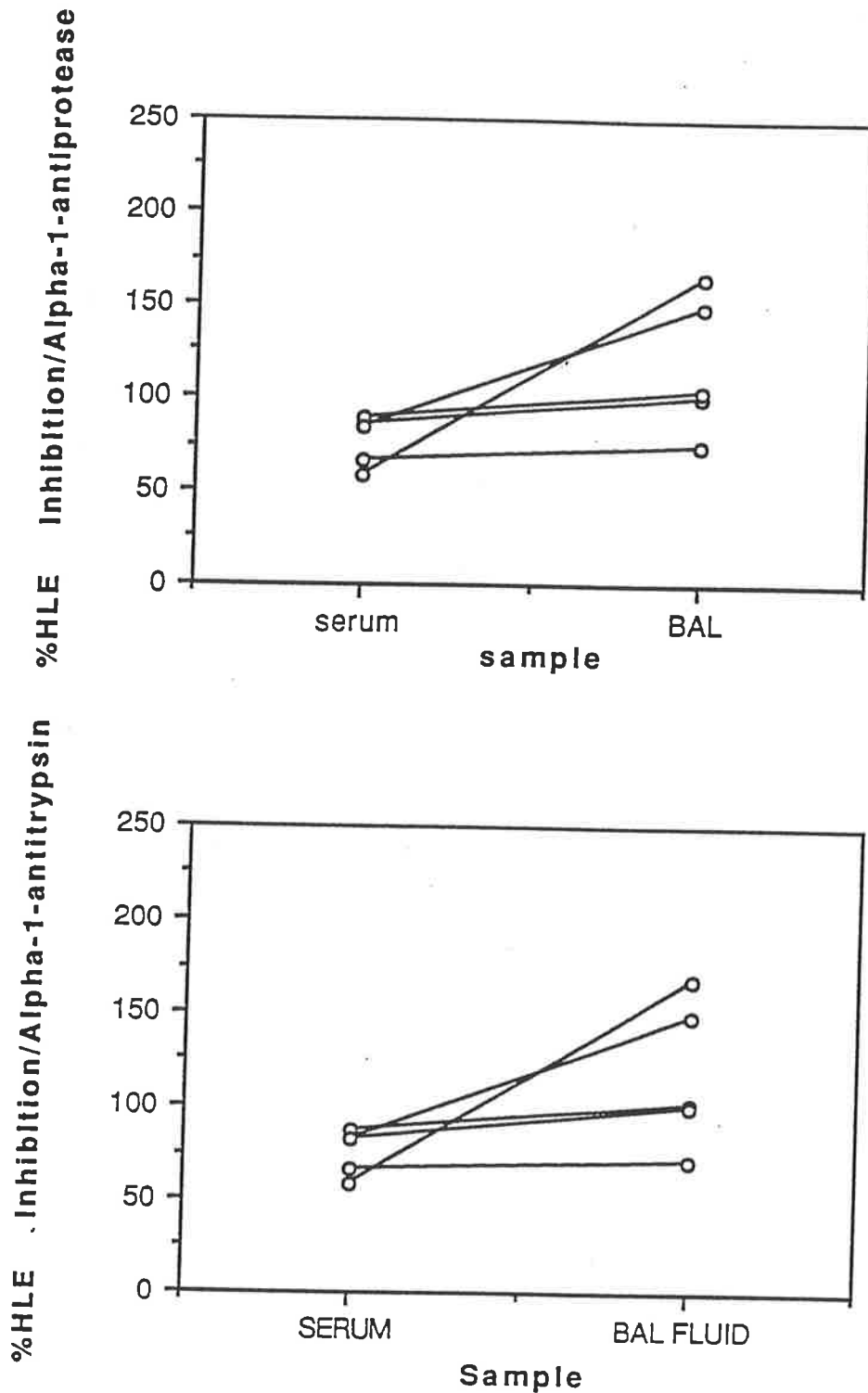


Figure 3.10 a & b: Comparison of % active α_1 -PI concentration in serum and BAL from ARDS patients (n = 5) using the HLE inhibition assay with 10 sec (a) or 15 min (b) incubation expressed as a ratio of the immuno-turbidimetric assay. The immuno-turbidimetric α_1 -PI concentrations are as for Figure 3.9. The HLE inhibitory capacity values for ARDS patients in serum and BAL respectively were: 3.86 ± 2.38 , 0.103 ± 0.166 . Assay procedures are as described in Sections 2.4.1.6 and 2.4.1.7.

While the incubation period of 10 sec, 5 or 15 min of either purified α_1 -PI or Protein Standard Plasma with HLE did not lead to significant differences in the HLE inhibition assay (Figures 3.1 and 3.2 respectively) there may be significant differences with serum and BAL samples from patients. To examine this possibility, the HLE inhibition assays on normal volunteers and ARDS patients as shown in Figures 3.8a and 3.10a respectively were also repeated with an incubation step of 15 minutes; the results of which are shown in Figures 3.8b and 3.10b respectively. However, no significant differences were seen whether the normal volunteer plasma samples from non-smokers and smokers combined ($n = 8$) were incubated for 10 sec (Figure 3.8a, 93.8% inhibition) or 15 min (Figure 3.8b, 93.5% inhibition). This was also the case for ARDS plasma samples ($n = 5$) whether incubated for 10 sec (Figure 3.10a, 76.4% inhibition) or 15 min (Figure 3.10b, 75.2% inhibition). With BAL fluid, where other HLE inhibitors are present, there could theoretically be differences in inhibition rates. However, no significant differences were seen whether the normal volunteer BAL samples from non-smokers and smokers combined ($n = 8$) were incubated for 10 sec (Figure 3.8a, 145.4% inhibition) or 15 min (Figure 3.8b, 133.4% inhibition) and similarly for ARDS plasma samples ($n = 5$) incubated for 10 sec (Figure 3.10a, 117.8% inhibition) or 15 min (Figure 3.10b, 117.5% inhibition).

3.6 DISCUSSION

In this chapter, a rapid method for the measurement of functional α_1 -PI (HLE enzyme inhibition) which uses as little as 10 μ L of 100-fold diluted plasma or serum is described. This assay has a shorter combined incubation and assay time (180 sec) than the immuno-turbidimetric (401 sec) and the PPE inhibition assay (960 sec) as shown in Table 2.1. The imprecision is acceptable and falls between that of the immuno-turbidimetric and PPE inhibition assays (Table 3.1). The only disadvantage is that heparinised plasma cannot be used (Figure 3.4).

Inhibition of PPE has previously been used to assess the oxidative inactivation of α_1 -PI in serum (Beatty et al., 1982). In the present study we have used HLE in lieu of PPE because it is the enzyme against which α_1 -PI is primarily directed (Beatty et al., 1980; Travis and Salvesen, 1983). The HLE inhibition assay, like the assay using PPE, has an advantage in serum or plasma in that only the protease inhibitory role of α_1 -PI is being measured and not inactive α_1 -PI such as the complexed, oxidised or fragmented forms which may be measured in the immuno-turbidimetric assay. For example, the oxidised form of α_1 -PI is approximately 2000-fold less able to inhibit HLE (Johnson and Travis, 1978). This partially explains the data in Figure 3.5 where HLE functional values were always slightly lower although not significantly less than the corresponding immuno-turbidimetric values. However, the difference is clearly shown in Figure 3.6 where the HLE value is expressed as a percentage of the immuno-turbidimetric value. All groups except the ARDS group had approximately 90% functional α_1 -PI activity towards HLE whereas the ARDS group had only 76% active α_1 -PI. This is not surprising as ARDS is an acute disease in which an elevated plasma α_1 -PI is expected (Figure 3.5) because of the inflammatory response. It is likely that much of the α_1 -PI is oxidised or complexed as has been shown in previous studies (Lee et al., 1981; McGuire et al., 1982; Cochrane et al., 1983) and this is consistent with the data presented in Figure 3.6.

The mean immuno-turbidimetric and HLE inhibition assay concentrations in the children, the emphysema and the ARDS groups were raised when compared to the means of adult normal non-smokers and smokers, although many individual values in the children and the emphysema group still fell in the reference range of 1.6-3.5 g/L as used by our routine laboratory (see Figure 3.5). The adult normals did not show any significant differences between non-smokers and smokers in α_1 -PI concentrations (Figure 3.5) or percentage α_1 -PI activity (Figure 3.6).

α_1 -PI functional concentrations in BAL fluid are likely to reflect, more closely than serum, the inflammatory activity that occurs in the lung. This is shown with the PPE inhibition assay by the much higher inactivation of α_1 -PI in BAL fluid from some ARDS individuals when

compared to the patient's plasma (Figure 3.9). However, the HLE inhibition assay in BAL fluid from normal volunteer and ARDS patients reflects not only α_1 -PI inhibitory activity but also the inhibitory activity of other HLE inhibitors in the lung, as demonstrated by the increased HLE inhibitory activity (Figures 3.8 and 3.10). As with the PPE inhibition assay, there was also less functional activity with the HLE inhibition assay in the ARDS fluid (118%) than in the BAL fluid from normal volunteers (145%).

This increased BAL inhibitory activity towards HLE compared to PPE has been reported in another study (Wewers et al., 1989), although the authors did not relate the activity to α_1 -PI standards and thus did not infer that the increased inhibition is due to inhibitors other than α_1 -PI. They also reported that BAL from smokers took approximately 1.5 times longer to inhibit HLE than in non-smokers by using multiple incubation times from 10 to 80 sec. This study did not aim to specifically examine this difference as only three incubation times of 10 sec, 5 min, or 15 min were used, and no significant difference was detected between non-smokers and smokers (see Figure 3.8a and 3.8b). However, the results of short incubation period of 10 seconds demonstrate that α_1 -PI and the other inhibitors in BAL act rapidly.

The PPE inhibition assay, used as a comparison method, demonstrated in a volunteer sub-group (Figure 3.7) that there was no significant difference in functional α_1 -PI activity between either serum or BAL fluid in smokers or non-smokers. This observation supports other reports (Boudier et al., 1983; Stone et al., 1983) which show similar active α_1 -PI levels in BAL from both smokers and non-smokers. These results suggest that the development of emphysema in smokers is not simply due to an acquired deficiency of active α_1 -PI as has been found in individuals with the ZZ phenotype inherited form of α_1 -PI deficiency, as has been proposed by Travis (1988).

In conclusion, both inhibition assays as described here on the Cobas Bio centrifugal analyser measure functional α_1 -PI in serum, but the HLE inhibition assay is preferable as it uses less sample, requires less assay time and is more precise (Tables 2.1 and 3.1). Whilst this assay is suitable for undiluted and diluted samples in the 5-50 mg/L assay range, the assay has also been used to measure unconcentrated BAL from normal volunteers in the 0.05-1 mg/l assay range by increasing the sample volume (x10), decreasing the HLE concentration (x10) and increasing the assay time from 3 to 30 min. It also has the advantage over other α_1 -PI assays in that it is able to detect not only functional α_1 -PI in BAL but also the inhibitory action of other BAL inhibitors towards HLE. This assay may have a role in monitoring the effectiveness of various treatments developed to augment the anti-elastase defences in acute inflammatory disease such as ARDS, and in chronic inflammatory diseases such as emphysema.

CHAPTER FOUR

ELASTASE INHIBITORS

4.1 INTRODUCTION

There is considerable evidence suggesting that HLE is the main protease responsible for the protease-antiprotease imbalance that leads to the development of emphysema. The evidence is based primarily on the plausible role of HLE in generating emphysema in individuals carrying an inherited deficiency of α_1 -PI, the major endogenous serine proteinase inhibitor for HLE, and its ability to mimic emphysema when administered as a purified enzyme in animal lungs (Janoff, 1985, Snider, 1992). This has led to studies with endogenous inhibitors and the development of many low molecular weight elastase inhibitors with the eventual aim of clinically supplementing the naturally occurring antiproteases in the lung.

Despite its very low elastolytic activity, cathepsin G is considered to be the other major inflammatory serine protease to HLE, which is secreted in neutrophil degranulation. Its occurrence in cells essentially mirrors that for HLE but in addition is found in mast cells (Caughey, 1994). However its physiological action and its role in disease states has not been established although a number have been proposed. One proposal is that cathepsin G acts as a mediator for the function of HLE and other elastase-related proteases secreted from the azurophil granules of neutrophils. Some examples are as follows. Cathepsin G could assist HLE function by allowing more efficient elastolysis because of its different substrate specificity to HLE, which allows it to bind to and act on different protein sequences (Lonky and Wohl, 1983). There have been reports of HLE activity toward elastin being enhanced in the presence of cathepsin G (Boudier et al., 1981; Reilly et al., 1984). There is evidence that cathepsin G is required for HLE to be released from neutrophils and that it may be required for HLE to be activated (Kubes et al.,

1993). Cathepsin G has also been reported to be able to convert the latent form of neutrophil collagenase to the active form (Capodici et al., 1989).

As well as α_1 -PI, another proteinase inhibitor that may have a role in the development of emphysema is α_2 -M. α_2 -M appears to be a non-specific proteinase inhibitor, which is able to bind to the majority of all proteinases, including HLE. When complexed to α_2 -M, proteinases typically retain 80-100% of their hydrolytic activity against low molecular weight substrates but less than 10% against large proteins (Barrett, 1981). On the basis of this residual proteolytic activity, it has been proposed that α_2 -M-HLE complexes may play a role in the development of pulmonary emphysema (Galdston et al., 1979; Stone et al., 1979), which is supported by the detection of α_2 -M-PPE complexes in BAL from the lungs of hamsters with PPE-induced emphysema (Stone et al., 1982). Clinically, because of its size (MW 725,000), α_2 -M cannot readily diffuse into the lung alveolus, where it is usually at such low concentrations that it is difficult to detect in normal BAL. Large amounts of α_2 -M have been detected in BAL from patients with ARDS in this laboratory (McLennan et al., 1984) and by others (Wewers et al., 1988) and in BAL from patients with cystic fibrosis (Bruce et al., 1985). However, the specific physiological role of α_2 -M as an antiprotease has not been clearly established, let alone a role in the development of pulmonary emphysema.

Central to the proteolytic mechanism in the development of emphysema is the ability of HLE to bind to and degrade lung elastin. Although HLE has a very broad specificity, studies with oligopeptide substrates show that the enzyme preferentially binds to sequences rich in amino acids with short hydrophobic side chains close to lysine derived cross-links (Yasutake et al., 1981) with cleavage occurring at carboxyl side of residues, particularly valine and alanine (Stein et al., 1987). Thus, studies of HLE inhibitors may be dependent on the relative affinity of HLE for the type of substrate and inhibitor used. For example, HLE has been shown to bind so

strongly to insoluble elastin that its inhibition by specific inhibitors is markedly impaired (Morrison et al., 1990, Padrines and Bieth, 1991) which is also likely to be influenced by binding of HLE to elastin derived peptides released during elastinolysis (Tyagi and Simon, 1993). An elastin peptide preparation (κ -elastin) has also been reported to inhibit HLE activity (Bonnaure-Mallet et al., 1995).

A number of compounds, such as GAGs, have been shown to interact with HLE and have the potential to modify the effectiveness of HLE inhibitors. GAGs such as heparin and the structurally similar heparan sulphate are components of proteoglycans which are found both in mast cells (Langunoff et al., 1964, Bergqvist et al., 1971), as part of the luminal surface of cell membranes (Hook et al., 1984) and as part of tissue surfaces such as the vascular endothelium (Marcum and Rosenberg, 1984). The most studied form is the commercially available standard clinical heparin, which is extracted from porcine mucosa and consists of a pool of sulphated polysaccharide chains of varying lengths with an average molecular weight of 14 ± 1 kDa. Heparin has been shown to interact with numerous biological systems (Lane and Lindahl, 1989) including a number of PMN functions, where it has been shown to have an anti-inflammatory role such as the inhibition of HLE activity (Redini et al., 1988a; Walsh et al., 1988; Walsh et al., 1991a). Heparin is able to bind to a large number of proteins (Jackson et al., 1991) because of its polyanionic nature and because it contains iduronic acid residues which allows the carbohydrate backbone of heparin sufficient flexibility to fit many relatively fixed chemical configurations that are electropositive (Casu et al., 1988). The binding of heparin to HLE is essentially by electrostatic interactions, which probably involves the negatively charged sulphate groups on heparin and some of the positively charged guanidinium groups of 19 arginine residues located in clusters on the surface of HLE (Bode et al., 1989; Navia et al., 1989).

In this chapter the effectiveness of a number of endogenous and exogenous protease and HLE inhibitors towards HLE and cathepsin G is examined. Inhibitors evaluated include α_1 -PI, α_2 -M, elastin peptides, sulphated GAGs, and the low molecular weight inhibitors, ICI 186756 and 200355.

4.2 DETERMINATION OF HUMAN LEUKOCYTE ELASTASE ACTIVITY

The HLE used throughout this study was a chromatographically pure preparation from human sputum supplied by Elastin Products. It was stated to be free of cathepsin G activity which was confirmed by assay for enzymic activity (see Section 2.4.3.1 for assay details). Elastin Products stated the product to be at least 90% active. This has been confirmed in two reports; one using active site titrants (Williams et al., 1987) and the other using previously published kinetic constants of Nakajima et al (1979) to determine enzyme activity (Burnett et al., 1988). Because the active site titrants were not readily available, the activity of the enzyme was determined using the published kinetic constants. The assay conditions used were exactly those reported by Nakajima et al (1979) and are shown in Section 2.4.8. The kinetic constants K_m and V_{max} were determined by varying the concentration of the substrate, Suc-A-A-A-NA using the reciprocal form of the Michaelis-Menten equation :-

$$1/V = 1/V_{max} + K_M/V_{max} \cdot 1/[S]$$

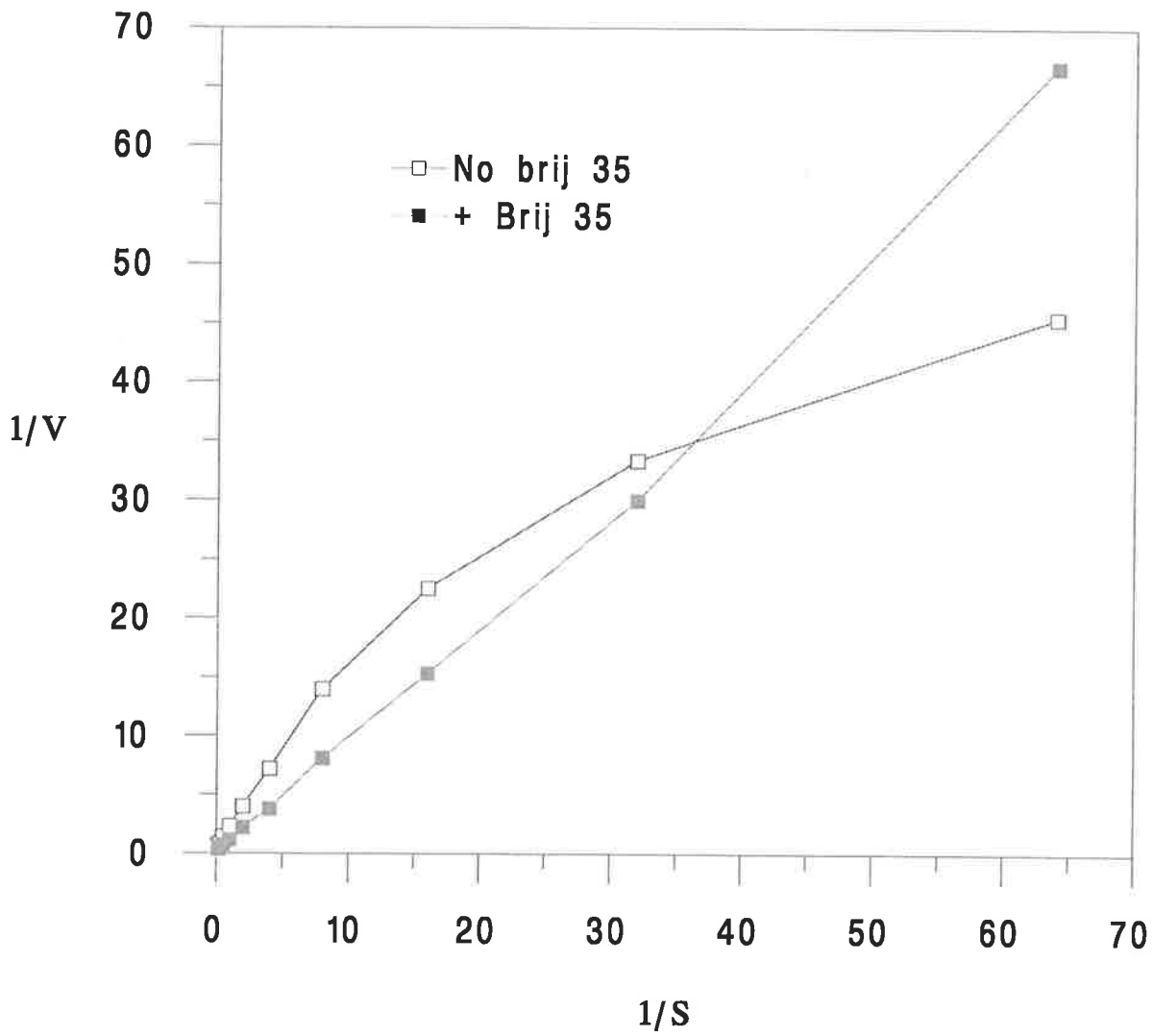


Figure 4.1: Determination of kinetic constants for HLE with 0.0156-8 mM of the substrate, Suc-A-A-A-NA. Assay conditions as described in Section 2.4.8.

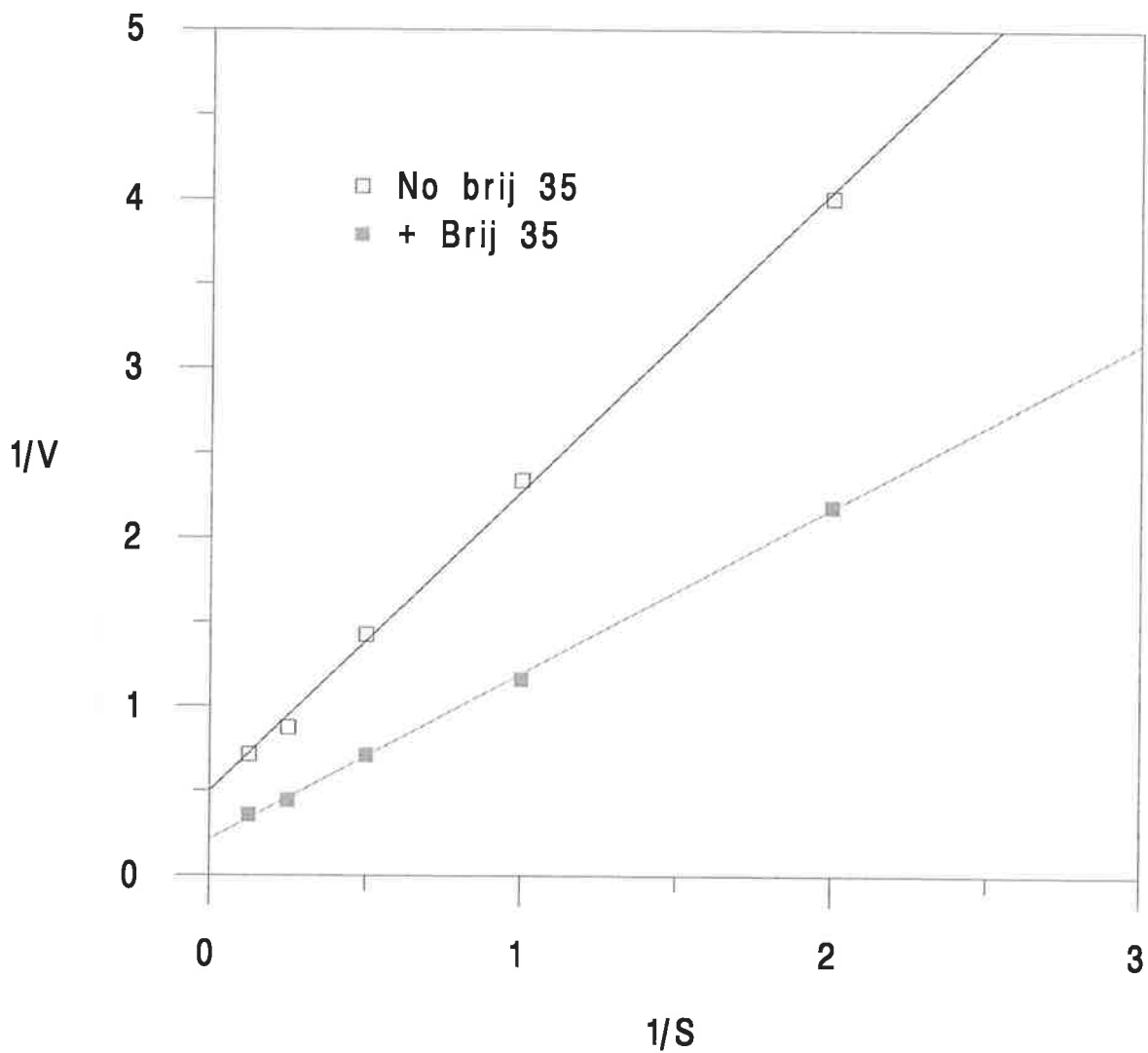


Figure 4.2: Determination of kinetic constants for HLE with 0.5-8 mM of the substrate, Suc-A-A-A-NA. Assay conditions as described in Section 2.4.8.

Linear regression equations:

$$\begin{aligned} \text{No Brij 35: } y &= 0.498 + 1.774x & r^2 &= 0.998 \\ \text{+ Brij 35: } y &= 0.212 + 0.980x & r^2 &= 0.999 \end{aligned}$$

The overall catalytic rate constant k_{cat} is obtained by dividing the maximal rate V_{max} by the total concentration of active enzyme $[E^0]$. Initial studies with a substrate concentration range of 0.0156-8 mmol/L of Suc-A-A-A-NA (Figure 4.1) showed that the reciprocal of the substrate concentration (ie. $1/[S]$) only gave a linear plot in the presence of Brij 35 which is routinely included in assays used in this thesis for HLE activity (see Section 2.4). The kinetic constants derived by Nakajima et al (1979) were obtained with a substrate concentration range of 0.4-7.6 mmol/l in the absence of Brij 35. Replotting Figure 4.1 using the substrate range of 0.5-8 mmol/l gave reproducible linear plots (Figure 4.2) in the absence of Brij 35. The kinetic constants found in the absence and presence of Brij 35 and those obtained by Nakajima et al (1979) are shown in Table 4.1.

Table 4.1 Kinetic constants for HLE with Suc-A-A-A-NA as substrate.

	K_M (mmol)	k_{cat} (s^{-1})	k_{cat} / K_M ($\text{mol}^{-1} \text{s}^{-1}$)
This study (no Brij 35)	3.6	1.9	501
This study (with 0.01% Brij 35 v/v)	4.6	4.5	987
Nakajima et al (1979, no surfactant)	3.7	2.1	570

The kinetic constants obtained in the absence of surfactant are very close to those obtained by Nakajima et al (1979) and would support the manufacturer (Elastin Products) statement that the product is at least 90% active enzyme as also found by Williams et al (1987) using active site titrants. In the presence of Brij 35 the plot is essentially linear to a very low substrate

concentration of 0.0156 mmol/L (ie. 1/S of 64) of Suc-A-A-A-NA (Figure 4.1), which is not the case in the absence of Brij 35 where non-linearity is seen below 0.125 mmol/L (ie. 1/S of 8). What is most interesting is the approximately 2 fold activation of HLE by 0.01% Brij 35 with an increase (27%) in the K_m . An increase in k_{cat} of 40% was reported by Cook and Ternai (1988a) using the substrate MeO-Suc-A-A-P-V-NA which is consistent with the findings using this substrate as reported and discussed further in Chapter 5.

4.3 PROTEIN INHIBITION

4.3.1 INHIBITION OF HUMAN LEUKOCYTE ELASTASE

In this chapter and chapter 5 the actual mass of the inhibitor added to the reaction cuvette was chosen in preference to mass/volume so as to emphasise the mass ratio of inhibitor to enzyme in the cuvette. This allowed a direct comparison of the effectiveness of inhibitors towards an enzyme and overcame the problem of comparing different assay systems using different sample and assay volumes. A number of proteins were evaluated for their ability to inhibit HLE (0.5 μ g) with MeO-Suc-A-A-P-V-NA as substrate (see Section 2.4.2.2 for assay conditions; note that instead of Suc-A-A-A-NA, the higher affinity substrate MeO-Suc-A-A-P-V-NA was used here, as it only became available prior to the commencement of this part of the study). Albumin, included as a control, showed almost no inhibitory effect compared with the mild inhibitory effect of α_2 -M and the strong inactivation by α_1 -PI (Figure 4.3). However, if sufficient albumin is present there is a non-specific inhibition of HLE activity with 50% inhibition seen with 6 g/l and almost 90% inhibition seen at normal serum albumin concentrations (routine laboratory reference range; 39-48 g/l), as shown in Figure 4.4. This would indicate that any free HLE in blood would be bound by the albumin present in addition to the protease inhibitors present such

as α_1 -PI and α_2 -M. However, this would not be the case in the lung where the albumin concentration is much lower (eg. alveolar lining fluid, 0.08-0.25 g/l; Baughman et al., 1983).

The complete inhibition of HLE activity (0.5 μ g present in assay cuvette) required 1.0 μ g of α_1 -PI which is consistent with the fact that HLE (MW 26,000) and α_1 -PI (MW 52,000) bind in equimolar amounts. This inhibition was independent of pH within the pH range tested, of 6-9 (data not shown; The assay conditions were as used for heparin inhibition in Section 4.5.1.4). This also supports the data that the HLE used in this study is at least 90% active (see Section 4.2). However, the weak inhibition of HLE by α_2 -M is surprising in that other studies have shown little if any inhibitory effect when using low molecular weight substrates, with one report showing activation of HLE activity (Twumasi et al., 1977).

The effect of elastin peptides on HLE may be dependent on molecular weight. A soluble high molecular weight elastin peptide preparation (α -elastin, approx. MW 70,000) strongly inhibited HLE (50% inhibition with approximately 2.6 μ g and 92% inhibition with 19 μ g) whereas, a synthetic elastin peptide (VGVAPG, MW 588) showed no significant inhibitory activity (Figure 4.3). VGVAPG is a sequence that is repeated multiple times in the elastin molecule (Sandberg et al., 1981) and has been shown to be a chemoattractant for monocytes, elastin-producing fibroblasts (Senior et al., 1984) and tumour cells (Blood et al., 1988). Another repeating sequence in the elastin molecule is VPGVG, which appears to be an essential sequence for the autoregulation of elastin synthesis (Wachi et al., 1995). This pentapeptide was also found not to inhibit HLE activity (data not shown).

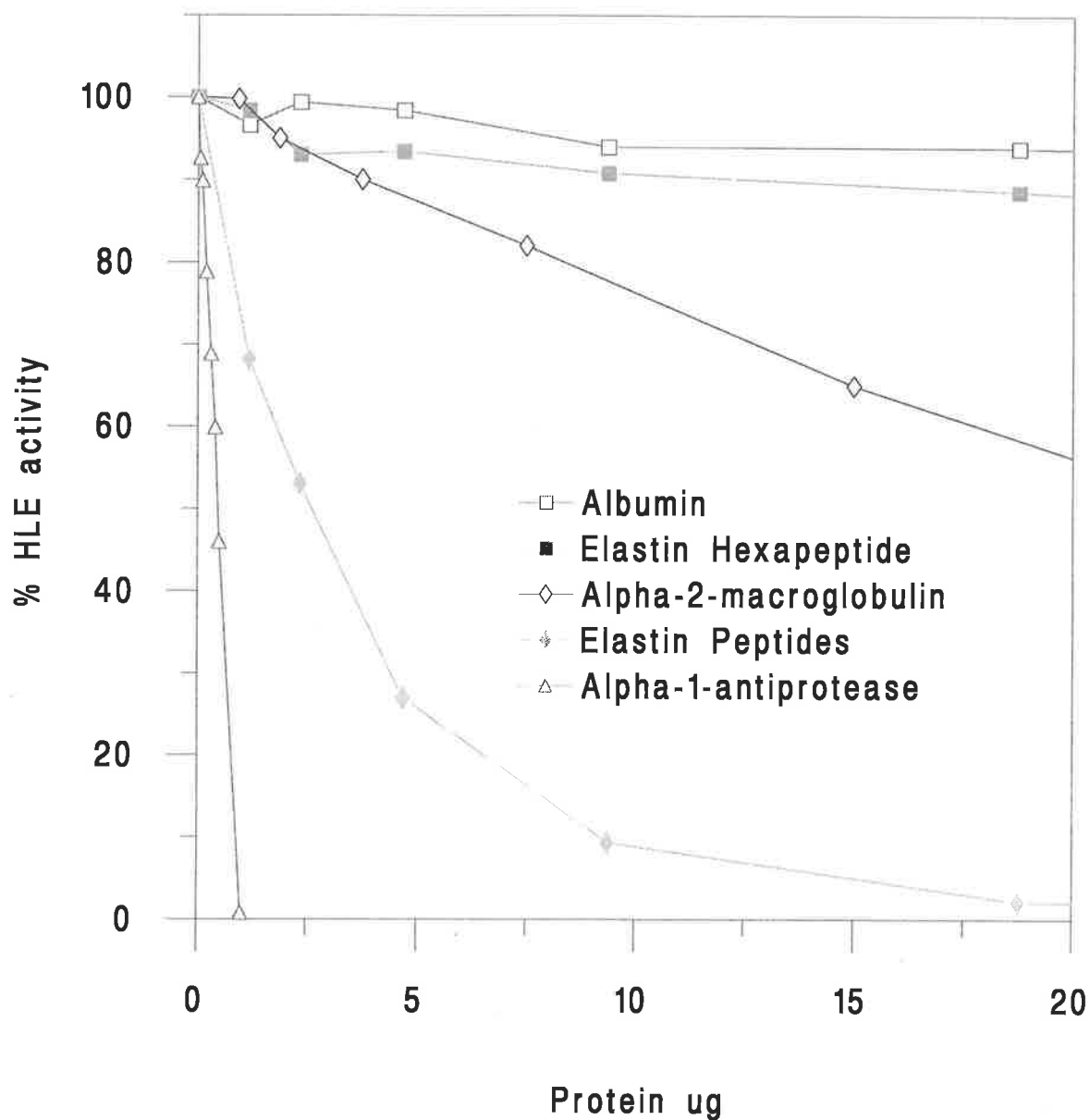


Figure 4.3: The inhibitory effect of proteins on HLE (0.5 ug) activity with MeO-Suc-A-A-P-NA as substrate. Assay conditions as described in Section 2.4.2.2.

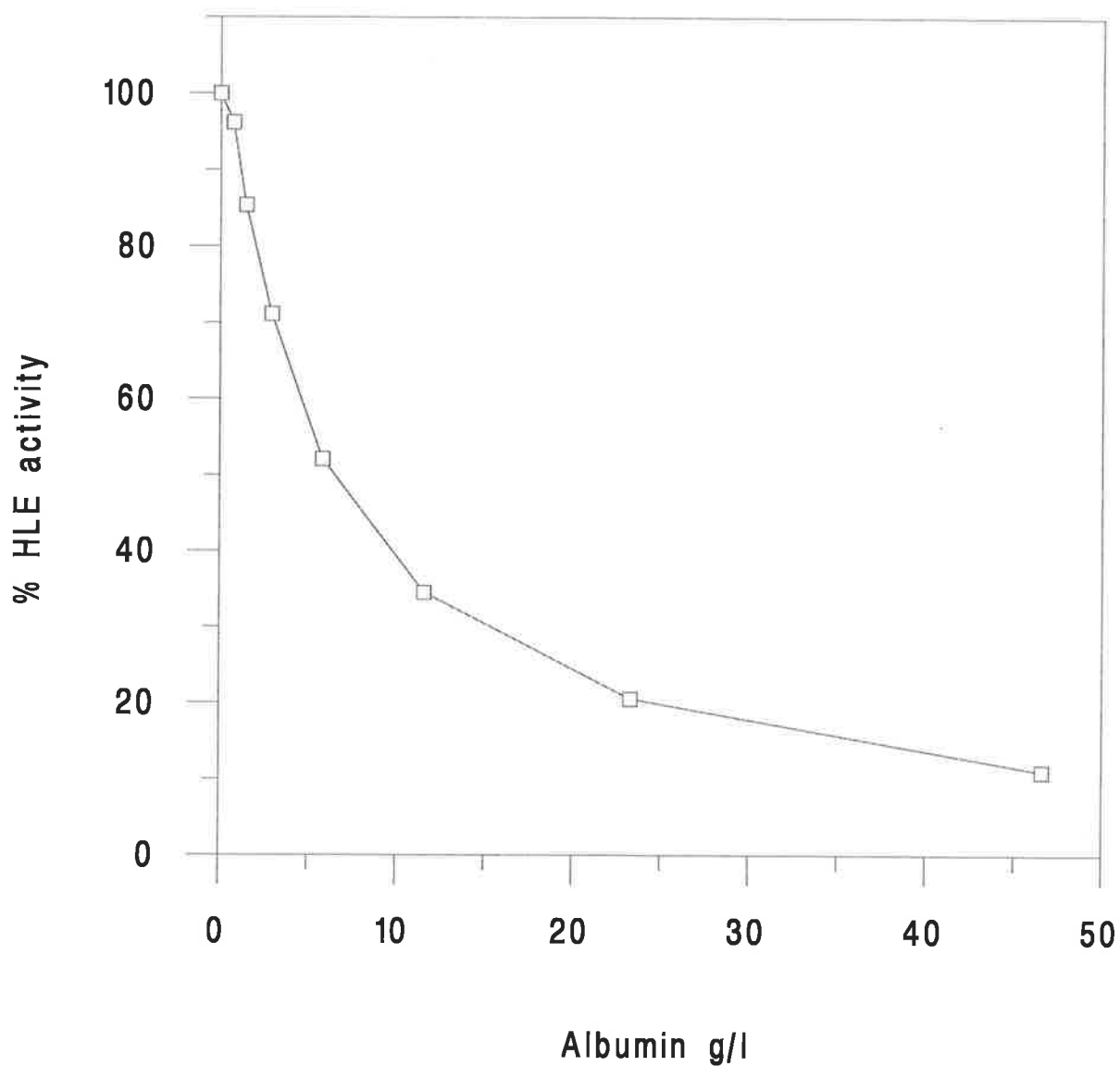


Figure 4.4: The inhibitory effect of albumin on HLE (0.5 ug) activity with MeO-Suc-A-A-P-V-NA as substrate. Assay conditions as described in Section 2.4.2.2

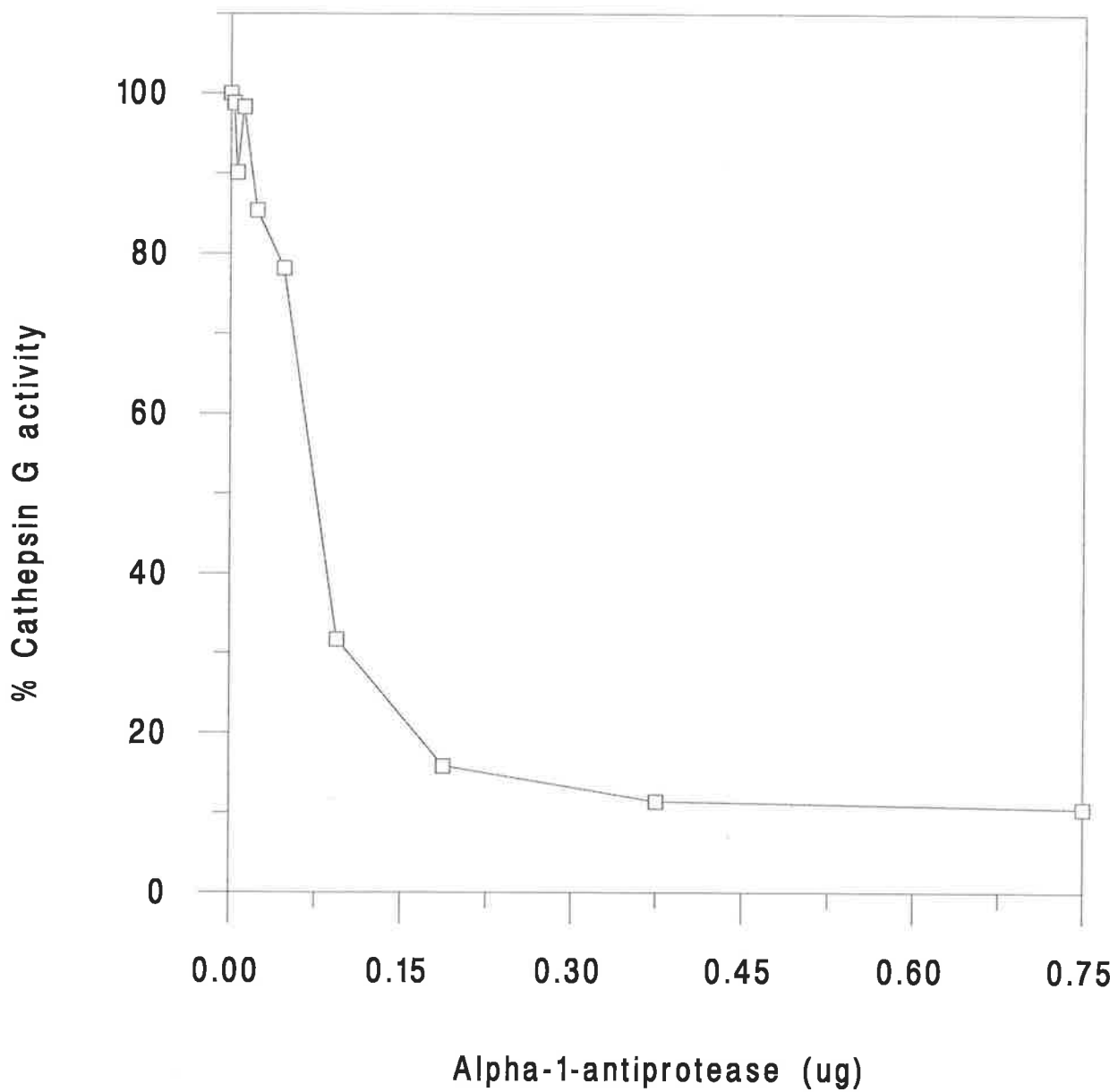


Figure 4.5: The inhibitory effect of alpha-1-antiprotease on cathepsin G (0.1 ug) activity. Assay conditions as described in Section 2.4.3.1.

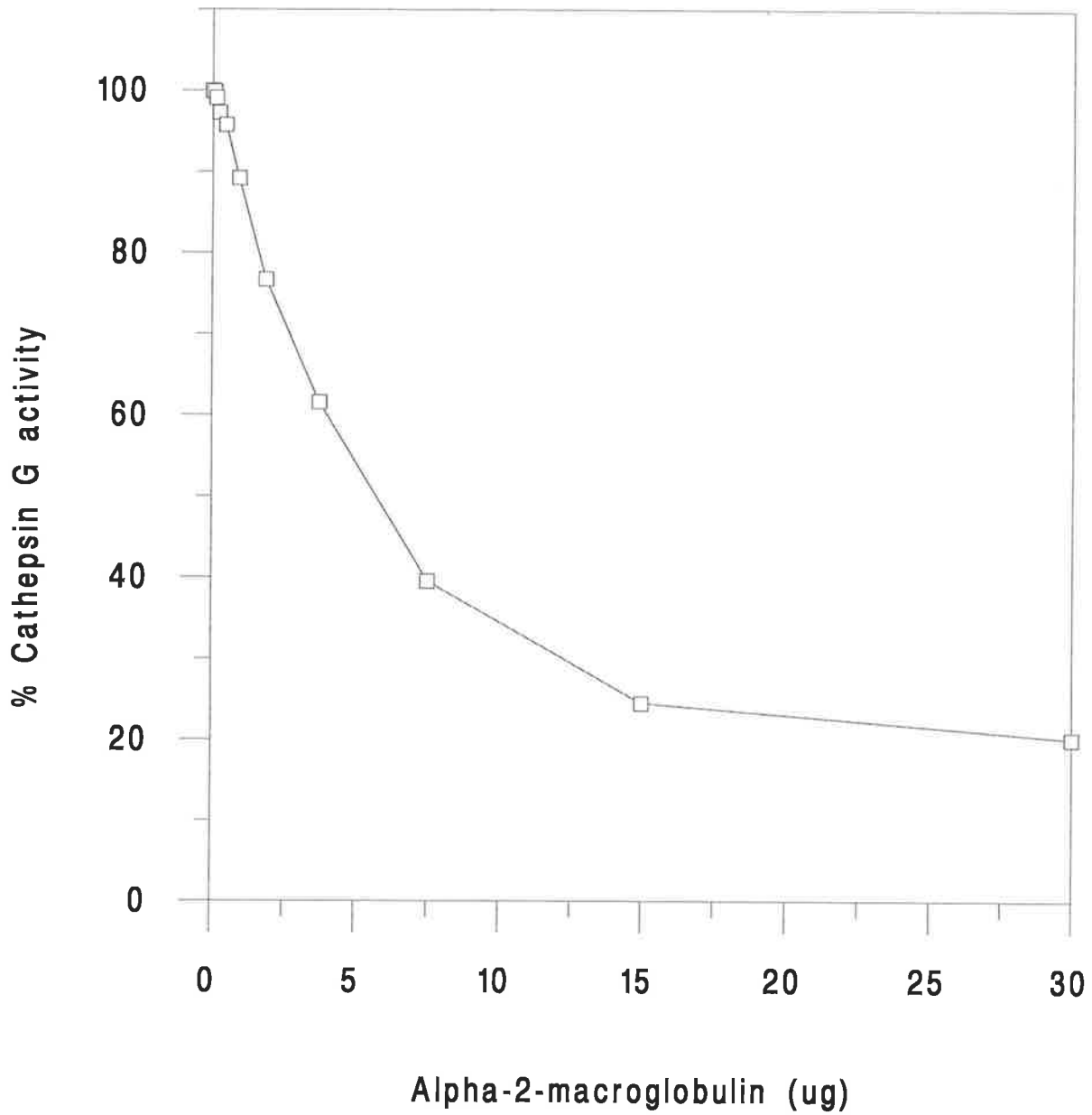


Figure 4.6: The inhibitory effect of alpha-2-macroglobulin on cathepsin G (0.1 ug) activity. Assay conditions as described in Section 2.4.3.1.

4.3.2 INHIBITION OF CATHEPSIN G

α_1 -PI and α_2 -M were evaluated for their ability to inhibit cathepsin G (0.1 μg), with n-Succinyl-(L-alanine)₂-proline-L-phenylalanine-p-nitroanilide as substrate, with an assay described in Section 2.4.3.1. α_1 -PI is a very effective inhibitor of cathepsin G with 50% inhibition seen with approximately 0.075 μg (Figure 4.5). However, unlike HLE (Figure 4.3), complete inhibition of cathepsin G is not seen (10% residual activity found, Figure 4.5).

α_2 -M is also a very effective inhibitor towards cathepsin G (50% inhibition seen with approximately 6.0 μg , Figure 4.6), which contrasts with its relative ineffectiveness towards HLE (Figure 4.3). As is the case for α_1 -PI inhibition of cathepsin G, α_2 -M did not completely inhibit cathepsin G (20% residual activity found, Figure 4.6).

4.4 HUMAN LEUKOCYTE ELASTASE SPECIFIC SYNTHETIC INHIBITORS

4.4.1 INHIBITION OF HUMAN LEUKOCYTE ELASTASE WITH MeO-Suc-A-A-P-V-NA AS SUBSTRATE.

The effectiveness of two potent synthetic inhibitors (ICI 186756 & 200355) which selectively inhibit HLE was evaluated (assay as in Section 2.4.2.2). ICI 186756, a low molecular weight aldehyde inhibitor, and ICI 200355, a peptide trifluoromethyl ketone inhibitor were found to be very effective inhibitors of HLE (Figure 4.7, 50% inhibition of 0.5 μg HLE was achieved with approx. 0.065 μg and 0.035 μg respectively). ICI 200355 was the more effective inhibitor with complete inhibition of HLE activity seen with less than 0.1 μg whereas greater than 1.5 μg was required in the case of ICI 186756.

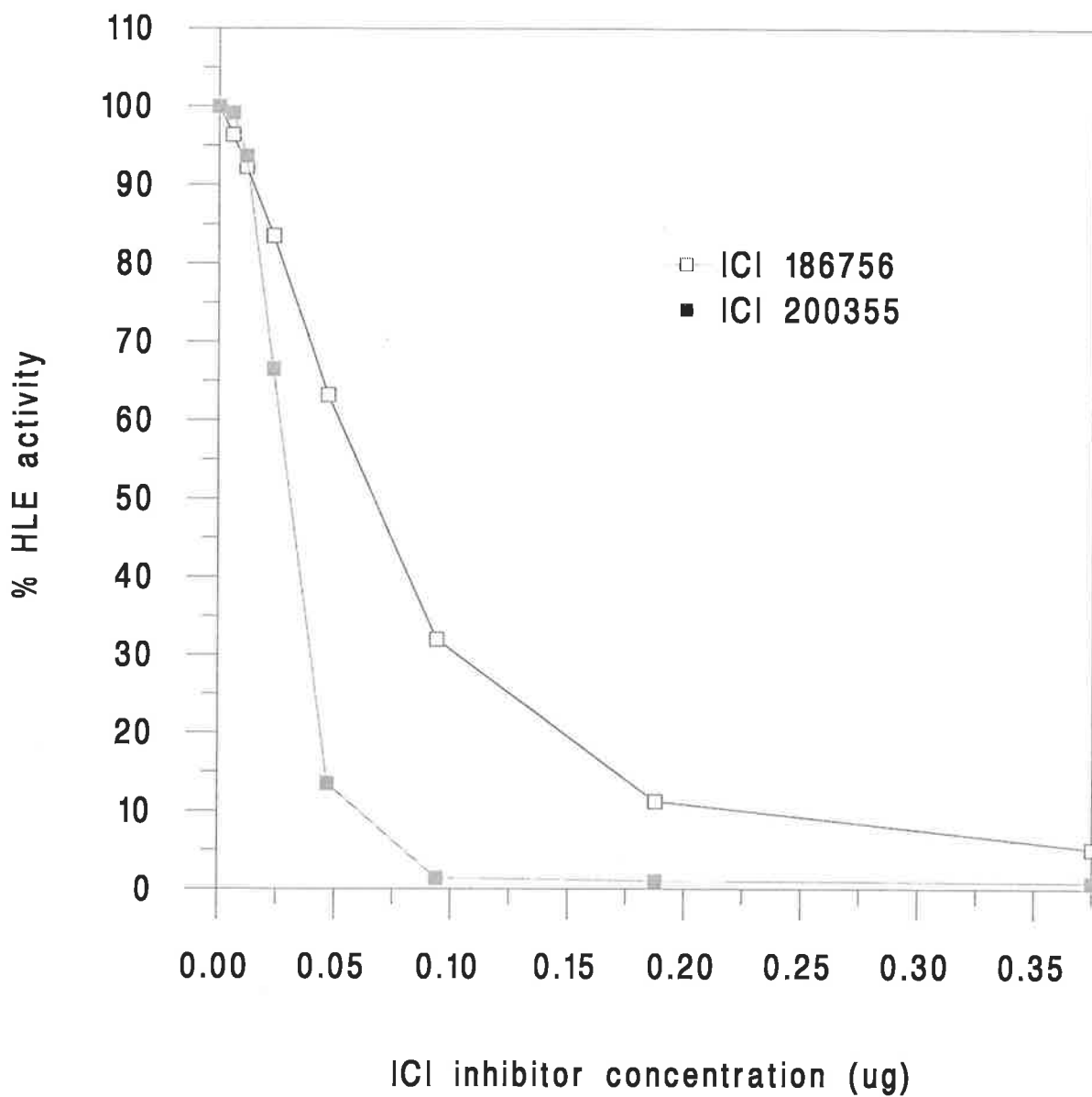


Figure 4.7: The inhibition of HLE with the ICI inhibitors 186756 and 200355. Assay conditions with MeO-Suc-A-A-P-V-NA as substrate as described in Section 2.4.2.2.

Table 4.2: Complimentary inhibitory effects of ICI 200355 towards α_1 -PI and heparin inhibition of HLE. Data were shown as actual % inhibition found towards HLE with calculated inhibition that would expected to be seen shown in brackets. HLE inhibition assay with MeO-Suc-A-A-V-P-NA is as described in Section 2.4.2.2.

	ICI 200355 (μg)			
	0	0.006	0.012	0.024
No inhibitor	0	6.0	16.7	33.0
Heparin (μg)				
0.0365	12.3	17.7 (18.3)	21.5 (29.0)	52.3 (45.3)
0.073	21.1	26.4 (26.1)	36.3 (37.8)	54.6 (54.1)
0.156	41.6	46.8 (47.6)	52.2 (58.3)	70.3 (74.6)
α_1-PI (μg)				
0.085	7.4	16.2 (13.1)	26.9 (24.1)	52.4 (40.4)
0.17	15.9	23.7 (21.9)	36.6 (32.6)	57.0 (48.9)
0.34	31.0	40.2 (37.0)	51.3 (47.7)	72.0 (64.0)

For a synthetic inhibitor to be fully effective *in vivo* it must not interfere with the activity of other inhibitors towards HLE and its inhibitory effect should be additive to other inhibitors. In particular, a synthetic inhibitor should be completely compatible with the activity of α_1 -PI, the main endogenous inhibitor of HLE. For example, ICI 200355 is found, in general, to be additive in inhibitory effect towards the inhibitors α_1 -PI and heparin (Table 4.2). This was also the case for ICI 186756 (data not shown).

4.4.2 INHIBITION OF HUMAN LEUKOCYTE ELASTASE WITH HUMAN LUNG ELASTIN AS SUBSTRATE.

The more effective ICI inhibitor, 200355, was also tested for its ability to block HLE hydrolysis of a more typical endogenous substrate, human lung elastin where the released elastin peptides were measured with an ELISA method for soluble elastin peptides (see Section 2.4.2.5 for assay procedure). As shown in Figure 4.8, 0.02 $\mu\text{g}/\text{ml}$ (ie. 2 ng added) of the inhibitor was required to obtain 50% inhibition of 5 ng of HLE in this assay system. Inhibition was essentially complete at concentrations greater than 1.25 $\mu\text{g}/\text{ml}$. This demonstrates that this inhibitor is not as effective when an endogenous substrate such as human lung elastin is used.

4.4.3 INHIBITION OF CATHEPSIN G

Two potent synthetic inhibitors of HLE, ICI 186756 & 200355, are reported to be highly specific for this enzyme. This is supported by the finding that they show almost no inhibitory activity towards cathepsin G (Figure 4.9).

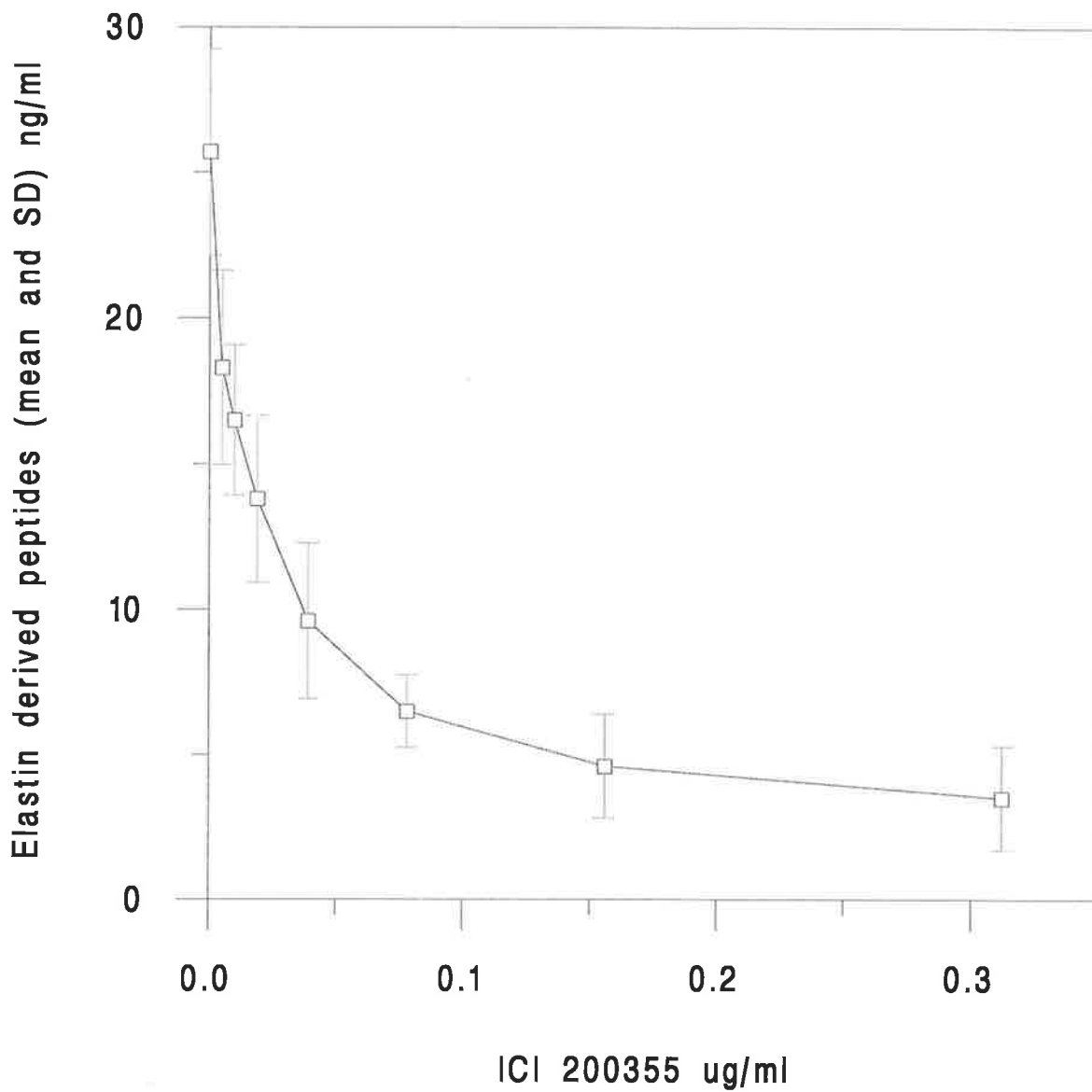


Figure 4.8: ICI 200355 inhibition of HLE hydrolysis of human lung elastin (n=9). Assay as described in Section 2.4.2.4.

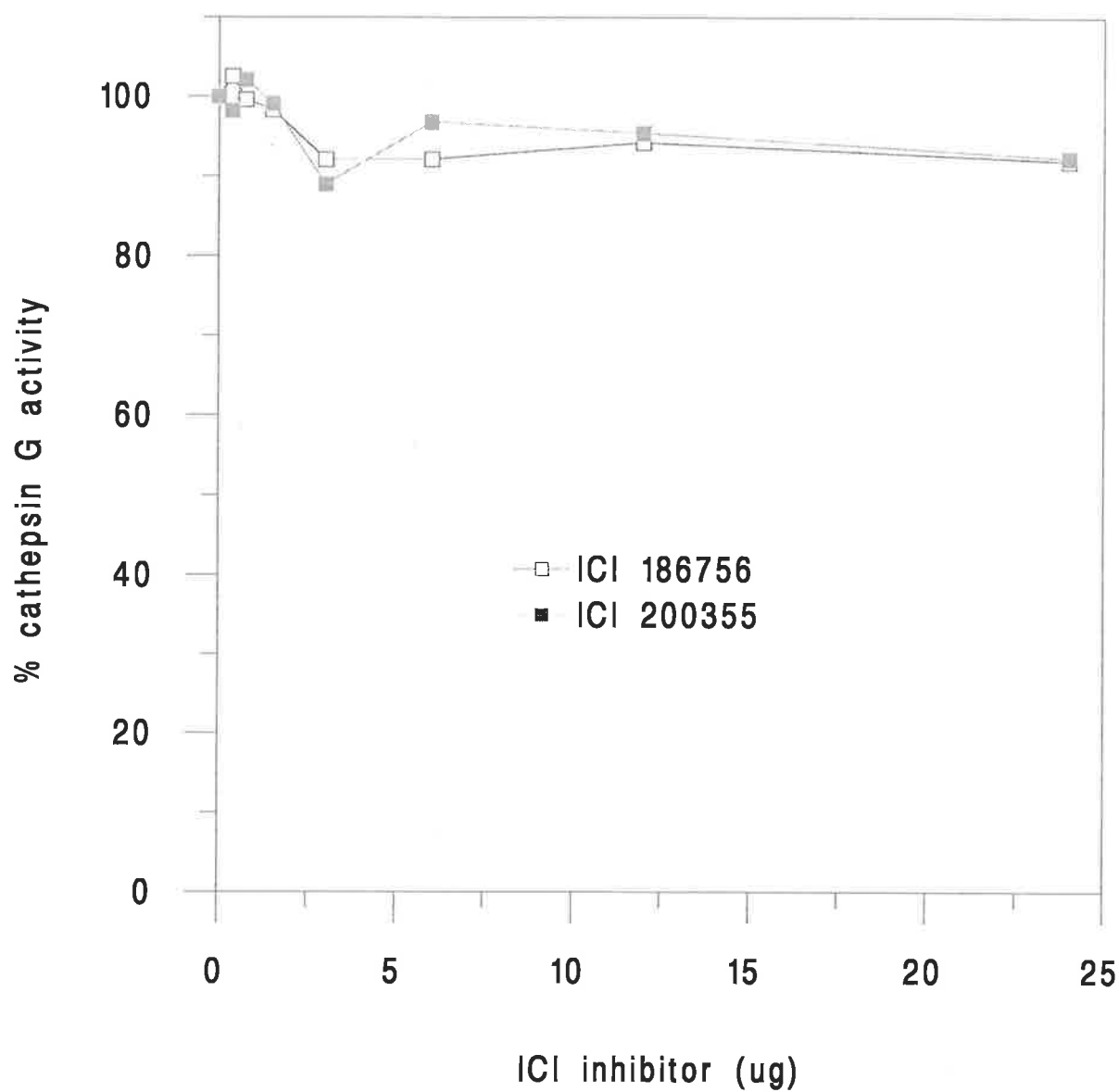


Figure 4.9: Effect of ICI inhibitors 186756 and 200355 on Cathepsin G activity. Assay procedure as in Section 2.4.3.1.

4.5 GLYCOSAMINOGLYCAN INHIBITION

4.5.1 INHIBITION OF HUMAN LEUKOCYTE ELASTASE WITH Suc-A-A-A-NA AS SUBSTRATE

The HLE inhibition assay in this Section used the method described in Section 2.4.2.1. This is an early study where Suc-A-A-A-NA was used as substrate before the more sensitive MeO-Suc-A-A-P-V-NA became available. Some of this material has already been published (Walsh et al., 1988; Walsh et al., 1991a). Preliminary studies showed that heparin strongly inhibited HLE but that the other leucocyte enzymes tested, cathepsin G and myeloperoxidase, and the pancreatic enzymes, pancreatic elastase, trypsin and chymotrypsin, were not inhibited by heparin concentrations to at least 250 $\mu\text{g/ml}$ (data not shown). This inhibition of HLE and cathepsin G by heparin and other GAGs was investigated further in this Section.

4.5.1.1 TYPE OF GLYCOSAMINOGLYCAN

The effect of a range of glycosaminoglycans on the hydrolysis of Suc-A-A-A-NA by HLE is shown in Fig. 4.10. The most effective inhibitor is heparin (0.22 μg or 80 μl of 2.8 $\mu\text{g/mL}$ gave 50 % inhibition of 2.5 μg of HLE) followed by heparan sulphate (0.64 μg), chondroitin sulphate B (2.4 μg), chondroitin sulphate C (>2.4 μg), chondroitin sulphate A (>2.4 μg), with hyaluronic acid having almost no inhibitory activity. Complete inhibition of HLE activity is not seen with any of the glycosaminoglycans. For example, the maximum inhibition for heparin and heparan sulphate of 85.5% and 70% is achieved with concentrations of 1.2 μg and 2.5 μg respectively.

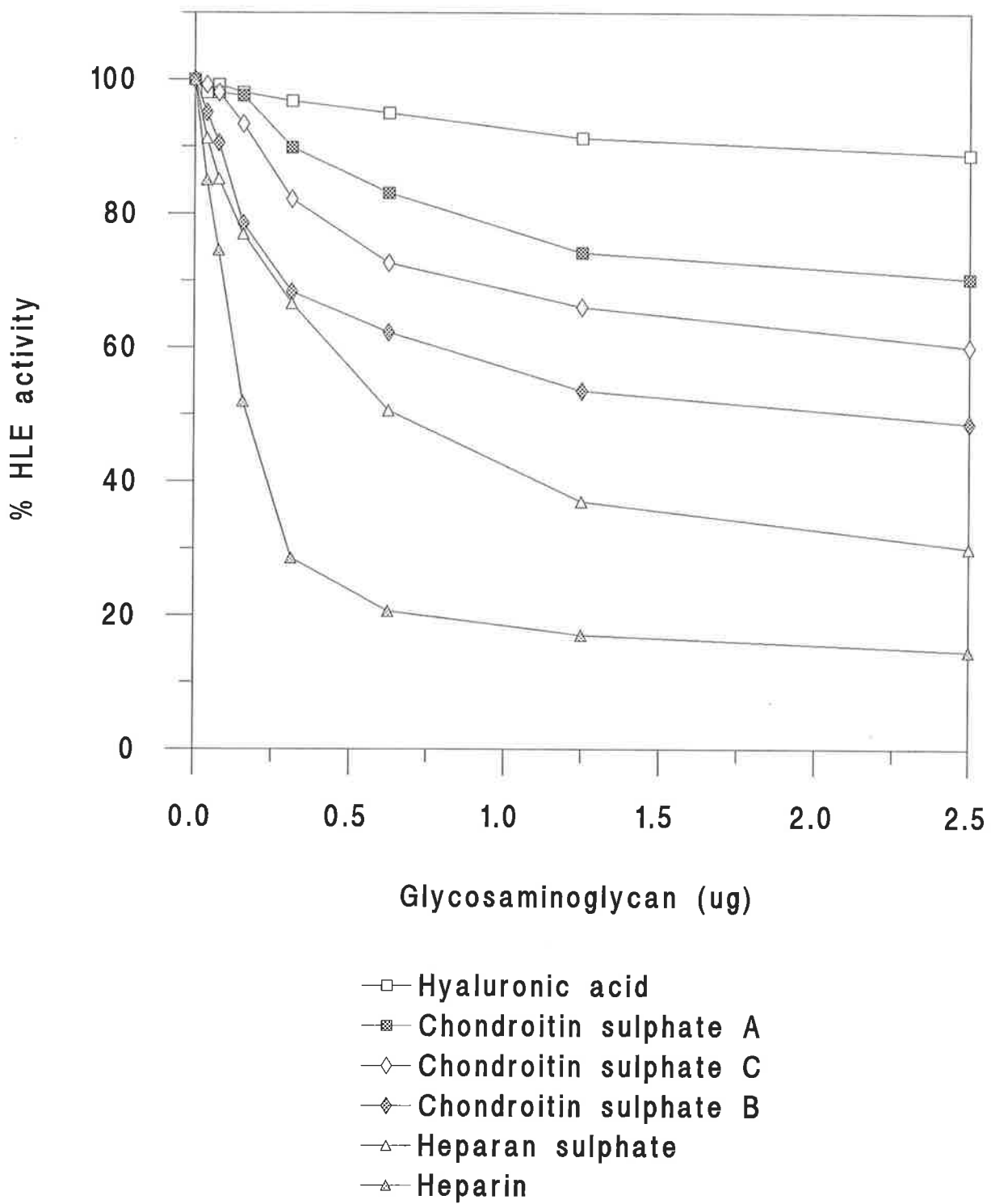


Figure 4.10: Glycosaminoglycan inhibition of HLE activity with Suc-A-A-A-NA as substrate. Assay conditions as in Section 2.4.2.1.

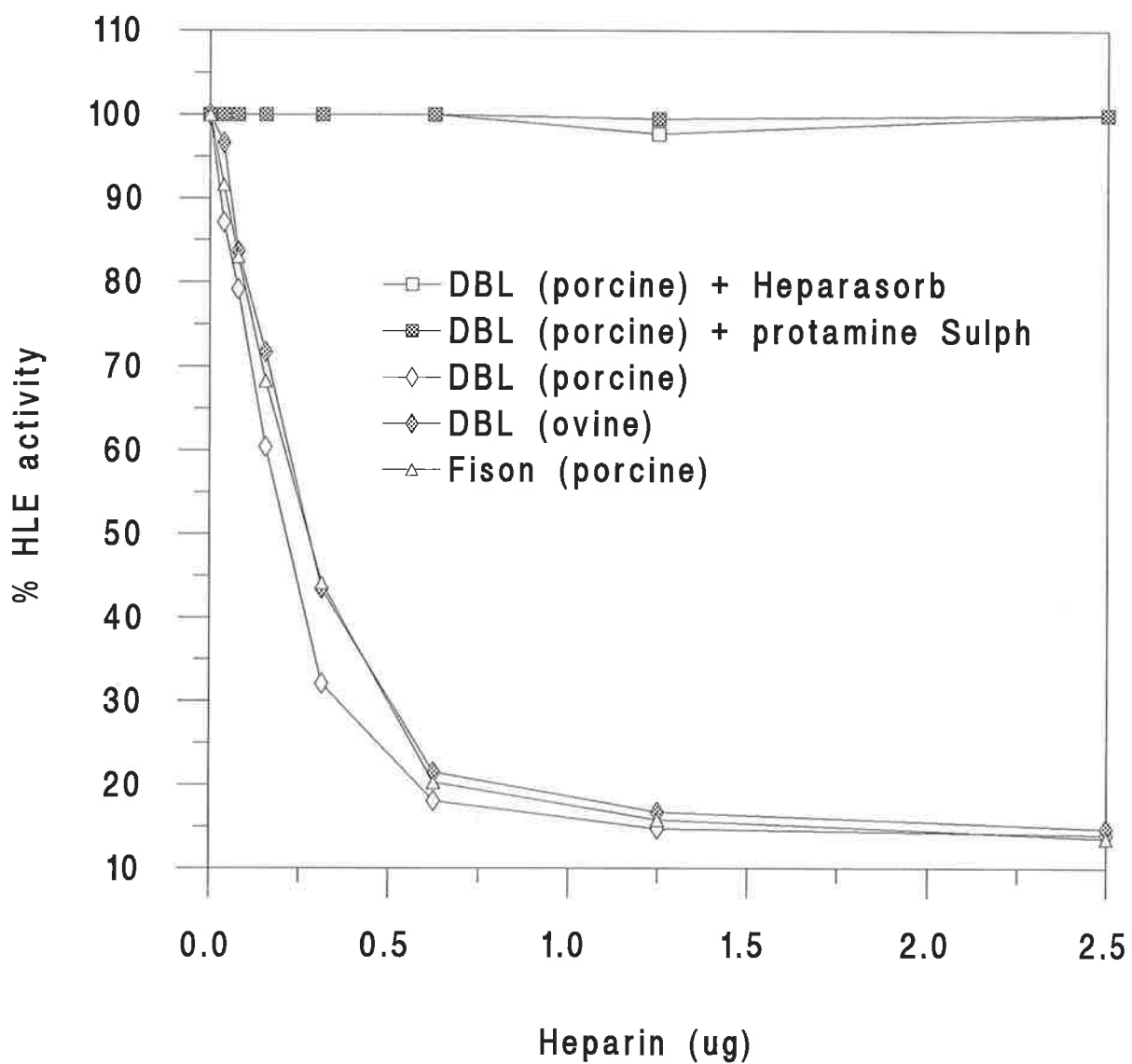


Figure 4.11: Effect of different sources of heparin on HLE activity with Suc-A-A-A-NA as substrate. Assay conditions as described in Section 2.4.2.1.

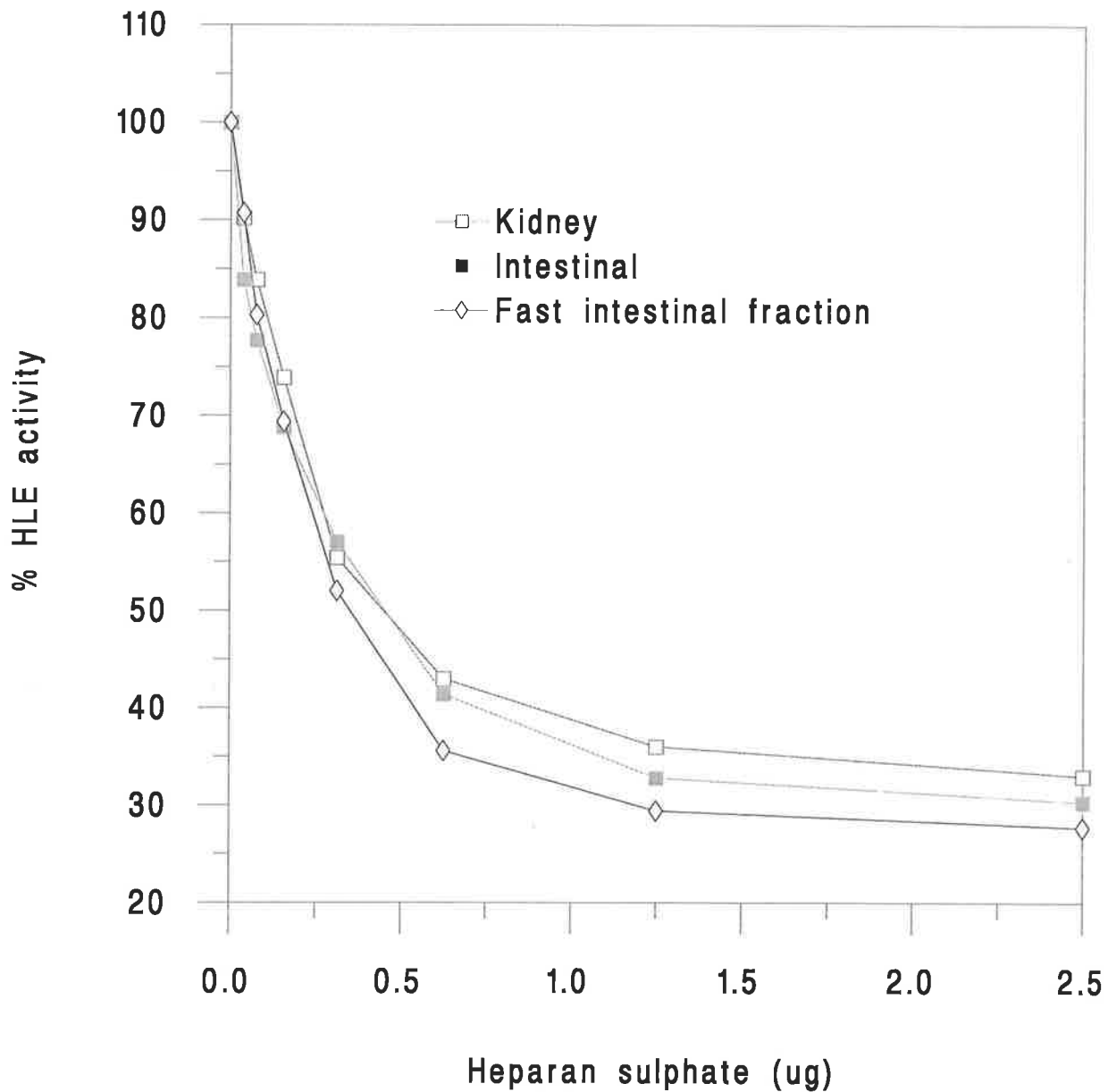


Figure 4.12: Effect of Heparan sulphate preparations from different sources on HLE activity with Suc-A-A-A-NA as substrate. Method as described in Section 2.4.2.1.

4.5.1.2 SOURCE OF HEPARIN AND HEPARAN SULPHATE

Heparin and heparan sulphate are the most effective GAG inhibitors of HLE (Figure 4.10). A number of heparin and heparan sulphate preparations were assessed for any variation in their ability to inhibit HLE activity. However, with heparin preparations from different commercial and animal sources, no variation was seen in the inhibition of HLE (Figure 4.11). This was also the case with heparan sulphate preparations where little variation in inhibition was seen (Figure 4.12). The inhibition of HLE by sulphated GAGs was completely removed by pretreating the assay sample with Heparasorb (cationic triethylaminoethyl cellulose) or protamine sulphate as demonstrated by treating heparin (Fig. 4.11). These reagents interfere in the assay by effectively binding heparin or HLE (ie. The cationic Heparasorb binds to the anionic GAG and the anionic protamine sulphate binds to the cationic HLE).

4.5.1.3 MOLECULAR WEIGHT OF HEPARIN

The effects of various low molecular weight heparins on the hydrolysis of Suc-A-A-A-NA by HLE are shown in Fig. 4.13. Opocrin 2133 (MW 4500) and Kabi 2165 (MW 4000-6000) inhibited HLE equally as well as heparin (typical average MW 15000). However Opocrin 381/1 (MW 2000) did not inhibit as well as heparin, with Opocrin 381/2 (MW 800-1000) showing little inhibition. This suggests that a molecular weight of greater than 2000 is required for maximum inhibition of HLE.

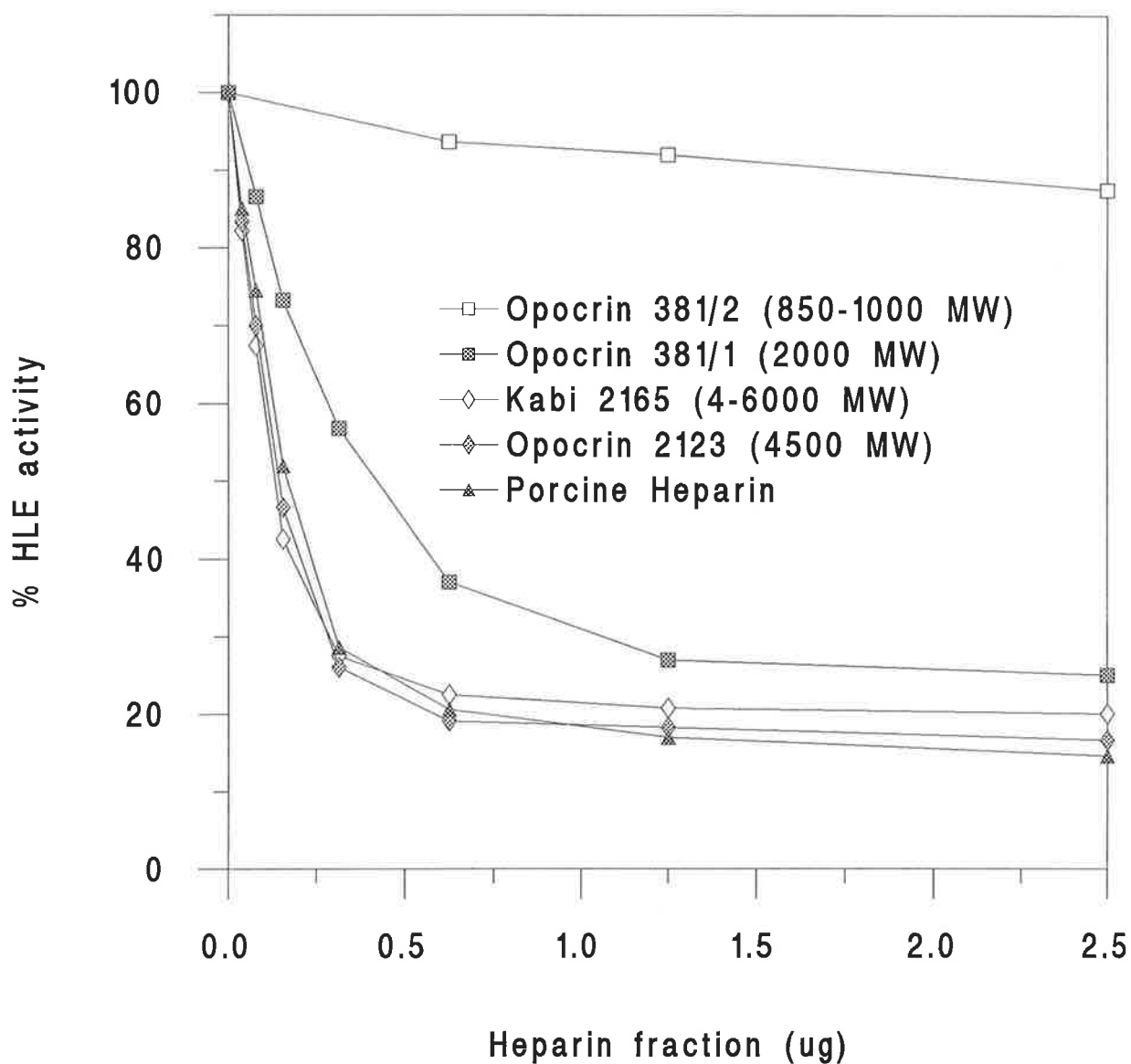


Figure 4.13: Effect of low molecular weight heparin fractions on HLE activity with Suc-A-A-A-NA as substrate. Assay conditions as described in Section 2.4.2.1.

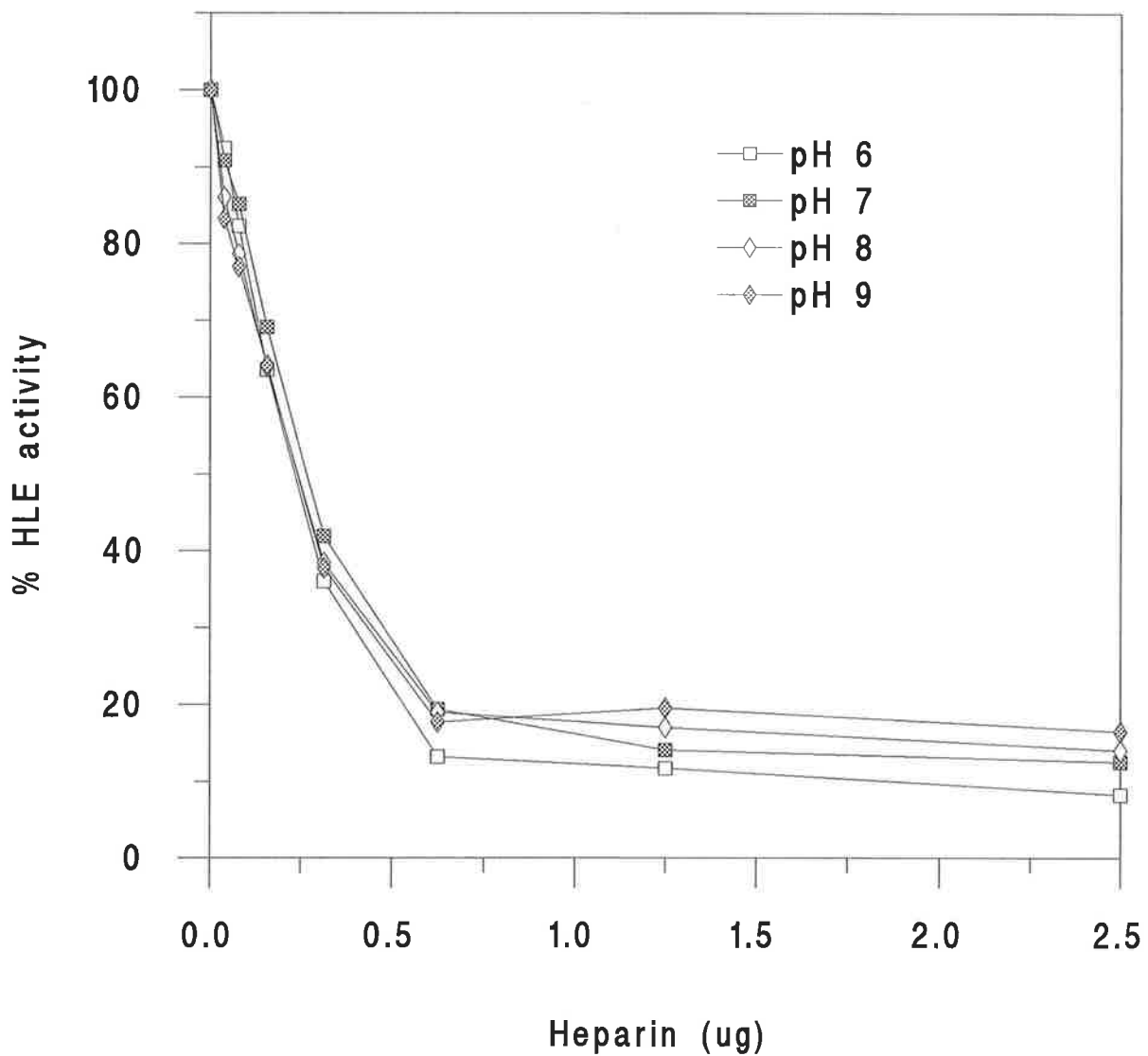


Figure 4.14: Effect of pH on heparin inhibition of HLE activity with Suc-A-A-A-NA as substrate. Procedure as described in Section 2.4.2.1.

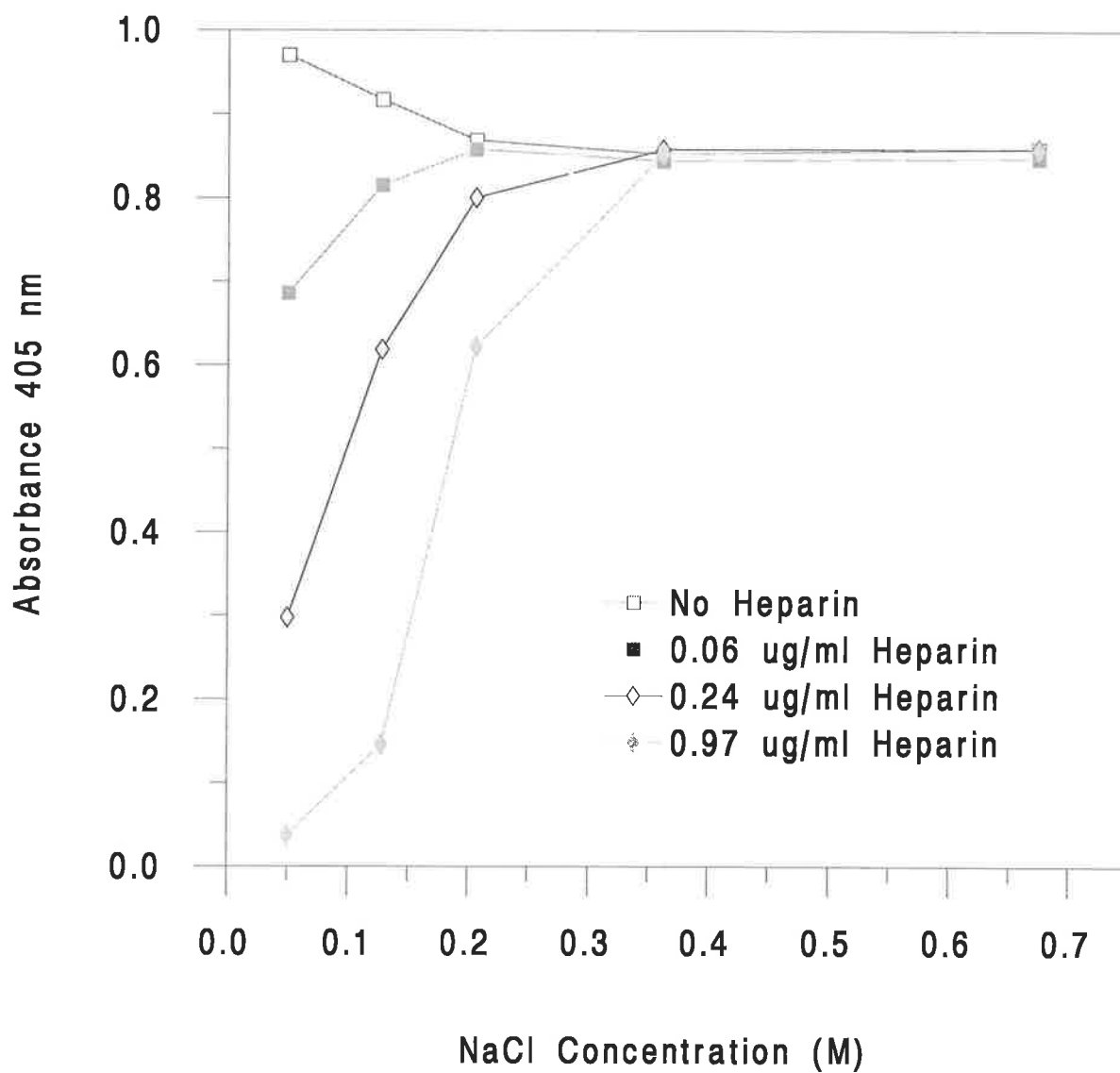


Figure 4.15: Effect of NaCl concentration on heparin inhibition of HLE activity with MeO-Suc-A-A-P-V-NA as substrate. Procedure is as described in Section 2.4.2.2.

4.5.1.4. ASSAY CONDITIONS

The binding of heparin to HLE is essentially by electrostatic interactions, which probably involves the negatively charged sulphate groups on heparin and some of the positively charged arginine residues on the surface of HLE. The relative charge on these groups may be altered by changing the pH of the assay buffer. It was found that while the rate of hydrolysis of Suc-A-A-A-NA by HLE changed with pH (optimum at pH 8.0), the degree of inhibition of HLE by heparin was found to be independent of pH within the 6-9 range of pH studied (4.14). Thus the binding of heparin to HLE appears to be unaffected by pH in the physiological pH range, as was also found for α_1 -PI (see Section 4.2).

However, it is likely that the ionic strength of the assay buffer will influence the degree of binding of heparin to HLE. This was examined by increasing the concentration of NaCl in the assay buffer above the 0.05M used as the standard concentration (see Section 2.4.1.5). NaCl interference in the inhibition of HLE activity by heparin increases progressively at NaCl concentrations above 0.05 M until 0.35 M and above, where NaCl completely blocks the inhibition of HLE activity by heparin (Figure 4.15). It is important to note that heparin is a strong inhibitor of HLE activity at the physiological NaCl concentrations of 0.14 M.

Other variations in assay conditions were examined and are reported elsewhere: ie. incubation time of GAG with HLE (Section 4.6); order of addition GAG, HLE and substrate (Section 4.7); presence of surfactant (chapter 5).

4.5.2 INHIBITION OF HUMAN LEUKOCYTE ELASTASE WITH HUMAN LUNG ELASTIN AS SUBSTRATE

Heparin and heparan sulphate, the most effective GAG inhibitors of the HLE hydrolysis of Suc-A-A-A-NA, also inhibited HLE activity towards human lung elastin where the released elastin peptides were measured with an ELISA method for soluble elastin peptides (Figure 4.16; see Section 2.4.2.4 for assay procedure). Whereas heparin was the more effective inhibitor in the assay with Suc-A-A-A-NA as substrate, heparan sulphate was more effective in the insoluble lung elastin assay. This is shown in Figure 4.16 where 50% inhibition of 5 ng of HLE was obtained with 4.5 μg of heparin/ml (ie. 0.45 μg added) or 0.8 μg of heparan sulphate/ml (ie. 0.08 μg added). This demonstrates that GAGs are much less effective inhibitors when an endogenous substrate such as human lung elastin is used than when the soluble peptide substrate, Suc-A-A-A-NA, is used (see Section 4.5.1.1). Like the inhibition seen using Suc-A-A-A-NA as substrate, complete inhibition of HLE activity was not seen; the highest GAG concentration used, 200 $\mu\text{g}/\text{ml}$, resulted in 85-95% inhibition which is similar to that obtained for heparin using Suc-A-A-A-NA as substrate (Figure 4.7).

To exclude the possibility that glycosaminoglycans may be directly interfering in the ELISA, standard curves were established in the presence and absence of heparin and heparan sulphate. As can be seen in Fig. 4.17, heparin did not cause a direct interference in the ELISA despite the fact that heparin at concentrations of 2 and 8 $\mu\text{g}/\text{ml}$ significantly inhibited HLE activity towards lung elastin (Fig. 4.16). This was also the case for heparan sulphate (data not shown).

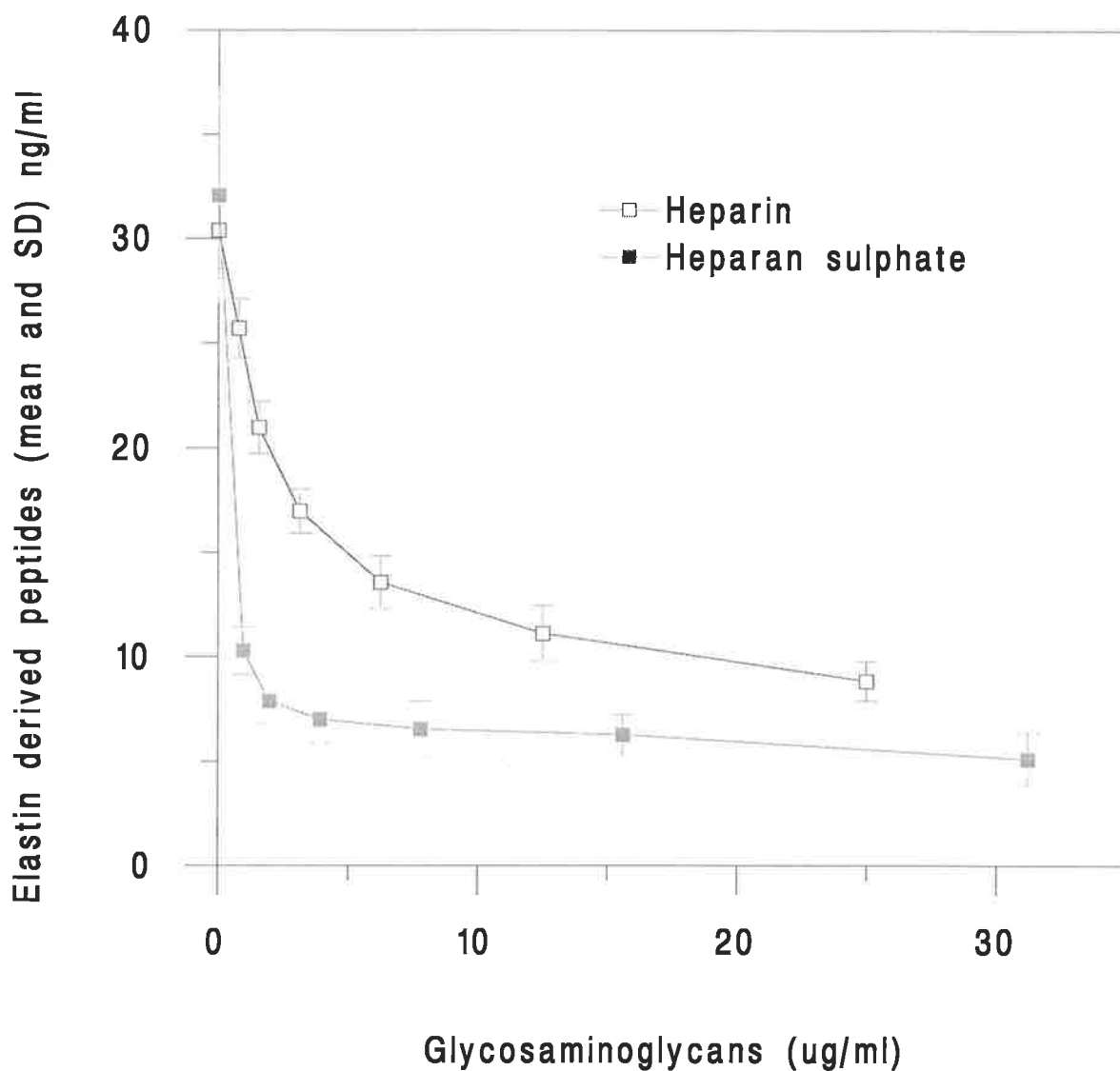


Figure 4.16: Effect of heparin and heparan sulphate on HLE hydrolysis of insoluble human lung elastin (n=12). Procedure is as described in Section 2.4.2.4.

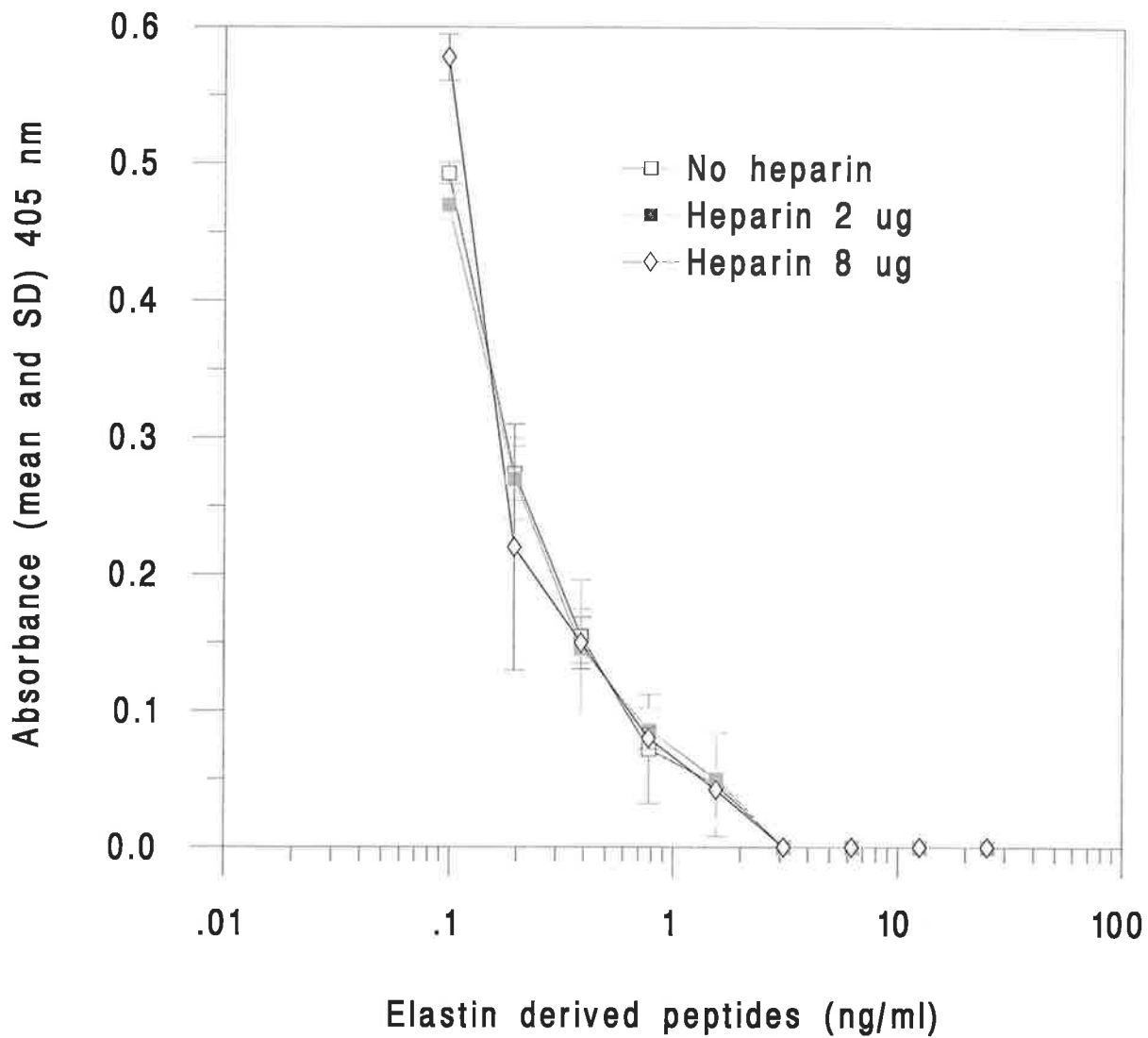


Figure 4.17: Effect of addition of heparin to the ELISA standard curve for elastin derived peptides (n=3). Procedure is as described in Section 2.4.4.

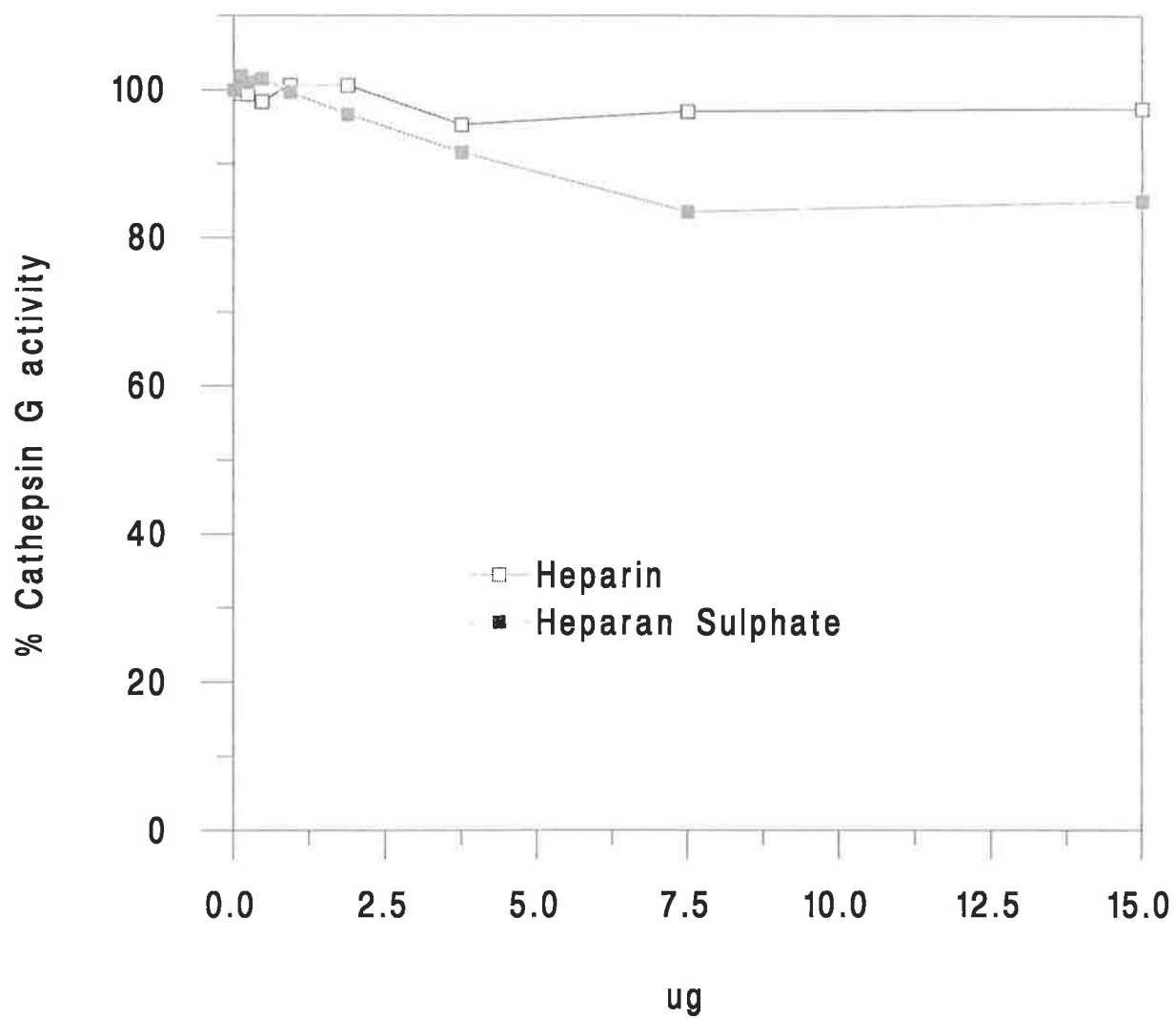


Figure 4.18: Effect of heparin and heparan sulphate on cathepsin G activity. Assay conditions as described in 2.4.3.1.

There was also the possibility that the size of the elastin-derived peptides generated by HLE activity towards lung elastin could change in the presence of heparin or heparan sulphate and that the different size peptides might not be recognized by the antibody in the ELISA. To examine this possibility, elastin peptides were prepared by HLE activity on elastin, both in the presence and absence of heparin, and were then separated according to size on a HPLC gel filtration column (see Section 2.3.13). The major peak, both in the presence and absence of heparin, was found to be of MW 4500 with shoulder peaks of MW 3500 and MW 9500 (data not shown). There was no evidence of other peaks or a shift in the molecular weight of the major and shoulder peaks. The peaks prepared with heparin were smaller than that without heparin, as would be expected from the heparin inhibition of HLE activity. Similar data was found for heparan sulphate.

4.5.3 INHIBITION OF CATHEPSIN G

While sulphated GAGs, such as heparin and heparan sulphate, are effective inhibitors of HLE (Figure 4.10), they are ineffective against cathepsin G, even at concentrations as high as 1 mg/ml (Figure 4.18).

4.6 INCUBATION TIME OF INHIBITORS

4.6.1 INHIBITION OF HUMAN LEUKOCYTE ELASTASE.

The evaluation of inhibitors that bind to an enzyme usually requires that the inhibitor be incubated with the enzyme for a period to achieve maximal binding between the inhibitor and the enzyme before measuring the reduced enzyme activity. The importance of this incubation period

was evaluated for five different types of inhibitors, α_1 -PI, α_2 -M, ICI 200355, ICI 186756 and heparin. Each inhibitor was incubated at 25^o C for 10 seconds (the shortest incubation time available on the Cobas-Bio centrifugal analyser), 5 minutes and 15 minutes with HLE (0.5 μ g) prior to the addition of the substrate, MeO-Suc-A-A-P-V-NA (see Section 2.4.2.2 for assay conditions). The inhibition plots show that the inhibitors, ICI 200355, ICI 186756 and heparin are fast acting with approximately 84%, 75% and 93% inhibition achieved after 10 seconds when compared to the same concentration of inhibitors respectively that gave 50% inhibition on each plot with 15 minutes incubation (Figures 4.19, 4.20). However, α_1 -PI was not as rapid with 42% inhibition achieved after 10 seconds when compared to 15 minutes incubation at the 50% inhibition point (Figure 4.21). α_1 -PI required at least 15 minutes for maximum inhibition (figure 4.21) unlike the other inhibitors tested, where maximum inhibition was achieved after 5 minutes (Figures 4.19, 4.20). However, in the presence of an excess of α_1 -PI, ICI 200355, ICI 186756 or heparin, incubation time was not a factor with complete inhibition seen in every case. α_2 -M, like α_1 -PI, required 15 minutes for maximum inhibitory effect but it was a much less effective inhibitor with only 65% inhibition of HLE (0.5 μ g) achieved with 30 μ g of α_2 -M (Figure 4.22).

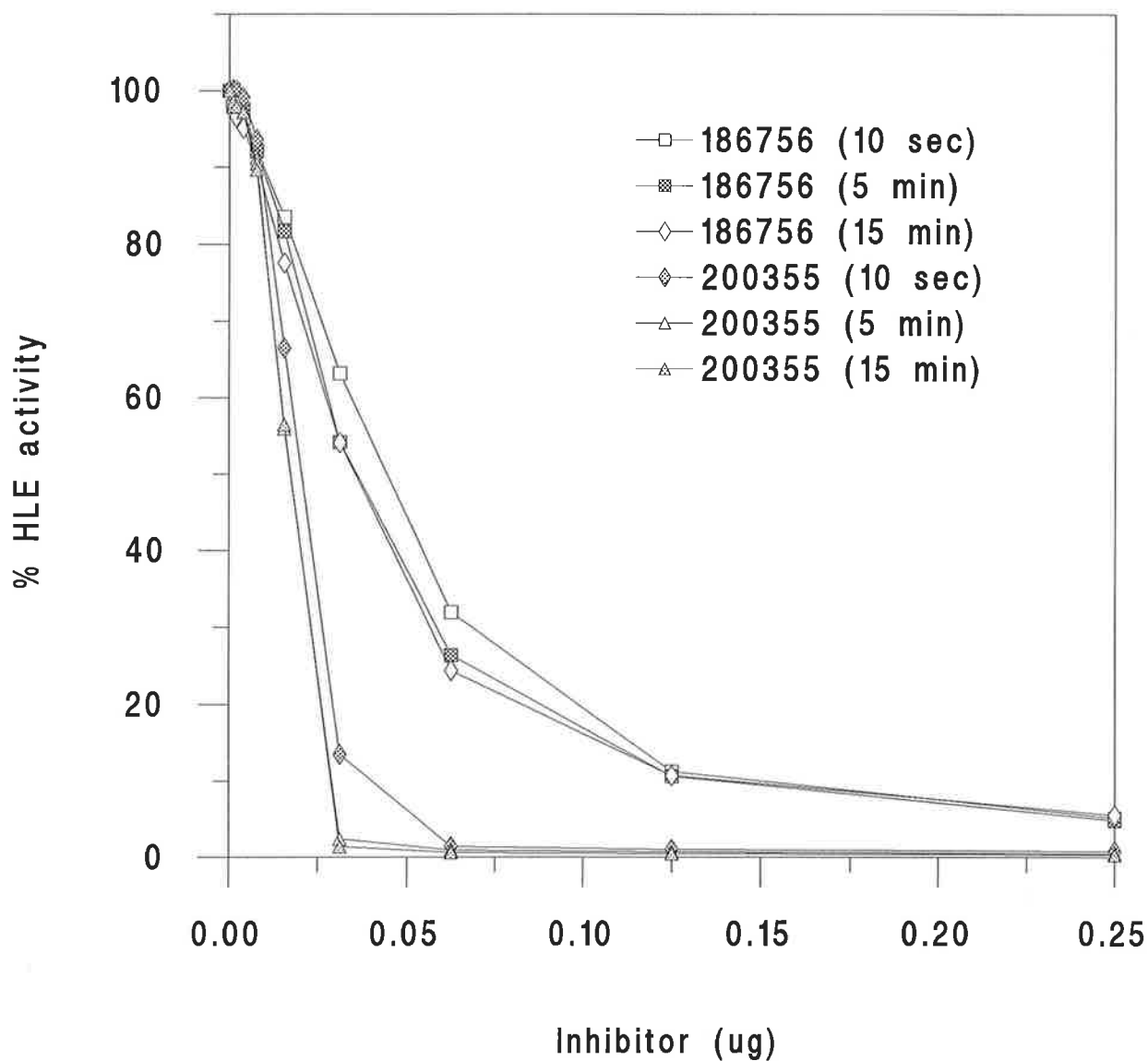


Figure 4.19: Effect of incubation time on ICI 186756 and 200355 inhibition of HLE with MeO-Suc-A-A-P-V-NA as substrate. Assay conditions as described in Section 2.4.2.2.

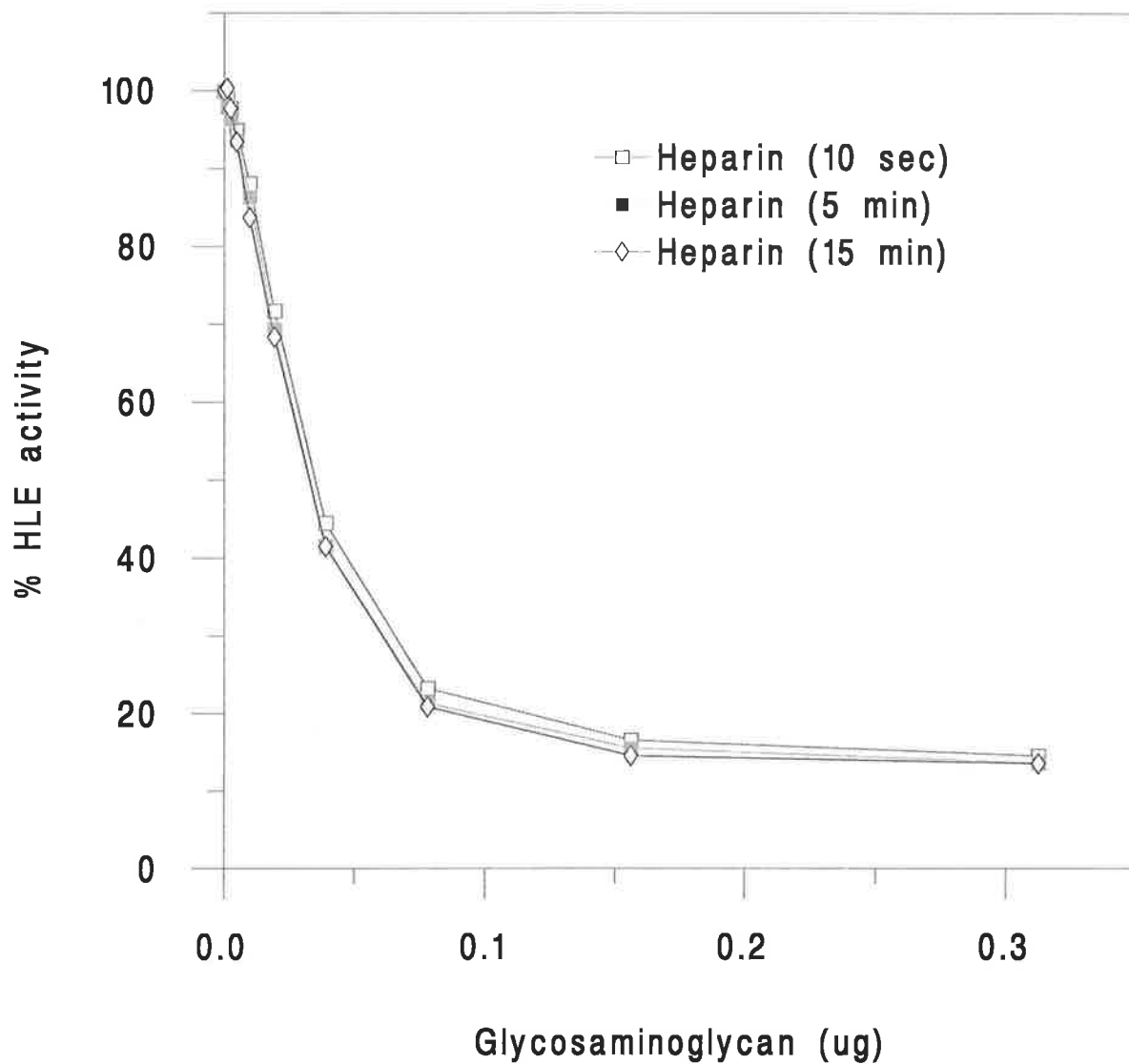


Figure 4.20: Effect of incubation time on heparin inhibition of HLE with MeO-Suc-A-A-V-P-NA as substrate. Assay conditions as described in Section 2.4.2.2.

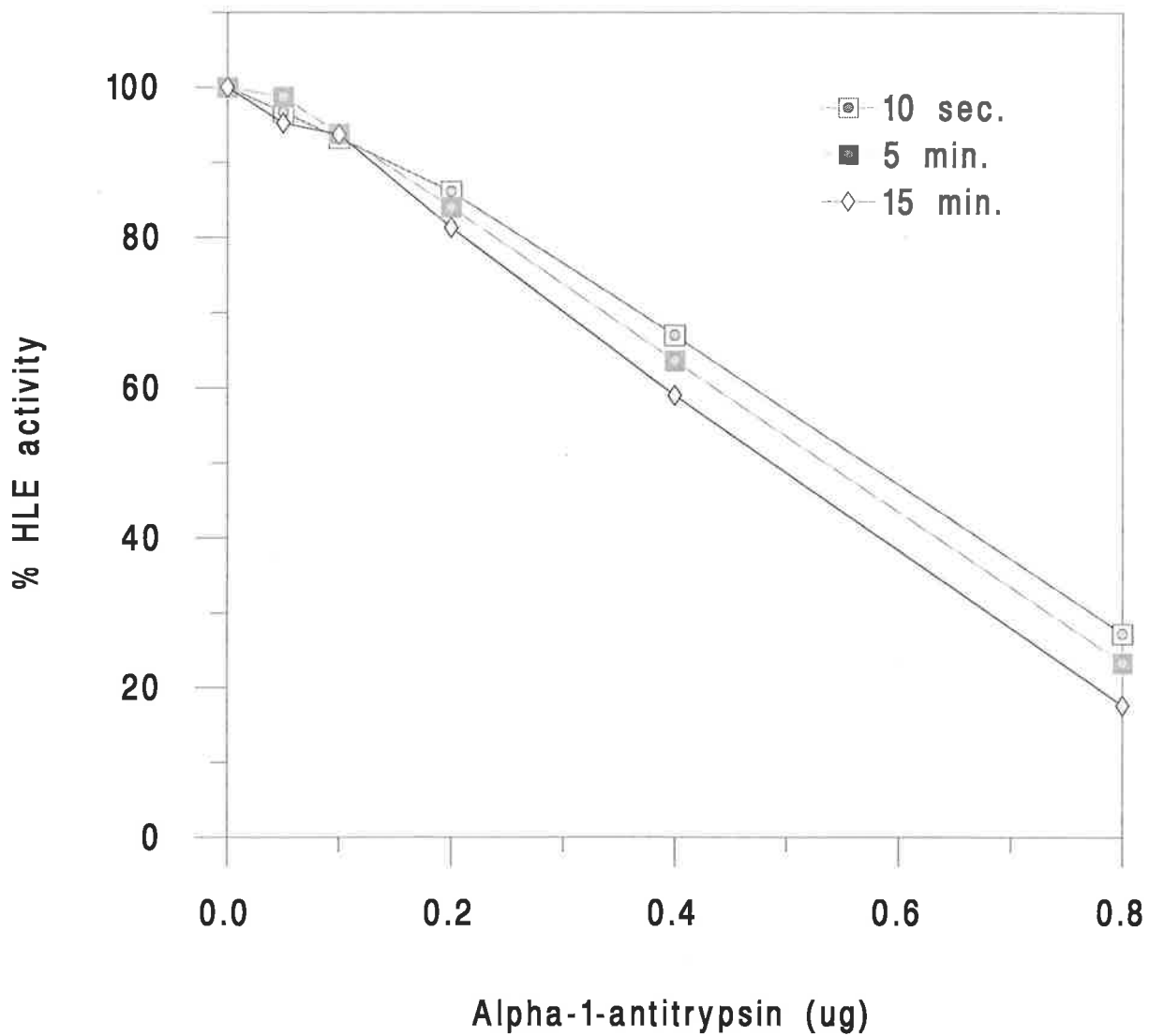


Figure 4.21: Effect of incubation time on alpha-1-antiprotease inhibition of HLE with MeO-Suc-A-A-P-V-NA as substrate. Assay as described in Section 2.4.1.6.

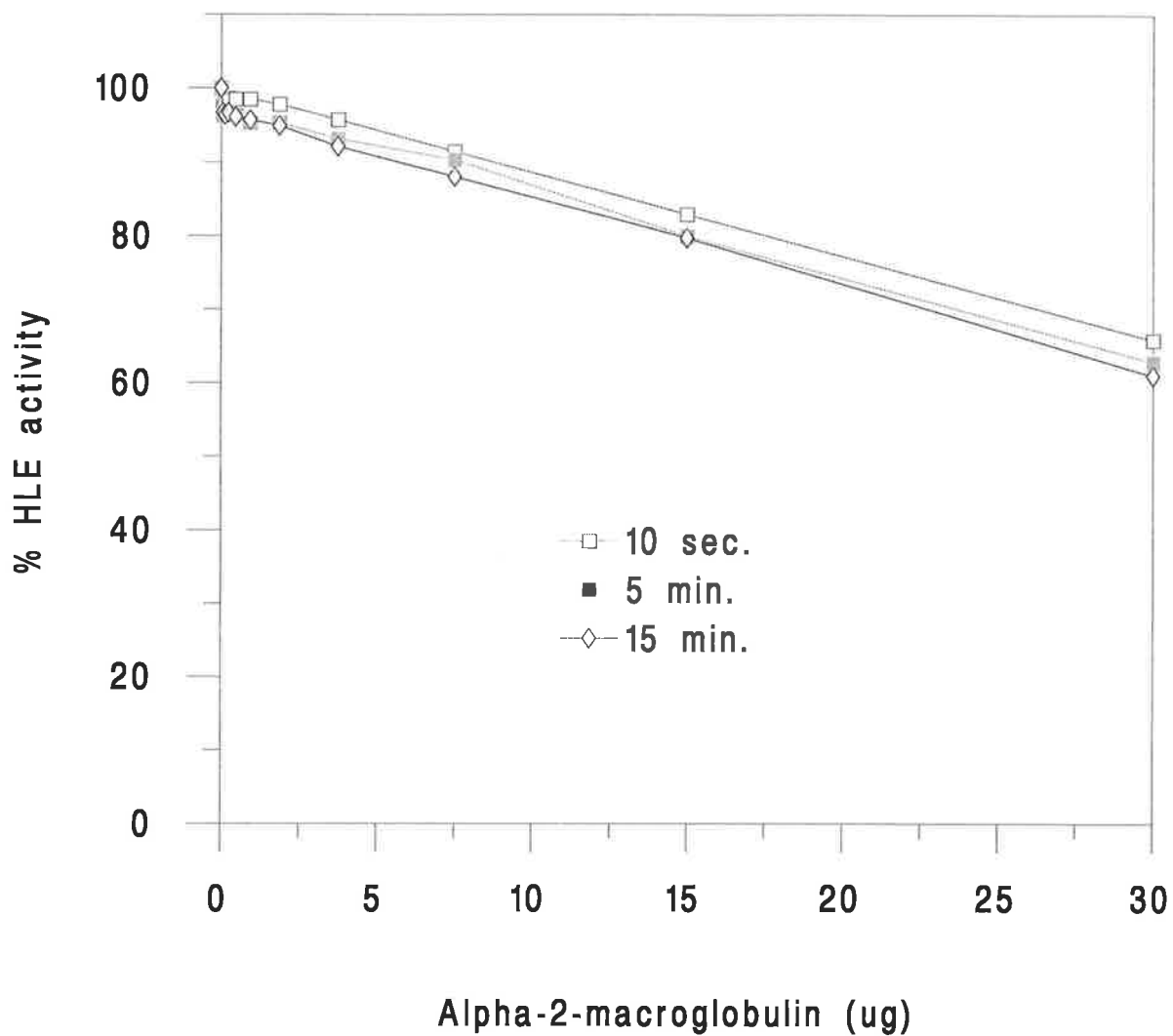


Figure 4.22: Effect of incubation time on alpha-2-macroglobulin of HLE with MeO-Suc-A-A-P-V-NA as substrate. Assay conditions as described in Section 2.4.2.2.

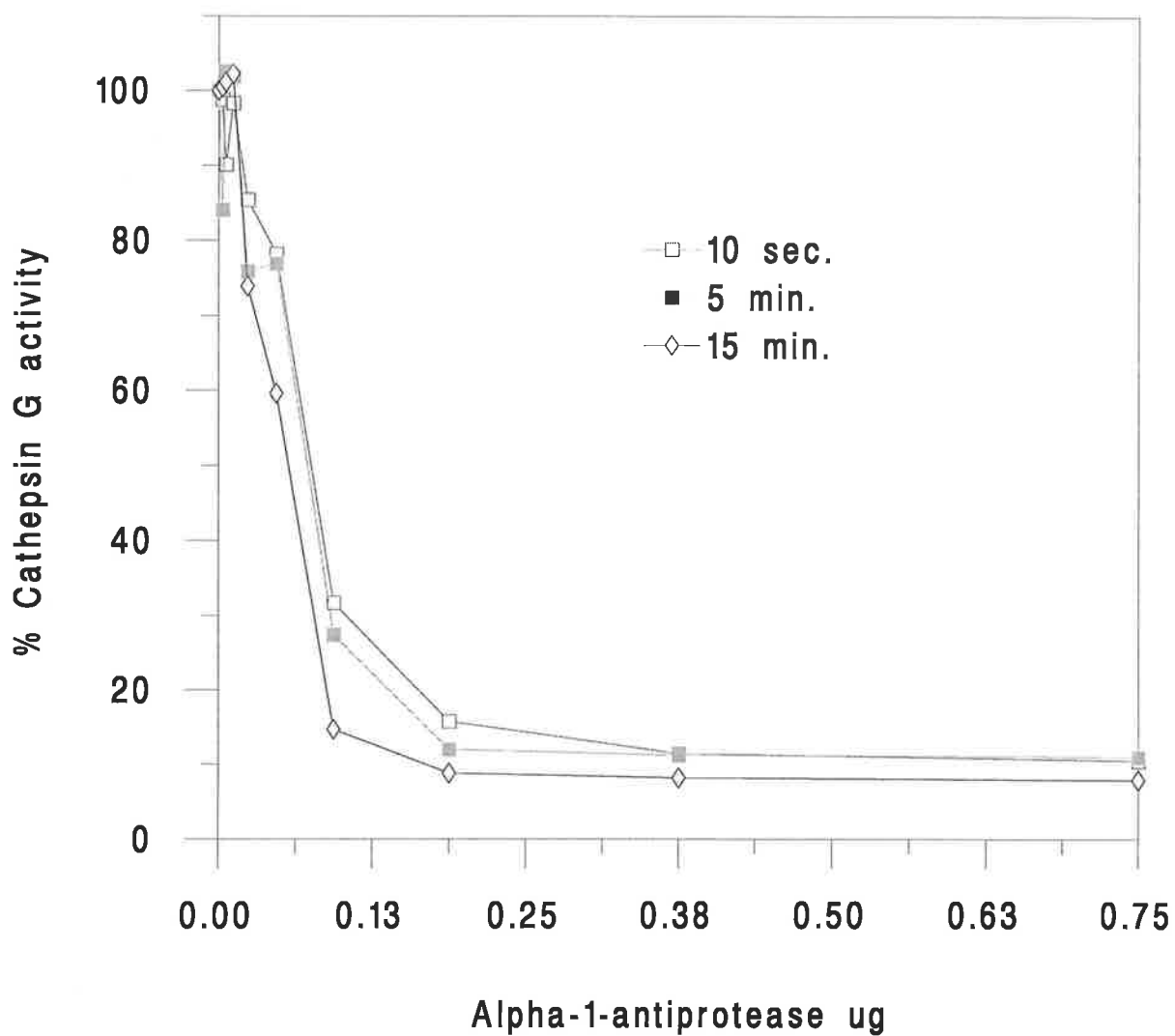


Figure 4.23: Effect of incubation time on alpha-1-antitrypsin inhibition of cathepsin G. Assay conditions as described in Section 2.4.3.1.

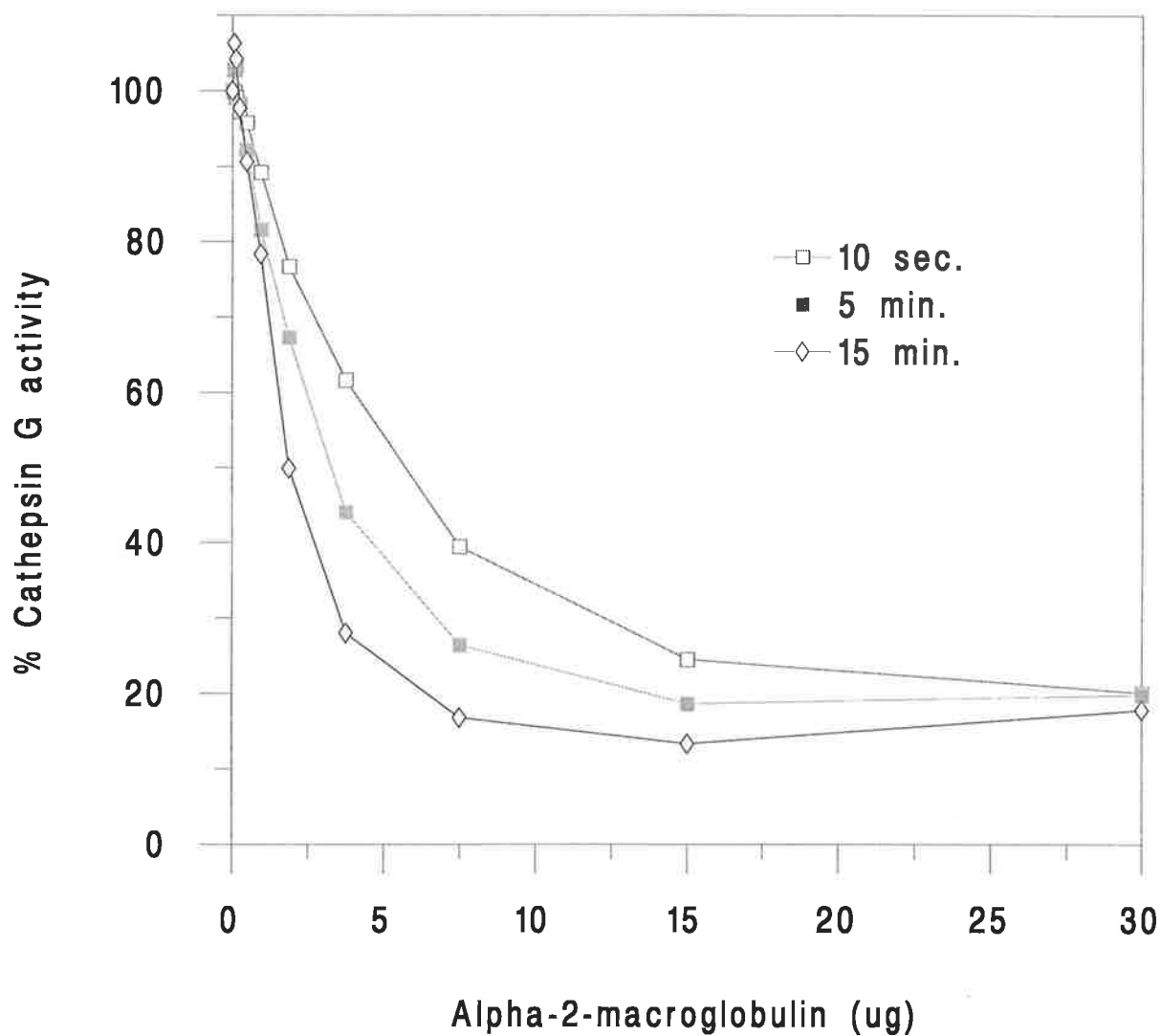


Figure 4.24: Effect of incubation time on alpha-2-macroglobulin inhibition of cathepsin G. Assay conditions as described in Section 2.4.3.1.

4.6.2 INHIBITION OF CATHEPSIN G

α_1 -PI and α_2 -M were the only two inhibitors evaluated that were effective against this enzyme (Section 4.3.2). The inhibition plots show that α_1 -PI is fast acting with approximately 75% inhibition achieved after 10 seconds, when compared to the same concentration of α_1 -PI that gave 50% inhibition with 15 minutes incubation (Figure 4.23). α_2 -M, on the other hand, was a slower acting inhibitor towards cathepsin G achieving only 33% inhibition after 10 seconds, when compared to the same concentration of α_2 -M that gave 50% inhibition with 15 minutes incubation (Figure 4.24). In the presence of an excess of α_1 -PI, incubation time was not a factor with complete incubation seen. This was not the case with α_2 -M until amounts greater than 2 mg/ml were added.

4.7 ORDER OF ADDITION OF INHIBITOR, ENZYME AND SUBSTRATE

To investigate the interaction of the inhibitors, α_1 -PI, ICI 200355 and heparin, with both HLE and the substrate, MeO-Suc-A-A-P-V-NA, the order of addition of reagents in the assay was varied, using a modified assay with equal volumes of inhibitor, enzyme, and substrate as described in Section 2.4.2.3. The assay was run three ways with the order of addition as follows:- Type 1: enzyme + inhibitor + substrate; Type 2: inhibitor + substrate + enzyme; Type 3: enzyme + substrate + inhibitor. The inhibition plots using the three assay variations are shown as Figures 4.25, 4.26 and 4.27 respectively for α_1 -PI, ICI 200355 and heparin. Both α_1 -PI and heparin are relatively unaffected by the order of addition of reagents, whereas maximum inhibition with ICI 200355 is only seen where the inhibitor is preincubated with HLE. Thus unlike α_1 -PI and heparin, ICI 200355 is not as an effective inhibitor as it is unable to effectively bind to and inhibit HLE, once HLE is bound to the substrate (Type 3 assay, Figure 4.26). ICI 200355 also appears to have a lower affinity for binding to HLE than the substrate, MeO-Suc-A-A-P-V-NA,

when the inhibitor and the substrate are added simultaneously to the enzyme (Type 2 assay, Figure 4.26).

4.8 INTERACTION OF HUMAN LEUKOCYTE ELASTASE AND CATHEPSIN G.

Previous reports have suggested HLE activity toward elastin was enhanced in the presence of cathepsin G (Boudier et al., 1981; Reilly et al., 1984). In this Section the possible interaction of HLE and cathepsin G was investigated using soluble peptide substrates with MeO-Suc-A-A-P-V-NA as the substrate in the assay for HLE activity (described in Section 2.4.2.1) and n-Succinyl-(L-alanine)₂-proline-L-phenylalanine-p-nitroanilide as substrate in the assay for cathepsin G activity (described in Section 2.4.3.1). In either assay the other enzyme was added in varying amounts as the sample. No activation or inhibition of either HLE or cathepsin G was observed in the presence of the other enzyme, as shown in Figures 4.28 and 4.29.

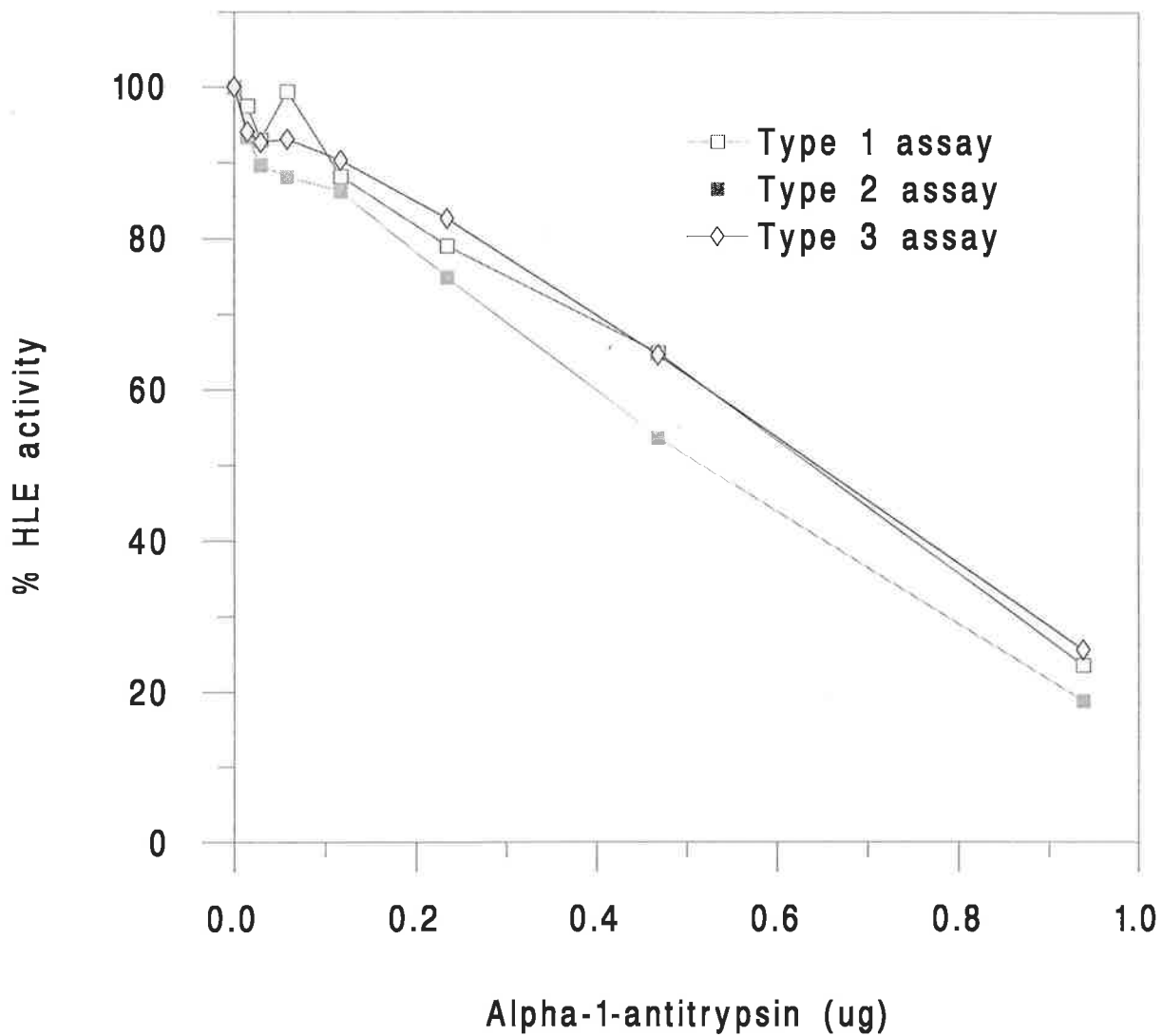


Figure 4.25: Effect of the order of addition of assay reagents on alpha-1-antitrypsin inhibition of HLE. Type 1: enzyme(E) + inhibitor(I) + Substrate (S); type 2: I + S + E; type 3: E + S + I. Assay conditions as described in Section 2.4.2.3.

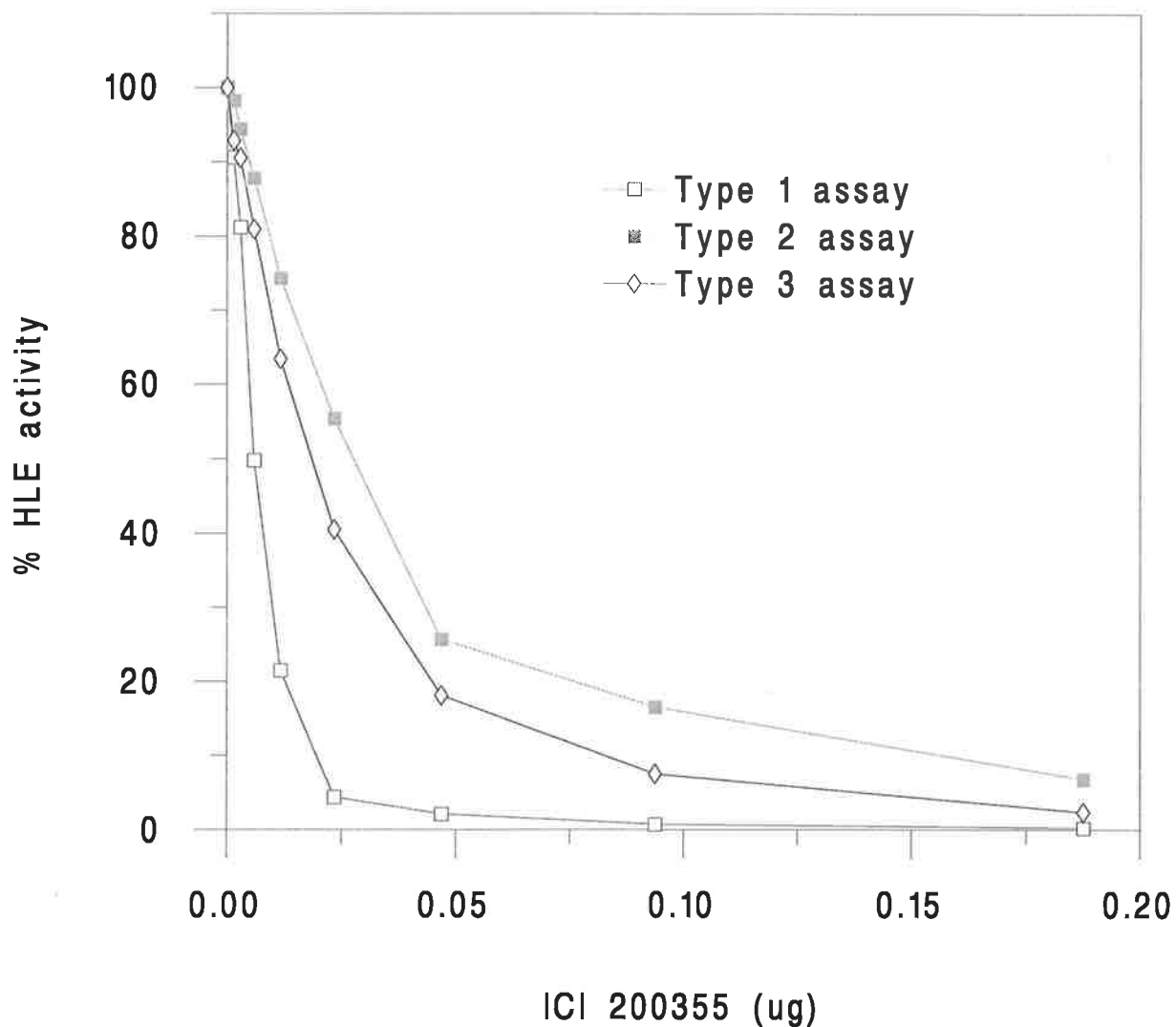


Figure 4.26: Effect of the order of addition of assay reagents on ICI 200355 inhibition of HLE. Type 1: enzyme (E) + inhibitor (I) + substrate (S); type 2: I + S + E; type 3: E + S + I. Assay conditions as described in Section 2.4.2.3.

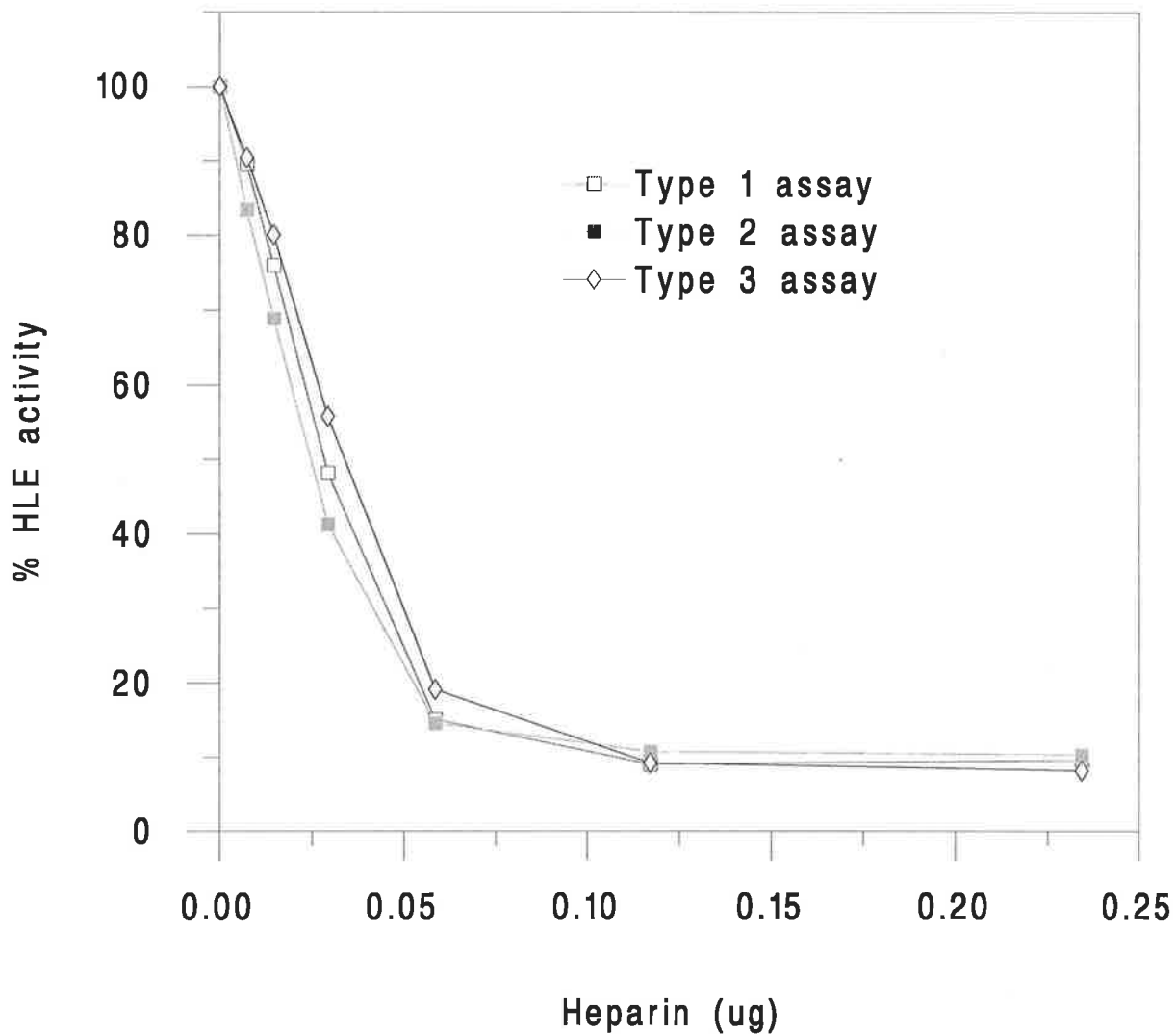


Figure 4.27: Effect of order of addition of assay reagents on heparin inhibition of HLE. Type1: enzyme (E) + inhibitor (I) + substrate (S); type 2: I + S + E; type 3: E + S + I. Assay conditions as described in Section 2.4.2.3.

4.9 COMPARISON OF EFFECTIVENESS OF INHIBITORS

A number of inhibitors were compared with the endogenous inhibitors, α_1 -PI and α_2 -M, for their ability to inhibit HLE activity with the synthetic low molecular weight substrate, MeO-Suc-A-A-P-V-NA. At inhibitor concentrations less than 8.8 $\mu\text{g/ml}$ (2.5 μg added, Figure 4.30), α_2 -M did not significantly inhibit HLE and was no more effective than albumin as an inhibitor. Elastin peptides were also effective at these low concentrations although not to the same extent as the specific endogenous HLE inhibitor, α_1 -PI. The synthetic inhibitor, ICI 200355 is the most effective inhibitor, with heparin being equally as effective to approximately 25% residual HLE activity. However if the inhibition is expressed as the number of moles of inhibitor that are required to inhibit 50% of the activity (IC₅₀) of one mole of HLE (Table 4.3), then heparin can be considered to be the most effective inhibitor, although with the proviso that complete inhibition is not found.

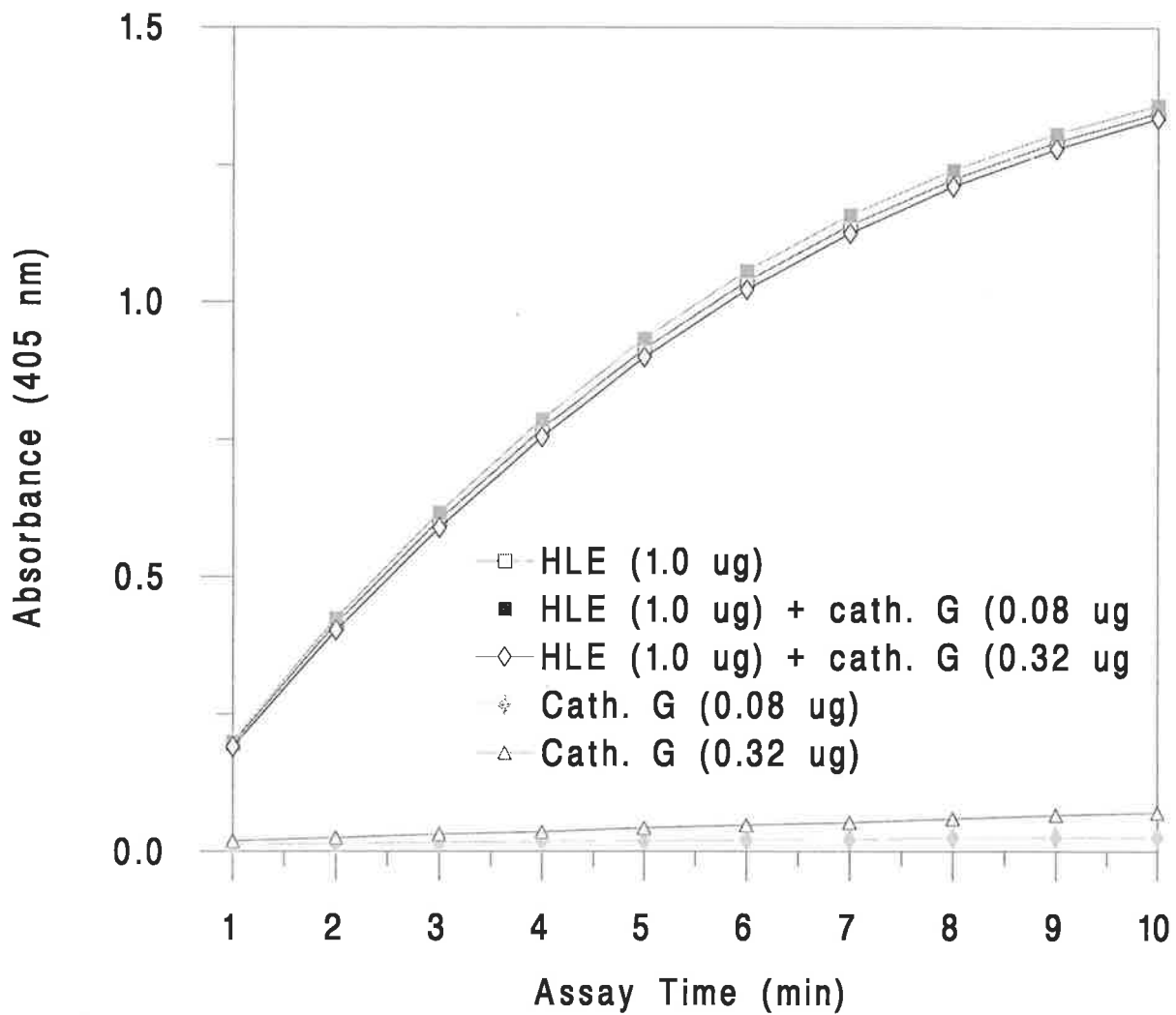


Figure 4.28: Effect of cathepsin G on HLE activity with MeO-Suc-A-A-P-V-NA as substrate. Procedure is as described in Section 2.4.2.2.

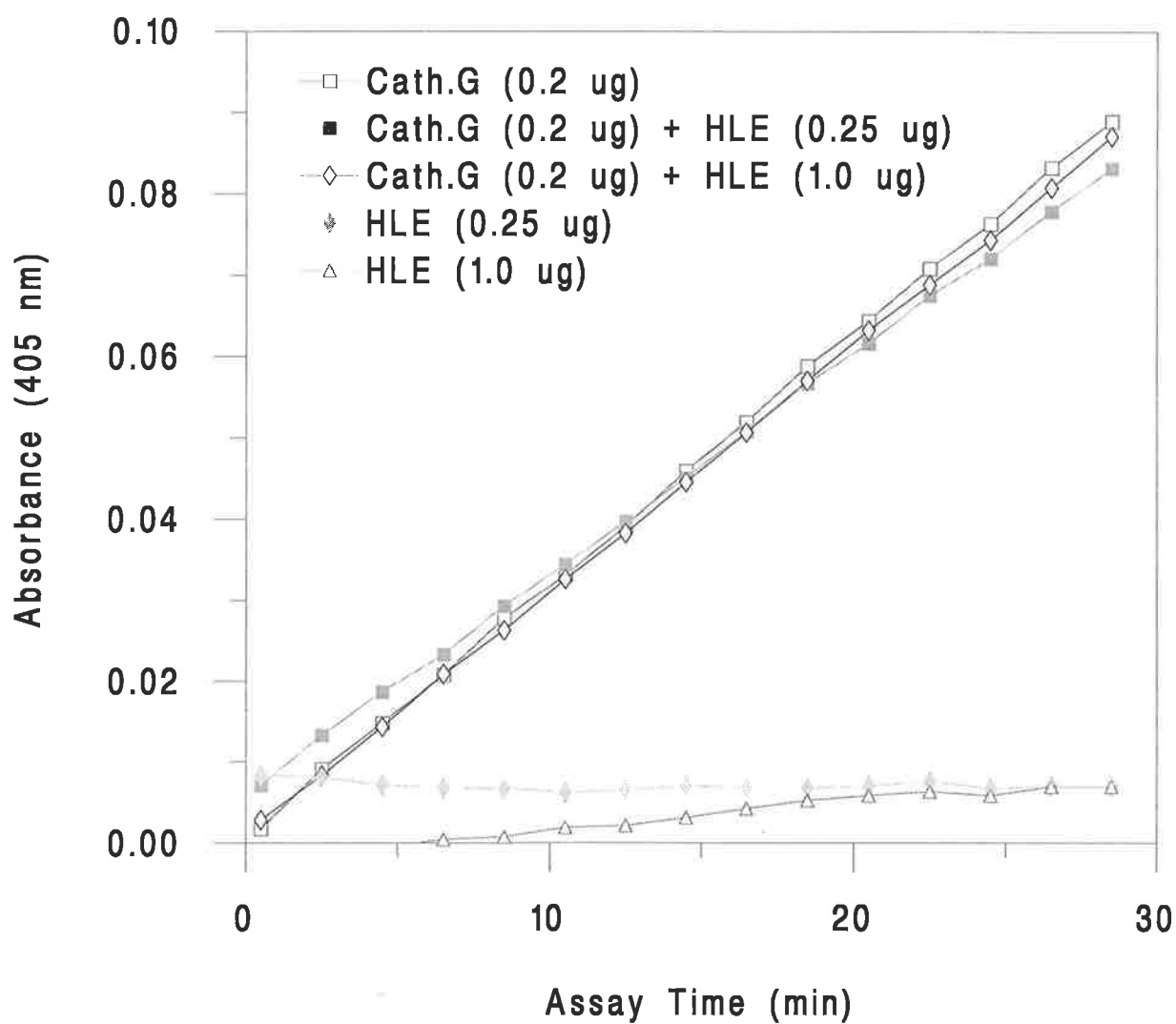
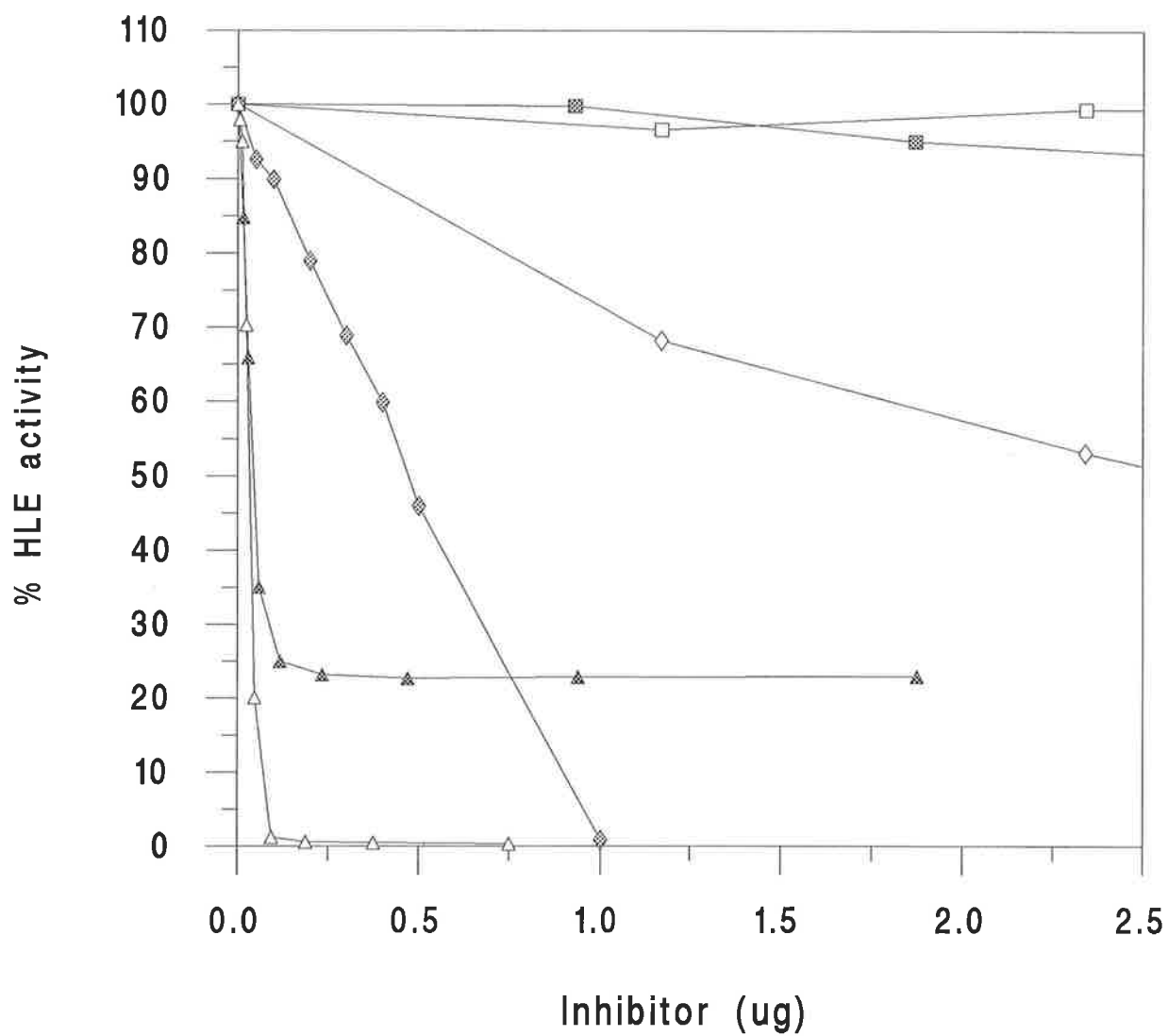


Figure 4.29: Effect of HLE on cathepsin G activity. Assay procedure as in Section 2.4.3.1.



- Albumin
- Alpha-2-macroglobulin
- ◇— Elastin Peptides
- ◆— Alpha-1-antitrypsin
- ▲— Heparin
- △— ICI 200355

Figure 4.30: Comparison of inhibitors towards HLE (0.5 ug) activity with MeO-Suc-A-A-P-V-NA as substrate. Assay conditions as described in Section 2.4.2.2.

**Table 4.3: COMPARISON OF INHIBITORS OF HLE
(0.5 ug of HLE added, av. MW of 30,000)**

Inhibitor	MW	IC50 (ug)	Inhibitor/HLE (molar ratio)
ICI 200355	732	0.035	2.87
Heparin	14,000 (av)	0.040	0.14
α_1 -PI	52,000	0.5	0.58
Elastin peptides	70,000 (av)	2.6	2.23
α_2 -M	725,000	23.0	1.90
Albumin	65,000	90.0	83.11

4.10 DISCUSSION

The primary function of α_1 -PI is considered to be to control the activity of HLE, since the k_{ass} is more than tenfold higher for this enzyme than that for other proteinases (Beatty et al., 1980). As reported elsewhere (Travis and Salvesen, 1983), the effectiveness of α_1 -PI towards HLE is confirmed in this study where complete inhibition of HLE is found, with equimolar amounts of α_1 -PI (Figure 4.3). α_1 -PI is equally effective even when added after HLE is preincubated with the low molecular weight substrate, MeO-Suc-A-A-P-V-NA (Figure 4.25). However α_1 -PI required at least 15 minutes incubation with HLE, prior to addition of the substrate, to achieve maximal binding between the inhibitor and the enzyme before measuring the reduced enzyme activity (Figure 4.21). This high specificity of α_1 -PI for HLE compared to inhibition of other proteases is demonstrated by the fact that α_1 -PI is not as effective against the other major inflammatory serine protease, cathepsin G, where incomplete inhibition was seen (10% residual

activity was found, Figure 4.5). α_1 -PI is also relatively unaffected by the order of the addition of reagents, such as adding α_1 -PI after the peptide substrate, MeO-Suc-A-A-P-V-NA was added to HLE (Figure 4.25) although this is not seen where elastin is used as substrate as reported elsewhere (Morrison et al., 1990, Padrines and Bieth, 1991).

In this study, with the low molecular weight substrate MeO-Suc-A-A-P-V-NA, α_2 -M is a very poor inhibitor of HLE (figure 4.3). This finding is consistent with the general finding that proteinases, when complexed to α_2 -M, typically retain 80-100% of their hydrolytic activity against low molecular weight substrates (Barrett, 1981). α_2 -M also required at least 15 minutes incubation HLE, prior to the addition of the substrate MeO-Suc-A-A-P-V-NA, to achieve maximum inhibition. Together with the poor inhibition achieved (65% inhibition of 0.5 μ g of HLE with 30 μ g of α_2 -M; Figure 4.22) this result suggests that the primary role of α_2 -M may not be to control HLE activity. In one study, α_2 -M was able to activate HLE activity from 1.1 to 15.3 fold the original activity, depending on the peptide substrate used (Twusami et al., 1977). However, α_2 -M is a more effective inhibitor towards cathepsin G, although as with proteases in general using low molecular weight substrates, α_2 -M did not completely inhibit cathepsin G (20% residual activity found, Figure 4.6). α_2 -M was even slower acting towards cathepsin G than towards HLE, as shown in Figure 4.24, where only 33% inhibition of cathepsin G was found after 10 seconds incubation of α_2 -M with HLE. What role α_2 -M has in regulating HLE and cathepsin G activity, if any, is not known. Any α_2 -M - protease complexes that do form *in vivo* are rapidly cleared by the reticuloendothelial system (Travis and Salvesen, 1983), so that any residual proteolytic activity of the complexes is likely to be minimal. A role in the development of pulmonary emphysema is unlikely as α_2 -M, because of its size, cannot readily diffuse into the lung alveolus where it is usually at such low concentrations that it is difficult to detect in normal BAL (McLennan and Walsh, 1995).

While cathepsin G is a relatively poor protease, its activity is not completely inhibited by the major protease inhibitors, α_1 -PI (Figure 4.5) or α_2 -M (Figure 4.6). In addition, α_1 -ACH, which appears to have a role in the regulation of cathepsin G activity, is specifically inactivated by the presence of HLE (Potempa et al., 1991). These findings suggest that cathepsin G may play a role in causing tissue damage in chronic inflammatory diseases such as emphysema where there is evidence of increased HLE activity in the lungs of emphysema patients, as discussed in chapter one of this thesis.

In this study, I demonstrate that soluble elastin peptides of high molecular weight can be effective inhibitors of HLE with over 90% inhibition found at 15 $\mu\text{g/ml}$ (a total of 9.37 μg added, Figure 4.3) and with over 99.5% achieved at 75 $\mu\text{g/ml}$ seen with an α -elastin preparation (approx. 70,000 MW). This finding suggests that the elastin peptide preparation appears to be so tightly bound to HLE that the enzyme is essentially unavailable to act on the peptide substrate, MeO-Suc-A-A-P-V-NA. Such high affinity binding between elastin peptides and HLE is also reported to occur during elastinolysis, with the formation of an unusually stable complex between HLE and the released elastin peptides rich in the alanine/glycine and hydrophobic domains of elastin (Tyagi and Simon, 1993). An elastin peptide preparation (κ -elastin) of average molecular weight of 57,000 has also been reported to be an effective inhibitor of HLE but that preparations of <10,000 MW did not show any significant inhibitory activity (Bonnaure-Mallet et al., 1995). While the elastin peptide sequences, VAGAPA and VPGVG have been suggested to have a number of biological functions (Sandberg et al., 1981; Senior et al., 1984; Blood et al., 1988; Wachi et al., 1995), the separate peptides did not show any inhibition of HLE activity in this study (Figure 4.3). While no inhibition is seen with the specific peptides used here, it is unlikely that inhibition would be seen with any other elastin peptides of such low

molecular weight as suggested by a report that elastin peptides with MW <10,000 had no major influence on elastase (Bonnaure-Mallet et al., 1995). This high affinity binding between elastin peptides and HLE reported here and by others has been suggested by Tyagi and Simon (1993) to have a regulatory role towards HLE activity at local sites of proteolysis during elastinolysis. How this proposal fits with normal remodelling of elastic tissue as distinct from the degradative mechanisms that occur in diseases such as pulmonary emphysema awaits further studies.

While a non-specific protein, such as albumin, shows almost no inhibitory effect at low concentrations (Figure 4.3), significant inhibition of HLE activity is seen if a relatively large concentration of albumin is present (50% inhibition seen with 6 g/l and almost 90% inhibition seen at normal serum albumin concentrations (routine laboratory reference range; 39-48 g/l) as shown in Figure 4.4). This would indicate that any free HLE in blood would be bound by the albumin present in addition to the protease inhibitors present such as α_1 -PI and α_2 -M. However there would not be any protective effect in the lung where the albumin concentration is much lower (eg. alveolar lining fluid, 0.08-0.25 g/L; Baughman et al., 1983). HLE has a very broad specificity allowing it to degrade a wide variety of proteins including a number of serum proteins (Bieth, 1986). Thus, it is likely that the inhibition of HLE by albumin can be explained by the large concentrations of albumin acting as a substrate and saturating all the available catalytic sites on HLE.

Two synthetic inhibitors (ICI 186756 & 200355) of HLE were found to be very effective with complete inhibition of HLE activity when MeO-Suc-A-A-P-V-NA was used as substrate (Figure 4.7). ICI 186756 & 200355 were also found to be fast acting with no significant differences noted whether the inhibitors were incubated with HLE for 10 seconds or 15 minutes prior to the addition of the peptide substrate (Figure 4.19). However, if the inhibitor (eg. ICI 200355) was not added to the enzyme first it was a less effective inhibitor of HLE (Figure 4.26) which

suggests that HLE has a lower affinity for ICI 200355 than for the substrate, MeO-Suc-A-A-P-V-NA. This type of inhibitor was less effective using human lung elastin as demonstrated with ICI 200355 (Figure 4.8) and as reported elsewhere for ICI 186756 & 200355 with bovine elastin as substrate (Williams et al., 1991; Sommerhoff et al., 1991). This reduced effectiveness of an inhibitor towards HLE having elastin as substrate compared to having a chromogenic peptide as substrate is similar to that found with heparin and heparan sulphate inhibition of HLE with Suc-A-A-A-NA (Figure 4.10) and human lung elastin (Figure 4.16) as substrates. This is common with a number of inhibitors due to the strong binding of HLE to insoluble elastin as has been reported by others (Morrison et al., 1990; Padrines and Bieth, 1991). Both inhibitors are reported to be highly specific for HLE with almost no activity towards cathepsin G (Williams et al., 1991; Sommerhoff et al., 1991) which was confirmed in this study (Figure 4.9). Importantly, ICI 186756 & 200355 were compatible and additive to the inhibitory activity of the main endogenous inhibitor of HLE, α_1 -PI, as well as another endogenous inhibitor, heparin, as shown for ICI 200355 in Table 4.2.

The binding of sulphated GAGs to HLE is rapid and occurs with a high affinity as shown by the binding of heparin to HLE which is relatively unaffected by incubation time of heparin with HLE prior to assay (Figure, 4.20; at least to the minimum time of 10 seconds studied) and the order of addition of assay reagents (Figure, 4.27). Evidence for ionic binding is demonstrated by the displacement of heparin from a HLE affinity column (see Section 6.2) which begins to occur at a NaCl concentration (approximately 0.4 M). This NaCl concentration is about the point (0.35 M and above) where NaCl completely blocks the inhibition of HLE activity by heparin (Figure 4.15). This agrees with another report that ionic strengths higher than 0.5 (0.4 in this study) completely abolished the inhibitory capacity of heparin towards leucocyte proteinases (Redini et al., 1988a). Alteration of pH could also change the degree of ionic binding between heparin and HLE. However, it was found that the inhibition of HLE by heparin was independent of pH, within the

6-9 pH range studied (Figure 4.14), which agrees with previous reports that inhibition is seen towards a chromogenic substrate at pH 6.0 (Marossy, 1981) and pH 8.0 (Redini et al., 1988a). However, two other studies have reported differing results, the first demonstrating inhibition only below pH 5.8 (Avila and Convit, 1976) and the second demonstrating that heparin stimulates enzyme activity towards native elastin (Lonky et al., 1978). The discrepancy in the data is most probably due to differences in assay conditions. The chromogenic assay data in this study are consistent with the most recently reported data on heparin (Drag and Petersen, 1994) and with the physiologically more relevant data obtained with human lung elastin as the substrate for HLE (Figure 4.16).

The inhibition of HLE activity is thought to occur by wrapping the linear polyanion chain of the sulphated GAG around the cationic enzyme (Baici et al., 1980). In general, maximum inhibition is seen with GAGs having long chain lengths and high sulphate content. The importance of sulphate content is demonstrated in this study where heparin, which has the highest sulphate content overall of GAGs (Lindahl and Hook, 1978), is the most effective inhibitor of HLE. This is in contrast to hyaluronic acid, a GAG with no sulphate content, which is ineffective as an inhibitor (Figure 4.10). These data are consistent with other studies using desulphated and oversulphated heparin derivatives (Redini et al., 1988a) and dextran and chitosan sulphates (Lentini et al., 1985). There is evidence that the position of sulphation on the GAG chain is also important (Redini et al., 1988; Lentini et al., 1985; *this study*, Chapter 5). The importance of chain length is shown with various low molecular weight heparins, where preparations with a MW of 4000-6000 inhibited equally as well as heparin (average MW 15000) but preparations of MW <2000 were not as effective. These data are consistent with other studies using varying chain lengths of heparin derivatives (Redini et al., 1988a) and dextran and chitosan sulphates (Lentini et al., 1985). However, this work, which has been previously reported (Walsh et al., 1991a) is not completely consistent with the later report of Drag & Petersen (1994) where a

gradual increase in HLE inhibition was shown with heparin preparations of MW from 2300 to 12700. The difference is likely to be due to the fact that the low MW fractions used in the Drag & Petersen (1994) study were a series of defined MW fractions prepared from one original heparin preparation whereas in the present study the preparations were obtained commercially from a number of different suppliers (Section 2.2.3). In this study there is the possibility of differences in original heparin source, method of preparation and differences in sulphate content and defined MW of the commercially supplied fractions.

In this study, heparin and heparan sulphate inhibit HLE activity towards insoluble human lung elastin (Figure 4.16) which differs from one earlier report (Lonky et al., 1978) where heparin is claimed to stimulate HLE activity towards elastin. However, the data in this thesis where heparin and heparan sulphate were found to inhibit HLE using elastin as substrate is consistent with a more recent report (Moczar and Hornebeck, 1991), where heparin inhibited rat leucocyte elastase with ³H-bovine elastin as substrate. The heparin and heparan sulphate inhibition of HLE found in this thesis using insoluble human lung elastin as substrate is also consistent with the inhibition seen where chromogenic peptide substrates have been used in this thesis and other studies. The inhibition seen in the assay system with insoluble human lung elastin as substrate is a true inhibition of HLE and is not due to heparin and heparan sulphate interference in the ELISA for elastin peptides (see Section 2.4.2.4 for assay procedure). Heparin and heparan sulphate do not produce a change in the size of the elastin derived peptides, another potential cause for apparent elastin peptide concentration changes.

Heparin and heparan sulphate were found to be ineffective as inhibitors of cathepsin G (Figure 4.18) which differs from the inhibition reported in two other studies (Redini et al., 1988a; Drag and Petersen, 1944). No inhibition of cathepsin G by heparin could be demonstrated even when the assay conditions of Redini et al (1988a) were used which included a 15-30 minute

preincubation step and an assay step at 37°C. This difference could not be readily explained at the time of publication (Walsh et al., 1991a) and has not been investigated further but is most likely to be due to differences in enzyme source.

Sulphated GAGs have been shown here and in other studies to be potent anti-elastase agents, as demonstrated for heparin (Redini et al., 1988a; Walsh et al., 1988; Walsh et al., 1991a; Drag and Petersen, 1994) and for a number of heparin derivatives and other sulphated GAGs (Barg et al., 1979; Baici et al., 1980; Baici et al., 1981; Baici et al., 1984; Lentini et al., 1985; Redini et al., 1988a; Redini et al., 1988b; Moczar and Hornebeck, 1991; Walsh et al., 1991a). However it has not been determined whether endogenous sulphated GAGs have an anti-elastase role *in vivo*. Although the occurrence and location of sulphated GAGs *in vivo* lends some support to this role. For example, heparin and heparan sulphate are found widely *in vivo* on cell surfaces and as components of proteoglycans (Staprans and Felts, 1985) in cell and basement membranes, where it is thought that they may have a role in the regulation of passage of substances and cells across membrane surfaces, including the proteolytic mechanisms involved. Some specific examples, include the evidence that heparin can prevent the passage of PMNs through subendothelial basement membrane by inhibiting the selectin family of cell adhesion receptors involved with granulocyte adhesion and activation (Parekh and Edge, 1994; Nelson et al., 1993) as well as inhibiting the hydrolytic enzymes released such as heparanase (Vlodavosky et al., 1992; Bartlett et al., 1995).

Overall heparin and ICI 200355 appear to be the most potent inhibitors of HLE with an IC₅₀ for HLE of 0.040 and 0.035 µg respectively (Table 4.3), using MeO-Suc-A-A-P-V-NA as substrate, although heparin is only most effective as a partial inhibitor (approx. 25% residual HLE activity, Figure 4.30). Whilst ICI 200355 is a synthetic inhibitor designed to be a potent inhibitor of HLE, it is surprising that heparin appears to be a better partial inhibitor of HLE than the primary

endogenous inhibitor, α_1 -PI, which suggests that heparin may have some regulatory role towards HLE. At the IC₅₀ point, heparin has a molar ratio of inhibitor to HLE of 0.14 in this study (Table 4.3) which indicates multiple HLE binding sites on the linear chain of heparin (approx. 7). The question as to whether there are specific high affinity binding sites for HLE along the heparin chain is examined in chapter 6.

CHAPTER FIVE

INTERACTION OF SURFACTANTS ON ELASTASE AND ELASTASE INHIBITORS

5.1 INTRODUCTION

A number of non-ionic surfactants, such as Brij 35, Triton X-100, Pluriol PE 6800 and Tween 20, have been widely used in the isolation of HLE, in assay systems using HLE and in kinetic assays for HLE (Spitznagel et al., 1974; Engelbrecht et al., 1982; Cook and Ternai, 1988a; Wenzel et al., 1990). Brij 35 has been reported to activate HLE activity towards elastin and oligopeptide substrates (Cook and Ternai, 1988a; Wenzel et al., 1990; Starkey and Barrett, 1976; Walsh et al., 1991b), but it was also found to reduce the inhibitory activity of the hydrophobic inhibitors; alkyl 2-pyrones, oleic acid and alkyl peptides (Cook and Ternai, 1988a). These observations suggest that Brij 35 binds to a hydrophobic site on the enzyme in competition with the hydrophobic inhibitors. I have described in a preliminary report that Brij 35 also modifies sulphated GAG inhibition of HLE (Walsh et al., 1991b). In contrast, the ionic detergents, sodium dodecyl sulphate and sodium linoleate have been shown to inhibit hydrolysis of Suc-A-A-A-NA by HLE (Finlay et al., 1982; Walsh et al., 1991b), but in one report to stimulate elastolytic activity of α_2 -macroglobulin-protease complexes (Finlay et al., 1982).

Because surfactants, such as Brij 35, are commonly added to *in vitro* HLE assay systems, it was the aim of this study to examine whether these surfactants could alter the effectiveness of a number of endogenous and exogenous HLE inhibitors. This study also examines the effectiveness of HLE inhibitors in the presence of pulmonary surfactant, a study that has relevance to the use of exogenous HLE inhibitors in the possible prevention of the development of pulmonary emphysema.

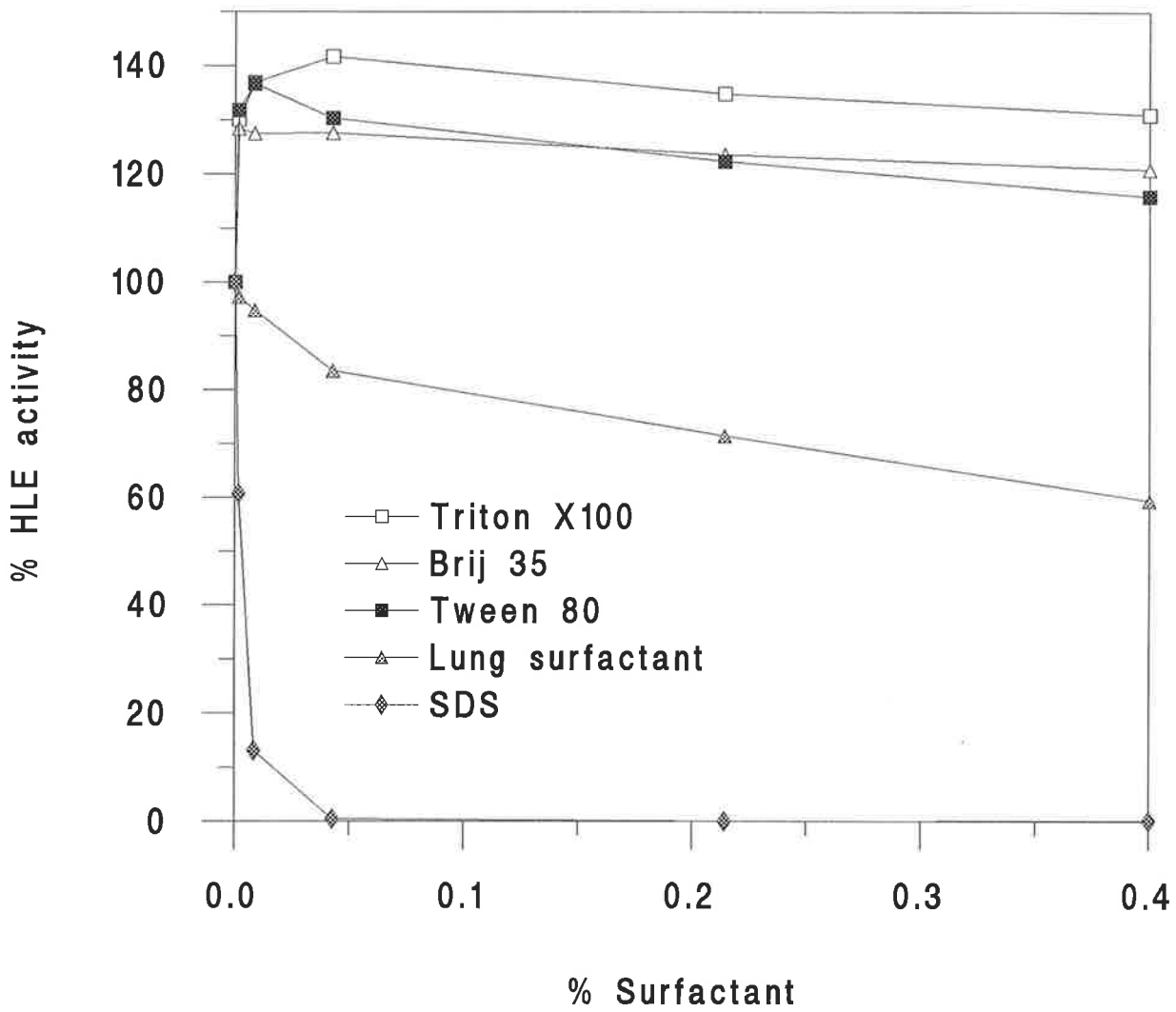


Figure 5.1: Effect of surfactants on HLE activity with MeO-Suc-A-A-P-V-NA as substrate. Assay procedure as described in Section 2.4.2.2.

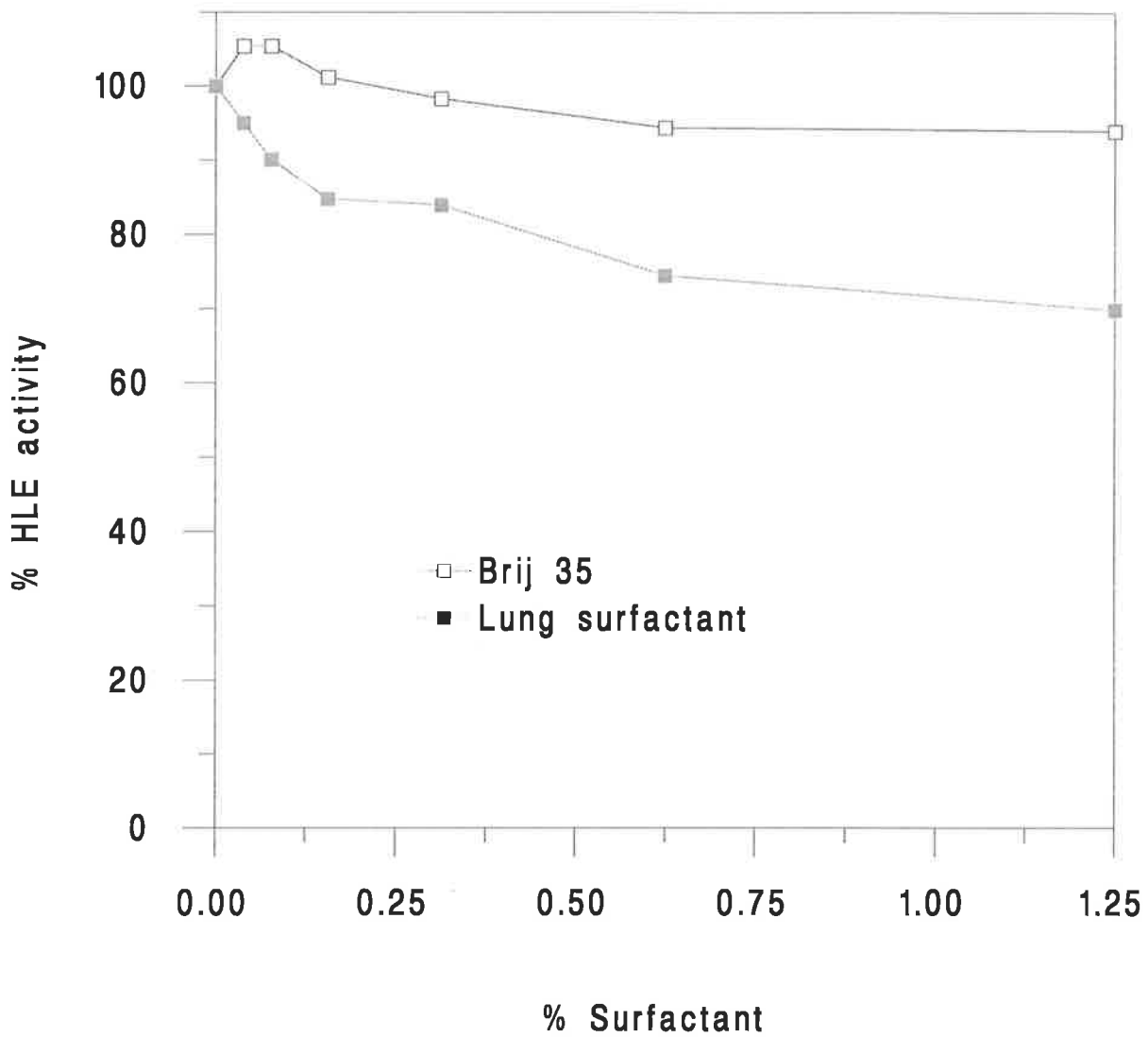


Figure 5.2: Effect of Brij 35 and lung surfactant on HLE hydrolysis of human lung elastin. Assay as described in Section 2.4.2.4.

The range of inhibitors examined are those evaluated in chapter 4 and include α_1 -PI, α_2 -M, elastin peptides, sulphated GAGs, and the low molecular weight inhibitors, ICI 186756 and 200355.

5.2 EFFECT OF SURFACTANTS ON HUMAN LEUKOCYTE ELASTASE ACTIVITY

5.2.1 MeO-Suc-A-A-P-V-NA AS SUBSTRATE

I have routinely included Brij 35 in my HLE preparations in order to prevent autolytic degradation of the enzyme and to minimise its adsorption to the surfaces of reaction container and cuvettes. Because of reports (Starkey and Barrett, 1976; Cook and Ternai, 1988a; Wenzel et al., 1990; Walsh et al., 1991b) that Brij 35 is able to activate HLE, I investigated its effect in a HLE assay using MeO-Suc-A-A-P-V-NA as substrate and compared its effect with two other commonly used non-ionic surfactants (Triton X100 and Tween 80), an ionic surfactant (SDS) and a pulmonary surfactant preparation. As seen in Figure 5.1, all the non-ionic surfactants activated HLE activity towards MeO-Suc-A-A-P-V-NA by 20-40%. Activation still occurred at a surfactant concentration of 0.001%, the lowest concentration tested in the assay system. HLE, a cationic enzyme ($pI > 9$), is completely inactivated by the anionic SDS, at a concentration as low as 0.04%. Pulmonary surfactant has a mild inhibitory effect (Figure 5.1) with almost 50% inhibition achieved with a surfactant concentration of 0.5%.

5.2.2 HUMAN LUNG ELASTIN AS SUBSTRATE

The effect of Brij 35 and a lung surfactant preparation on HLE activity, with insoluble human lung elastin as substrate, was investigated as a model of possible surfactant modification of HLE mediated damage of lung elastic tissue. Brij 35 showed a slight HLE activation of 6% in the

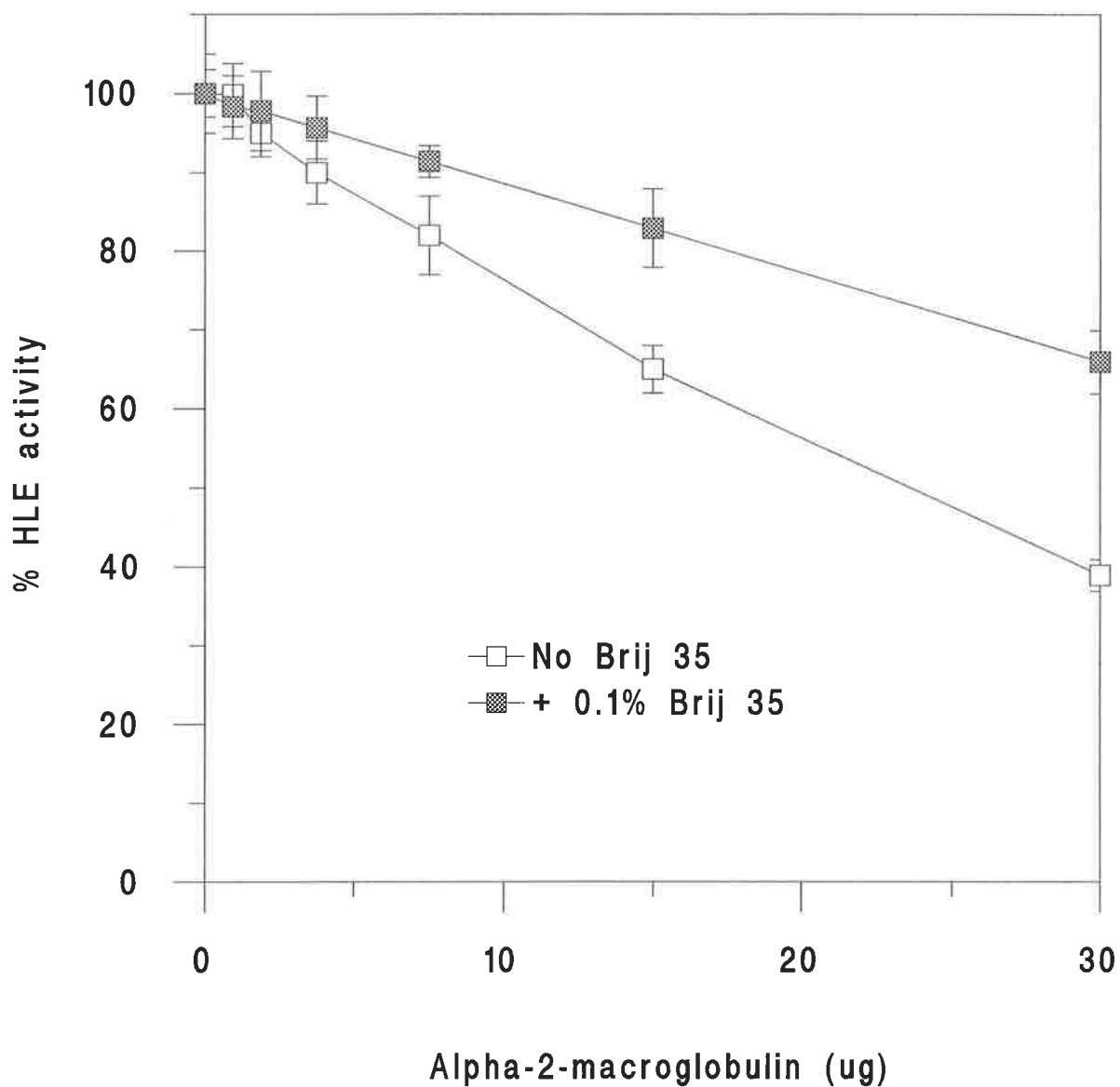


Figure 5.3: Effect of Brij 35 on alpha-2-macroglobulin inhibition of HLE activity with MeO-Suc-A-A-P-V-NA as substrate. Assay procedure as described in Section 2.4.2.2. Data interpretation as in Section 2.5.

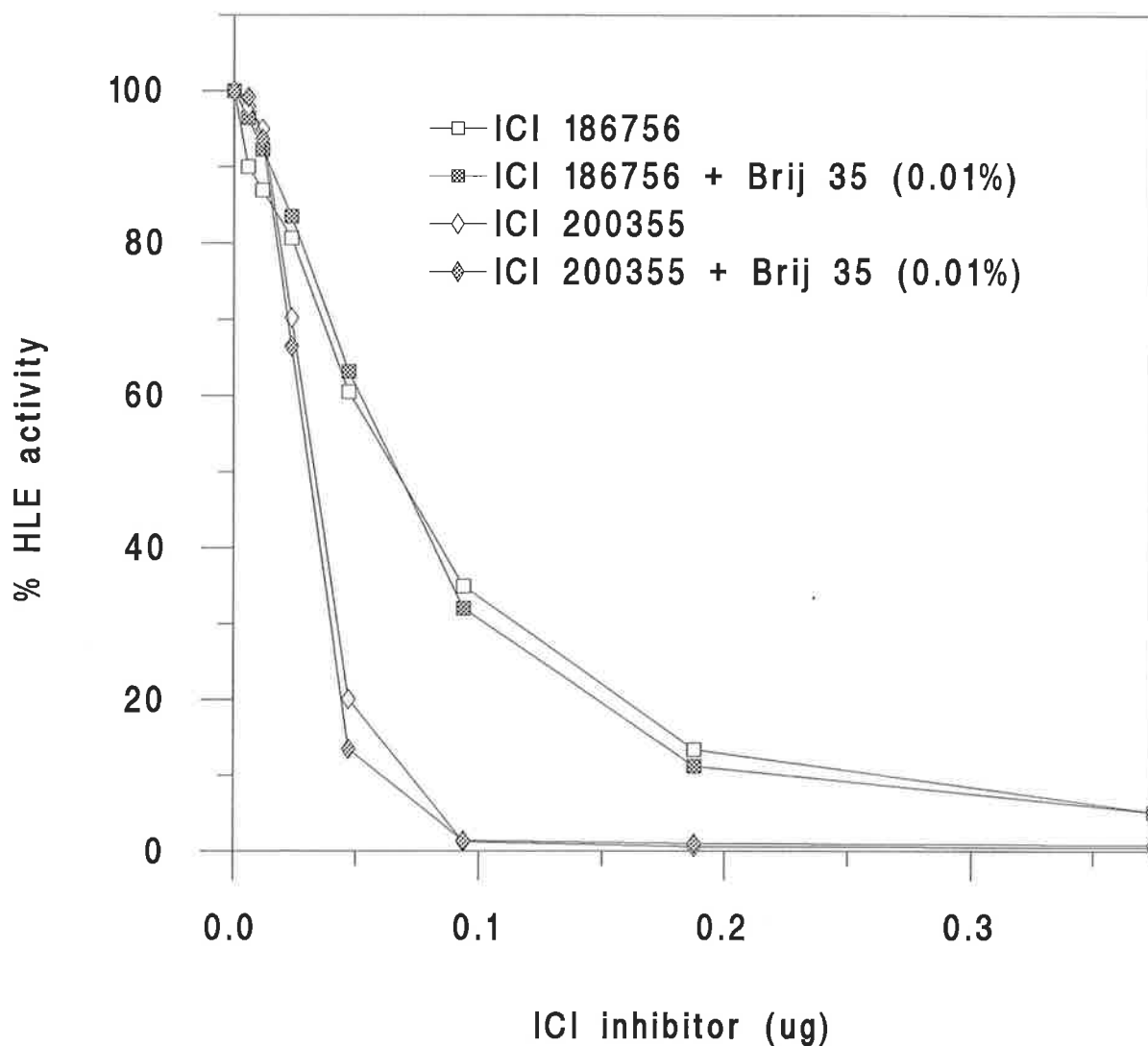


Figure 5.4: Effect of Brij 35 on ICI 186756 and 200355 inhibition of HLE activity with MeO-Suc-A-A-P-V-NA as substrate. Assay as described in Section 2.4.2.2.

region of 0.05 % Brij 35 concentration but there was no significant overall activation of HLE at other concentrations (Figure 5.2), as described for the soluble peptide substrate, MeO-Suc-A-A-P-V-NA in Figure 5.1. The slight inhibitory effect of lung surfactant towards HLE activity on insoluble elastin was consistent with its inhibitory effect towards MeO-Suc-A-A-P-V-NA (Figure 5.1).

5.3 EFFECT OF BRIJ 35 ON HUMAN LEUKOCYTE ELASTASE INHIBITORS

5.3.1 PROTEIN INHIBITION

Because of a previous report (Cook and Ternai, 1988a) of the deleterious effect of Brij 35 on some hydrophobic inhibitors, I investigated its effect on a number of HLE inhibitors and assay components. The modifying effect of Brij 35 on the ability of a range of proteins to inhibit HLE (0.5 μg) with MeO-Suc-A-A-P-V-NA as substrate was evaluated. The proteins examined were albumin, α_2 -M, α_1 -PI and the elastin peptide preparations (α -elastin, VGVAPG and VPGVG), all of which had been previously examined in the absence of surfactant in Section 4.3.1. Brij 35 had no significant effect on any of these proteins and peptides (data not shown) except that it reduced the ability of α_2 -M, a non-specific protease inhibitor, to inhibit HLE (Figure 5.3), such that a 50% inhibition of HLE (0.5 μg) required 44 μg of α_2 -M in the presence of Brij 35 compared with 23 μg in the absence of Brij 35 (Not all data is shown).

5.3.2 HUMAN LEUKOCYTE ELASTASE SPECIFIC SYNTHETIC INHIBITORS

The effect of Brij 35 on two potent synthetic inhibitors (ICI 186756 & 200355), which selectively inhibit HLE, was evaluated (Figure 5.4). As also shown in section 4.4.1, ICI 186756, a low molecular weight aldehyde inhibitor, and ICI 200355, a peptide trifluoromethyl ketone

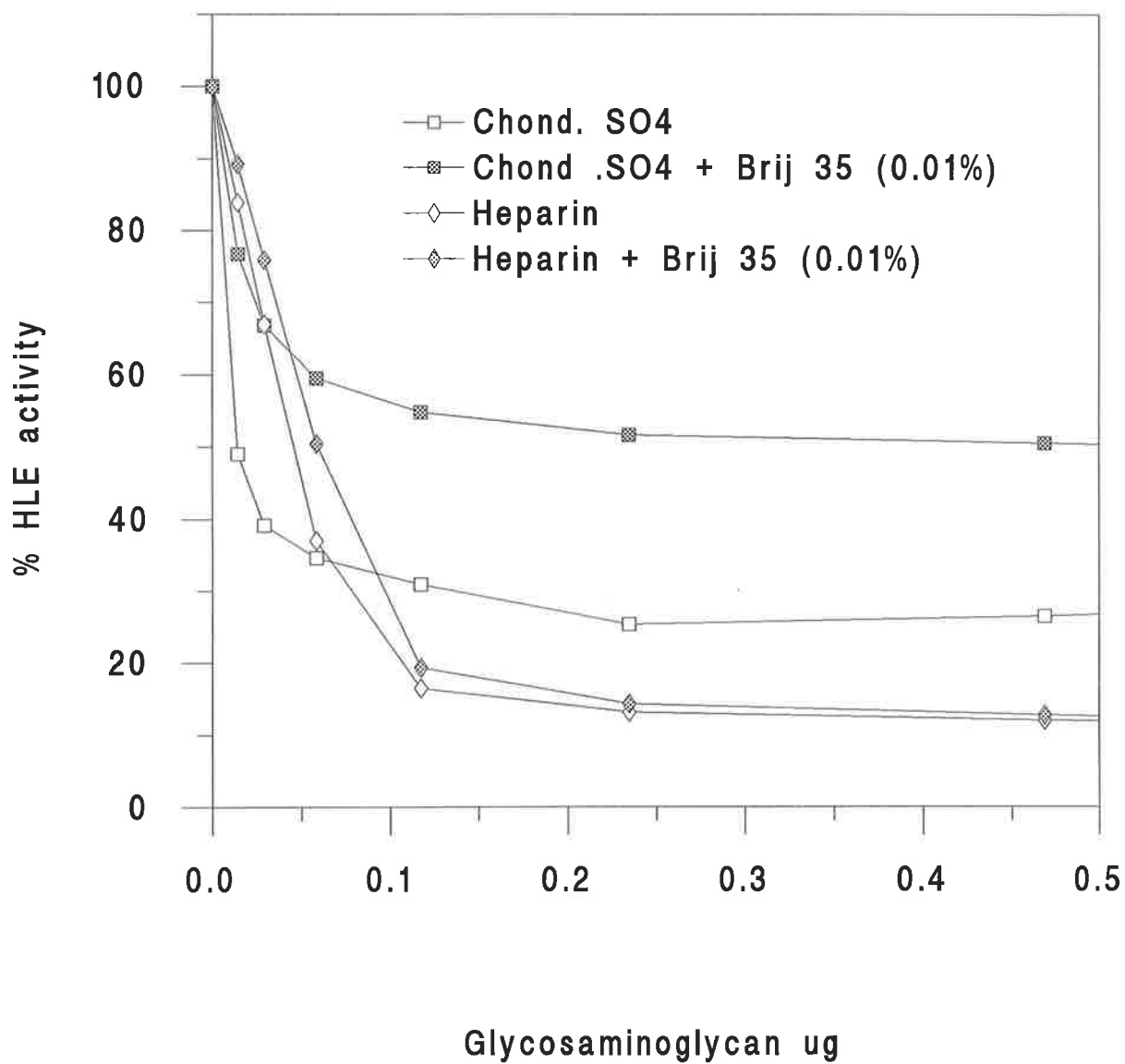


Figure 5.5: Effect of Brij 35 on heparin and chondroitin sulphate inhibition of HLE with Suc-A-A-A-NA as substrate. Assay procedure as described in Section 2.4.2.1.

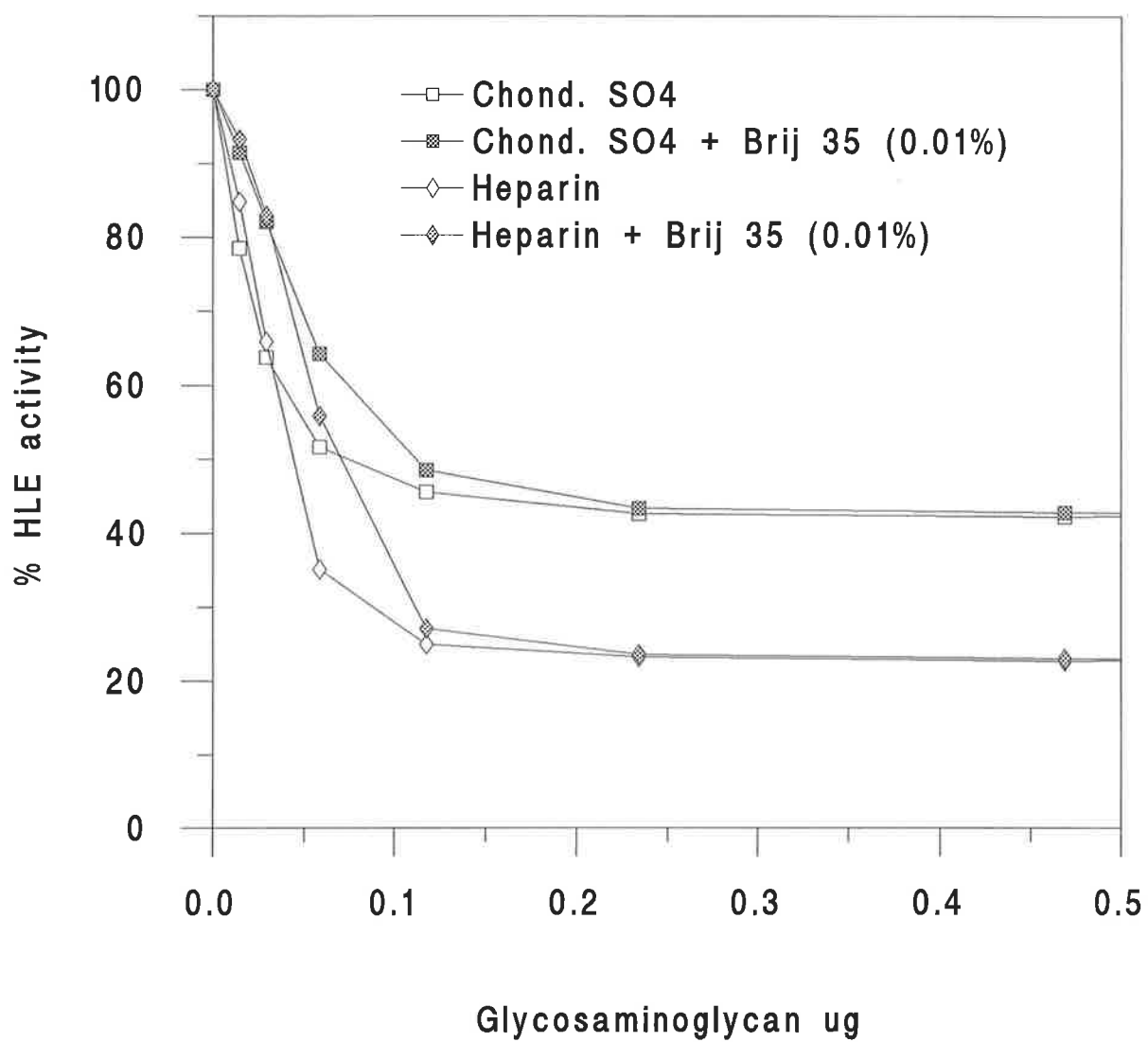


Figure 5.6: Effect of Brij 35 on heparin and chondroitin sulphate inhibition of HLE with MeO-Suc-A-A-P-V-NA as substrate. Assay procedure as described in Section 2.4.2.2.

inhibitor, were very effective inhibitors of HLE (50% inhibition of 0.5 μg HLE was achieved with 0.065 μg and 0.035 μg respectively). The tight binding of these inhibitors was not significantly modified by the presence of Brij 35 (Figure 5.4).

5.3.3 GAG INHIBITION

The most significant effects of Brij 35 on HLE inhibitors were seen with sulphated GAGs. An example of the effects of Brij 35 on the inhibition of HLE by sulphated GAGs is shown with heparin and chondroitin sulphate using Suc-A-A-A-NA (Figure 5.5) or MeO-Suc-A-A-P-V-NA (Figure 5.6) as substrate. Overall, heparin is a more effective inhibitor with Suc-A-A-A-NA as substrate (approx. 12% residual HLE activity, Figure 5.5) than with MeO-Suc-A-A-P-V-NA (approx. 23% residual HLE activity, Figure 5.6), but is a less effective inhibitor in the presence of Brij 35 at heparin concentrations below 0.1 μg with either substrate. Chondroitin sulphate, with MeO-Suc-A-A-P-V-NA as substrate, shows similar inhibition plots to those with heparin, although it is a less effective inhibitor than heparin (approx. 42% residual HLE activity, Figure 5.6). However, with Suc-A-A-A-NA as substrate, the inhibitory effect of chondroitin sulphate was drastically effected by the presence of Brij 35 (approx. 50% residual HLE activity with Brij 35 compared with 26% in its absence, Figure 5.5). Interestingly, in the absence of Brij 35, chondroitin sulphate at concentrations below 0.05 μg is a better inhibitor of HLE than heparin at similar concentrations where Suc-A-A-A-NA was used as substrate in both assays.

Brij 35 was found to reduce the ability of sulphated GAGs to inhibit HLE, the effect being dependent on the type of GAG, the substrate used and the order of addition of reagents (Table 5.1) using heparin, heparan sulphate and chondroitin sulphate as examples. This table only compares the 50% inhibition points for each GAG (ng) of HLE (0.5 μg) activity and not the maximum inhibition of HLE that can be achieved, as shown in figures 5.5 and 5.6 for heparin

Table 5.1: Summary of the effects of Brij 35 on heparin and chondroitin sulphate inhibition of HLE activity towards the substrates, Suc-Ala-Ala-Ala-NA and MeO-Suc-Ala-Ala-Pro-Val-NA.

Brij 35 was added to either the GAG (sample) or HLE (enzyme) prior to assay and compared to reagents with no Brij 35 present. The amount of GAG added to the assay to achieve a 50% inhibition of HLE activity is shown in the table as ng amounts with the factor (in brackets) indicating the increased amount required to achieve the same 50% inhibition with Brij 35 present compared to its absence. The 50% inhibition points of HLE activity were determined from plots similar to those shown in Figures 5.5 and 5.6. The HLE inhibition assays, with Suc-Ala-Ala-Ala-NA and MeO-Suc-Ala-Ala-Pro-Val-NA as substrate, are as described in Sections 2.4.2.1 and 2.4.2.2 respectively.

GAG (ng)	Brij 35	Suc-Ala-Ala-Ala-NA	MeO-Suc-Ala-Ala-Pro-Val-NA
Heparin	not present	45	45
	in sample	45 (x1)	45 (x1)
	in enzyme	60 (x1.3)	67 (x1.5)
Heparan sulphate	not present	15	110
	in sample	43 (x2.8)	110 (x1)
	in enzyme	65 (x4.3)	230 (x2.1)
Chondroitin sulphate	not present	15	52
	in sample	300 (x20)	75 (x1.4)
	in enzyme	460 (x30.6)	110 (x2.1)

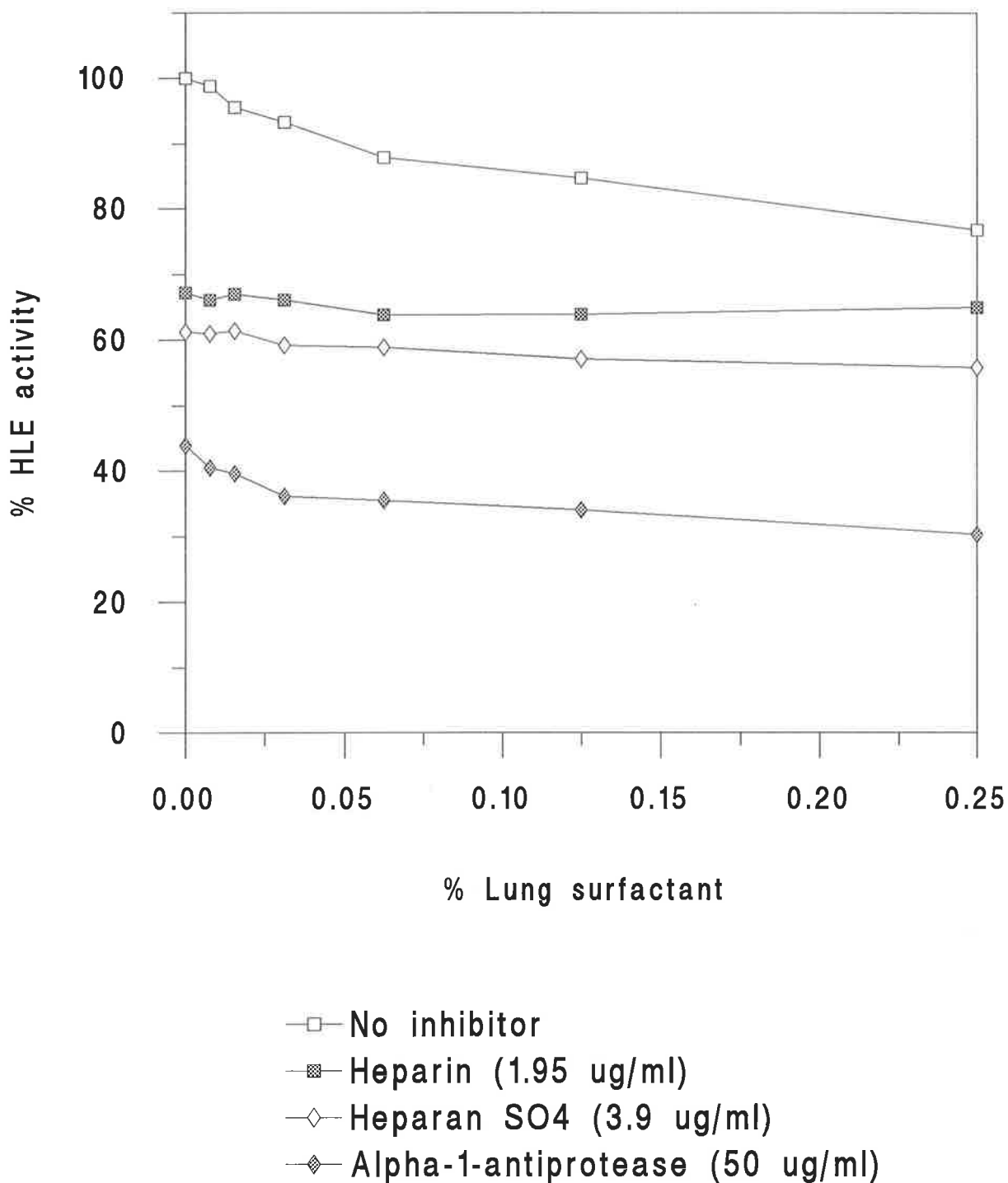


Figure 5.7: Effect of lung surfactant on heparin, heparan sulphate and alpa-1-antiprotease inhibition of HLE with MeO-Suc-A-A-P-V-NA as substrate. Assay procedure as described in Section 2.4.2.2.

and chondroitin sulphate. All the GAGs are effective partial inhibitors of HLE in the absence of Brij 35, with heparan sulphate and chondroitin sulphate being the most effective where Suc-A-A-A-NA was used as substrate. The maximum interference of Brij 35 in the inhibition of HLE by sulphated GAGs was seen only when it was added to the HLE prior to assay. The extent of the interference ranged from a minimal effect on heparin (x1.3) to a large effect on chondroitin sulphate (x30.6) both with Suc-A-A-A-NA as substrate. The addition of Brij 35 to sulphated GAGs prior to assay had less of an effect, ranging from no effect on heparin (Suc-A-A-A-NA or MeO-Suc-A-A-P-V-NA as substrate) or heparan sulphate (MeO-Suc-A-A-P-V-NA as substrate) to a 20 fold effect on chondroitin sulphate (Suc-Ala-Ala-Ala-NA as substrate).

5.4 EFFECT OF LUNG SURFACTANT ON HUMAN LEUKOCYTE ELASTASE INHIBITORS

As shown in Section 5.2, lung surfactant has an inhibitory effect towards HLE activity. This function of lung surfactant may have a protective function in the lung alveolus to minimise elastolytic lung damage. However, this inhibitory effect of lung surfactant should ideally be additive to, and not interfere with, the main HLE inhibitor, α_1 -PI and other endogenous inhibitors, such as heparin and heparan sulphate. It was found that the inhibitory effect of lung surfactant was additive to the inhibition by α_1 -PI, but appeared to have no additional inhibitory effect on the inhibition seen with heparin and heparan sulphate (Figure 5.7).

5.5 DISCUSSION

While the 20-40% activation of HLE by non-ionic surfactants, found in this study, with peptide substrates is consistent with other reports (Cook and Ternai, 1988a; Wenzel et al., 1990), the degree of activation varied depending on the substrate and assay conditions used. For example, I

have found approximately 125% activation with Brij 35 (Figure 5.1) using 350 $\mu\text{mol/l}$ MeO-Suc-A-A-P-V-NA in 0.05 mol/l NaCl compared with the other reports of 111% with 170 $\mu\text{mol/l}$ with the same substrate in 0.5 mol/l NaCl (Cook and Ternai, 1988a) and 167% with Suc-Ala-Ala-Ala-NA as substrate (Wenzel et al., 1990). With human lung elastin as substrate (Figure 5.2), only a slight activation of HLE (about 106% with 0.04-0.08% Brij 35) is reported here with no activation seen at Brij 35 concentrations of greater than 0.16%. The only other report in which elastin was used as substrate demonstrated a 14% increase in elastolysis in the presence of 0.1% Brij 35 using a gel plate assay (Starkey and Barrett, 1976). The results from this thesis are consistent with earlier reports that non-ionic surfactants activate HLE activity towards peptide substrates although, as demonstrated with Brij 35, there is little, if any, activation of HLE activity towards elastin as substrate. It has been proposed that certain agents which modify HLE activity, such as surfactants, bind to a regulatory site on HLE (Lestienne and Bieth, 1980). With hydrophobic compounds, such as non-ionic surfactants, the interaction probably occurs at an unusual hydrophobic binding site near the active site of HLE (Cook and Ternai, 1988b; Bode et al., 1989).

I have found that pulmonary surfactant has a mild inhibitory effect on HLE activity towards both a peptide substrate (Figure 5.1) and elastin (Figure 5.2). This inhibitory effect does not interfere with inhibitory activity of the endogenous inhibitors, α_1 -PI, heparin and heparan sulphate. However while the lung surfactant activity is additive to α_1 -PI, the main HLE inhibitor, it appears not to be additive to heparin and heparan sulphate. As pulmonary surfactant consists of 80-90% phospholipid (Hawgood et al., 1985) it is likely that the mild inhibitory effect could be due to electrostatic binding between the anionic phosphate groups on the phospholipid and the cationic arginine groups on HLE. This finding suggests that pulmonary surfactant *in vivo* may have an additional role in that it could partially protect the alveolar surface against elastolytic

enzyme induced injuries by direct inhibition of HLE. This proposal is consistent with other reports that the administration of pulmonary surfactant resulted in a significant inhibition of elastase-induced emphysema in mice (Otto-Verberne et al., 1992) and that neltenexine, a pulmonary surfactant inducing drug, showed a significant reduction in alveolar deformation in elastase-induced emphysema, when administered to rats prior to elastase treatment (Braga et al., 1995). However, HLE has also been reported to cleave surfactant proteins, which may alter surfactant function (Pison et al., 1989). Thus it would appear that replacement of the pulmonary surfactant layer on the alveolar surface is essential where HLE comes in contact with pulmonary surfactant so as to prevent elastolytic-induced injuries.

Brij 35 had little effect on the interaction of proteins and elastin peptides with HLE, except with α_2 -M (Figure 5.3) where the presence of Brij 35 required that the α_2 -M concentration be almost doubled to obtain the same inhibition of HLE (eg. 50% inhibition of HLE (0.5 μ g) required 44 μ g of α_2 -M in the presence of Brij 35 compared with 23 μ g in the absence of Brij 35). α_2 -M is a non-specific protease inhibitor with poor inhibition shown towards HLE as shown in this thesis with the low molecular weight substrate MeO-Suc-A-A-P-V-NA and in another report where α_2 -M showed little or no inhibitory activity towards a variety of proteases using low molecular weight substrates (Barrett and Starkey, 1973). Thus it is not surprising that the effectiveness of a poor inhibitor, such as α_2 -M in the presence of low molecular weight substrates, is further compromised by the presence of surfactants such as Brij 35. As discussed previously in this chapter, there is evidence that Brij 35 binds to a hydrophobic site on the enzyme. A possible mechanism is that the presence of Brij 35 on the surface of HLE may act by simply blocking or sterically hindering the binding sites on HLE required for binding to α_2 -M.

As discussed in chapter 4, the inhibition of HLE activity by sulphated GAGs is thought to occur by wrapping the linear polyanion chain of the sulphated GAG around the cationic enzyme (Baici et al., 1980). Non-ionic surfactants, such as Brij 35, probably interact with hydrophobic regions on HLE such as the unusual hydrophobic binding site near the active site of the enzyme (Cook and Ternai, 1988b; Bode et al., 1989). Thus Brij 35 binding to one of these nonpolar domains on HLE could sterically hinder the tight (essentially electrostatic) interactions between sulphated GAGs and HLE, with a consequent reduction in the inhibitory activity of sulphated GAGs towards the enzyme. It is likely that sulphated GAGs and substrates with a high affinity for HLE would be less affected by the binding of non-ionic surfactants to HLE than those sulphated GAGs and substrates with a low affinity for HLE. To test this proposal, HLE activity was measured in the presence of Brij 35 and three types of sulphated GAGs with a relatively low affinity substrate (Suc-A-A-A-NA) in comparison to a relatively high affinity substrate (MeO-Suc-A-A-P-V-NA). Three general conclusions can be made from the results (see Figures 5.5, 5.6 and Table 5.1) obtained from this study. The conclusions are listed as follows.

1. Maximum Brij 35 interference in the sulphated GAG inhibition of HLE requires that Brij 35 be bound to HLE prior to assay.
2. The HLE assay using the higher affinity substrate, MeO-Suc-A-A-P-V-NA, was less affected by Brij 35 than the assay using the lower affinity substrate, Suc-A-A-A-NA (a K_m of 0.14 and 3.7 mM respectively has been reported by Nakajima et al., 1979).
3. Sulphated GAGs (eg. heparin), which have a high affinity for HLE, generally are less affected by Brij 35 than those with a lower affinity (eg. chondroitin sulphate).

In conclusion, I have demonstrated in this chapter that non-ionic surfactants, such as Brij 35, can modulate the inhibitory effect of sulphated GAGs and α_2 -M towards HLE. However, no measurable effect of the presence of Brij 35 was found on the activity of other inhibitors

evaluated which include α_1 -PI, elastin peptides, and the low molecular weight inhibitors, ICI 186756 and 200355.

The other significant finding is that elastin peptides and pulmonary surfactant both inhibit HLE activity. This suggests that they may have a role in modulating elastolytic damage at the alveolar surface and that their influence may need to be taken into account in inhibitor studies using *in vivo* model assay systems.

CHAPTER SIX

ISOLATION OF HIGH AFFINITY HEPARIN FRACTIONS TOWARDS HLE

6.1 INTRODUCTION

Heparin is the commercially purified form of a sulphated GAG that is widely distributed throughout the human body in parallel with mast cell occurrence (Nader and Dietrich, 1989). The standard clinical heparin is extracted from porcine mucosa and consists of a pool of sulphated polysaccharide chains of varying lengths with an average MW of 14 ± 1 kDa. Heparin has been shown to interact with numerous biological systems (Lane and Lindahl, 1989) including a number of PMN functions, where it has been shown to have an anti-inflammatory role. Examples of the anti-inflammatory function of heparin include reduced PMN superoxide anion production, adhesion to endothelial cells, homotypic aggregation, lysosomal enzyme activity and PMN dependent activation of platelets (Bazzoni et al., 1993, Raitjar et al., 1993) as well as the inhibition of HLE activity (Redini et al., 1988a; Walsh et al., 1988; Walsh et al., 1991a). In particular, there is evidence to suggest that heparin can prevent the passage of PMNs through subendothelial basement membrane by inhibiting the selectin family of cell adhesion receptors involved with granulocyte adhesion and activation (Parekh and Edge, 1994; Nelson et al., 1993) and by inhibiting hydrolytic enzymes such as heparanase (Vlodavosky et al., 1992; Bartlett et al., 1995) and HLE (Redini et al., 1988a; Walsh et al., 1988; Walsh et al., 1991a; Drag and Petersen, 1994) which have been implicated in the degradation of the subendothelial basement membrane. These reports suggest that the isolation or synthesis of heparin fractions, particularly with an enhanced or specific anti-inflammatory function may prove to be useful in the prevention of the inflammatory components in the development or emphysema.

While heparin binds to a large number of proteins (Jackson et al., 1991), only one specific binding site on heparin for a protein has been well characterised. This binding site on heparin is a sulphated pentasaccharide sequence that tightly binds to antithrombin resulting in the acceleration of inhibition of thrombin which forms part of the anticoagulant effect of heparin (Casu, 1990). While heparin strongly binds to HLE by essentially electrostatic interactions which probably involves the negatively charged sulphate groups on heparin and some of the positively charged guanidinium groups of 19 arginine residues located in clusters on the surface of HLE (Bode et al, 1989; Navia et al., 1989), no specific binding sequences on heparin for HLE binding have been reported. In this chapter the binding between heparin and HLE is examined to determine if there is any evidence for preferential binding sequences in heparin. In a preliminary report (Walsh et al., 1993) and as explored further in this thesis, it was found that some heparin chains in unfractionated commercial heparin have a higher affinity than others for binding to HLE which is independent of total sulphate content. It was also found that there appears to be oligosaccharide sequences in heparin with high affinity binding for HLE.

6.2 AFFINITY CHROMATOGRAPHY OF HEPARIN ON HLE-SEPHAROSE

HLE-EAH- and HLE-ECH- Sepharose 4B gels were prepared (see Section 2.3.2) to assess the degree binding of sulphated GAGs to each gel. Initial binding studies with heparin showed that it strongly binds to HLE attached through the EAH spacer arm, resisting elution even with high salt concentrations. However, there was no binding of heparin to HLE attached through the ECH spacer arm. The latter finding indicates that the positively charged groups on HLE, such as the guanidinium groups of some of the arginine residues, when bound to the ECH spacer arm, are unavailable for binding to the anionic groups on heparin, either through binding to the carboxylic

group of the ECH spacer arm on the Sepharose gel or through being sterically hindered. Thus only HLE-EAH-Sepharose was used for heparin binding studies.

As Brij 35, a non-ionic surfactant, had been shown to reduce the ability of heparin and heparan sulphate to inhibit HLE elastolytic activity (see Section 5.3.3 and Table 5.1), it was added to the Tris buffer used for affinity chromatography to reduce the high affinity binding of heparin towards HLE and to minimise possible non-specific binding of heparin towards HLE-EAH-Sepharose. Brij 35 enabled the elution of heparin from the HLE-EAH-Sepharose column with a lower salt concentration, such that all the heparin was eluted with less than 1.5 M NaCl.

Affinity chromatography of heparin was performed as described in Section 2.3.9. Four major fractions were eluted with a 0.05-2 M NaCl gradient as shown in the typical profile reproduced in Figure 6.1. The first and the last peaks were eluted with approximately 0.4 and 1.0 M NaCl respectively. A different profile was seen for heparan sulphate. Here too the most tightly bound peak was eluted at approximately 1.0 M NaCl (Figure 6.2). The displacement of heparin begins to occur at a concentration of NaCl (approximately 0.4 M) which is about the point (0.35 M and above) where NaCl completely blocks the inhibition of HLE activity by heparin (see Section 4.5.1.4 and Figure 4.15). However, the salt interference in the inhibition of HLE activity by heparin increases progressively at physiological NaCl concentrations (0.14 M) and above, well before heparin is displaced from HLE (Figure 4.15). There is minimal interference in HLE activity with increasing salt concentration in the absence of heparin, as shown in Fig. 4.15, where 11.4% inhibition of activity was seen on increasing NaCl concentration from 0.05 M to 0.36 M NaCl. No change of inhibition was seen with a further increase to 0.67 M.

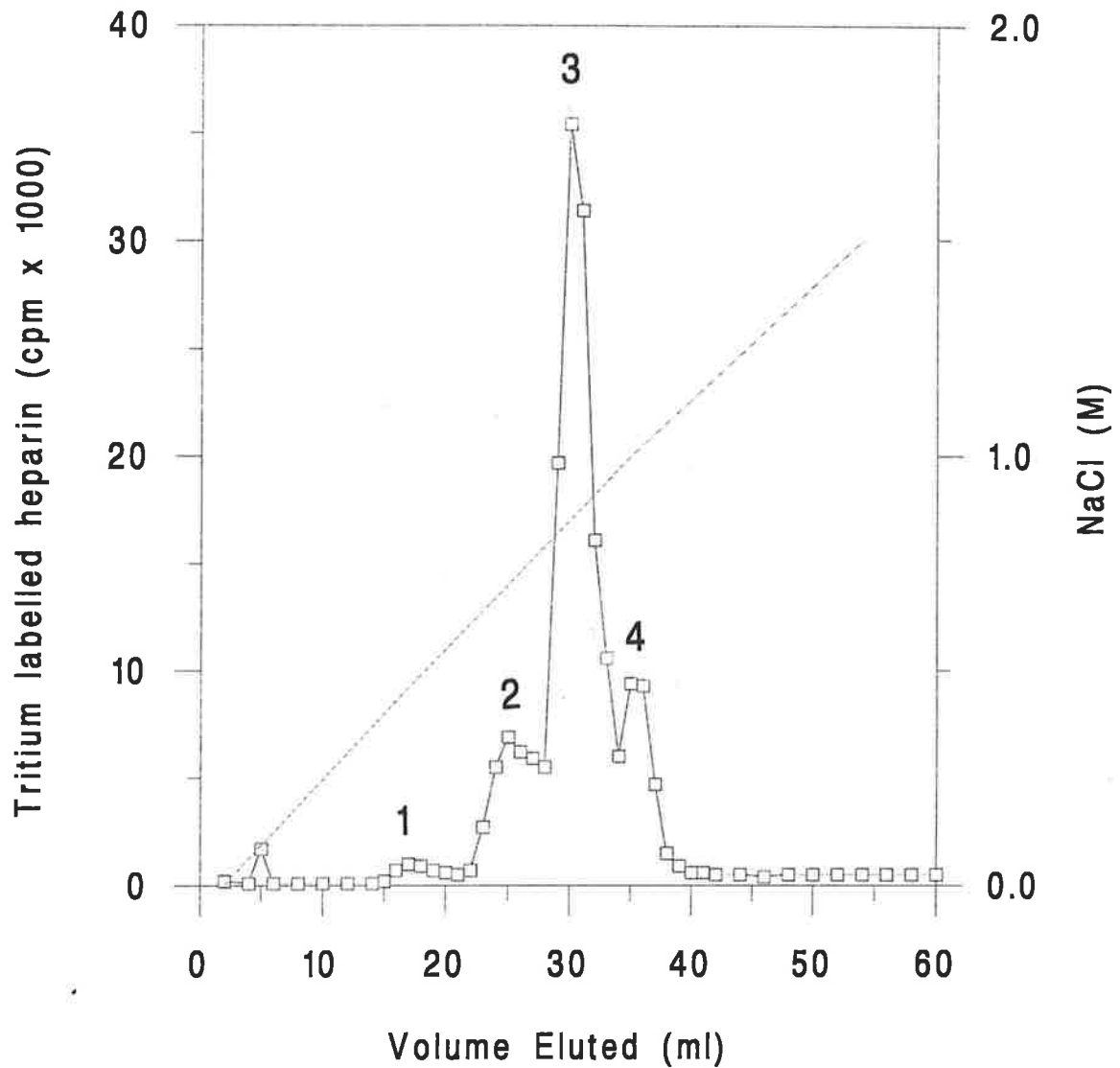


Figure 6.1: Affinity chromatography of heparin on HLE-Sepharose with a NaCl gradient in the presence of Brij 35. Preparation of gel and chromatography procedure are as described in Sections 2.3.2 and 2.3.9 respectively.

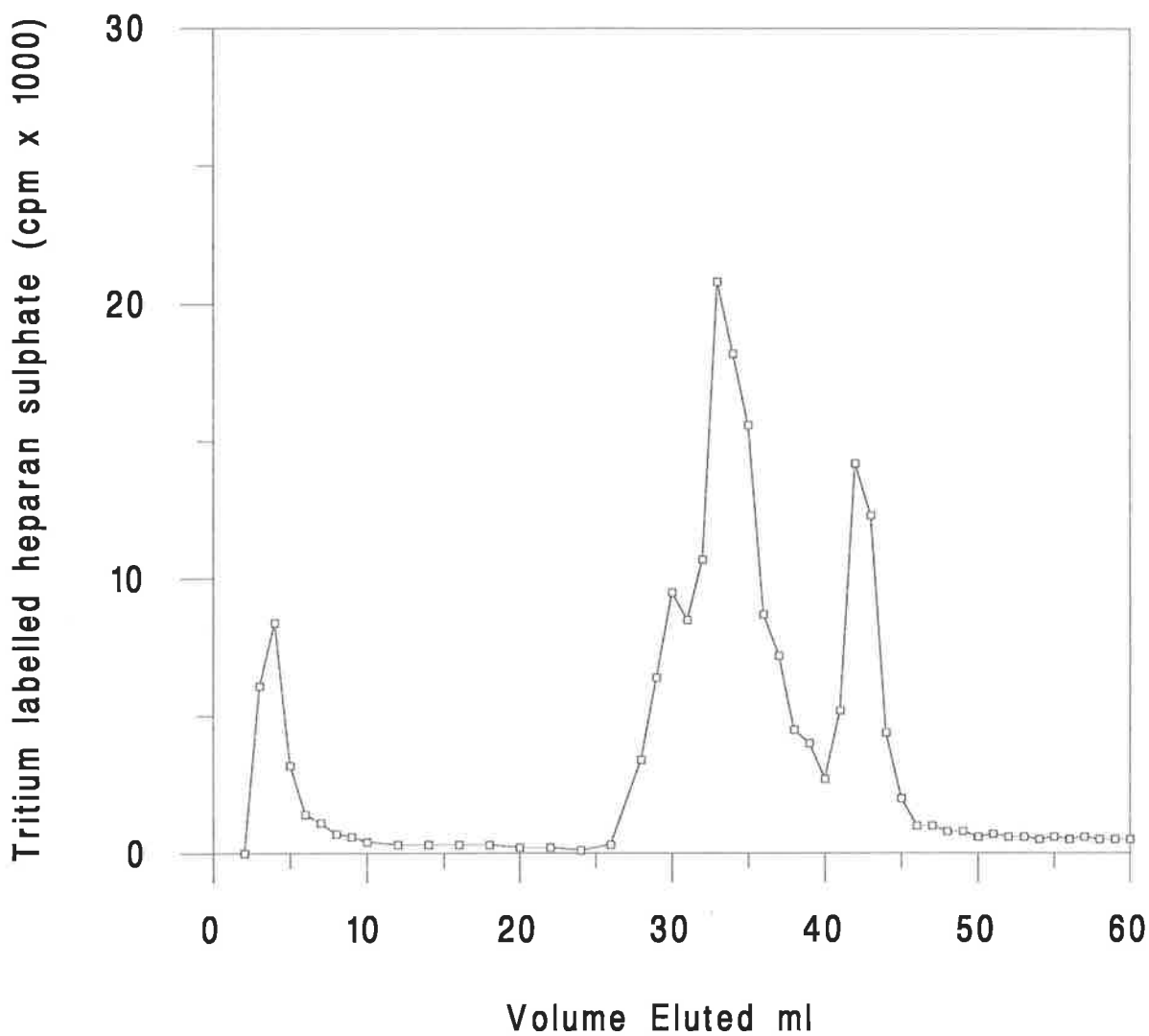


Figure 6.2: Affinity chromatography of heparan sulphate on HLE-Sepharose with a NaCl gradient in the presence of Brij 35. Preparation of gel and chromatography procedure are as described in Sections 2.3.2 and 2.3.9 respectively.

The ability of the isolated heparin fractions to inhibit HLE elastolytic activity increased progressively from the first to the last eluted fraction as shown in Figure 6.3a, where heparin content of the fraction is based on uronic acid content. This inhibition pattern was also seen, where heparin content of the fraction is based on sulphated GAG content as estimated with a 1,9-dimethylmethylene blue (DMB) dye binding assay (Figure 6.3b). The most tightly bound fractions of heparin, peaks 3 and 4 in Figure 6.1, are better inhibitors of HLE than the original unfractionated commercial heparin preparation which is represented as peak 0 in Figures 6.3a and b. Thus higher affinity binding is consistent with higher enzyme inhibitory activity.

Total sulphate content of the eluted heparin fractions fell with increased binding (Figures 6.4a and b) and inhibition of HLE (Figures 6.3a and b). This is a surprising finding in view of the fact that the presence of sulphate on heparin (an anionic GAG) appears to be essential for its high affinity binding to a cationic enzyme, such as HLE (Redini et al., 1988a; Walsh et al., 1988; Walsh et al., 1991a). This finding demonstrates that maximum binding and inhibition of HLE by heparin is not simply due to the total sulphate content of the heparin.

Using [³H]-end labeled heparin it was shown that the most tightly bound fractions (3 and 4) appear to have a longer average saccharide chain length when expressed on the basis of uronic acid (Figure 6.5a) or sulphated GAG content (Figure 6.5b). Fraction 1, the least tightly bound fraction, appears to have the same average chain length as unfractionated heparin whereas surprisingly fraction 2 appears to consist of shorter chain length heparin. HPLC was performed on unfractionated and affinity separated heparin fractions as described in Section 2.3.14. The HPLC retention times found (eg. unfractionated heparin, 10.6 min.; fraction 3, 10.6 min.; fraction 4, 10.0 min.) give an approximation of the molecular size of the fractions and lend some support to this finding in that while fraction 3 did not show an apparent mean molecular weight difference from unfractionated heparin, fraction 4 was shown to have the largest average molecular

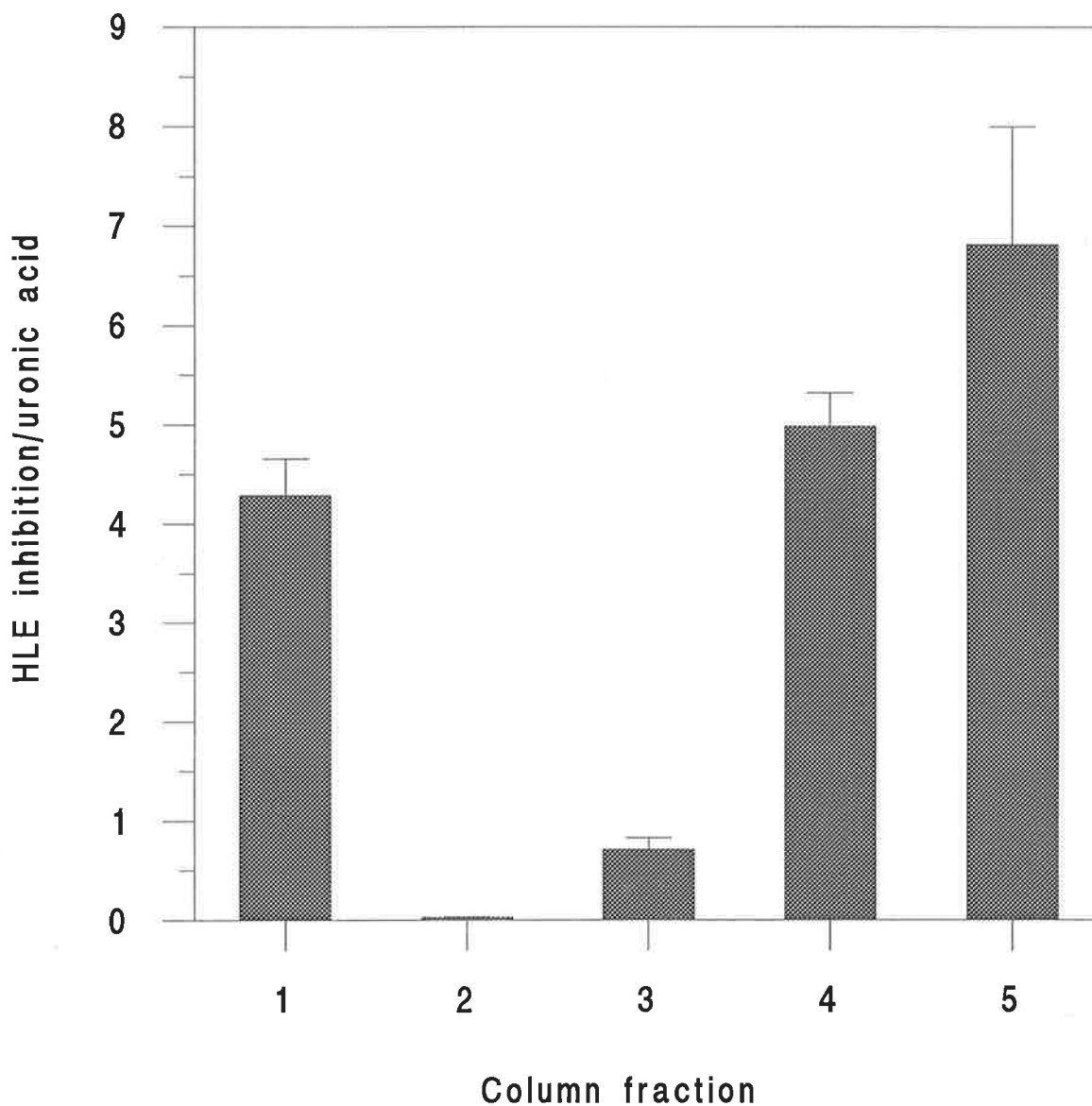


Figure 6.3a: Inhibition of HLE by unfractionated heparin (0) and heparin fractions (1-4) separated by HLE-Sepharose chromatography as shown in Figure 6.1. HLE inhibition and uronic acid assays are as described in Sections 2.4.2.3 and 2.4.5 respectively.

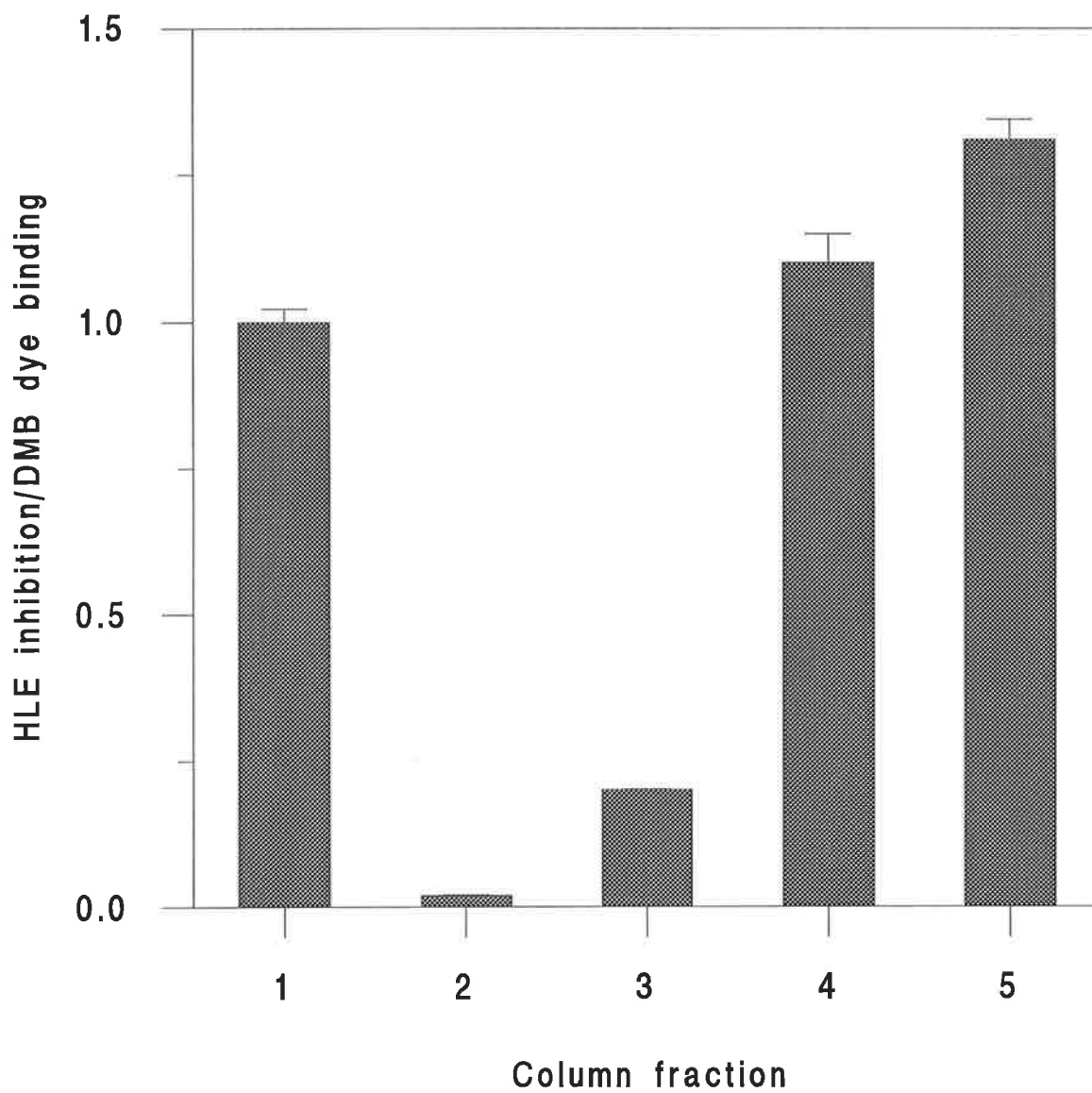


Figure 6.3b: Inhibition of HLE by unfractionated heparin (0) and heparin fractions (1-4) separated by HLE-Sepharose chromatography as shown in Figure 6.1. HLE inhibition and DMB dye assays are as described in Sections 2.4.2.3 and 2.4.7.

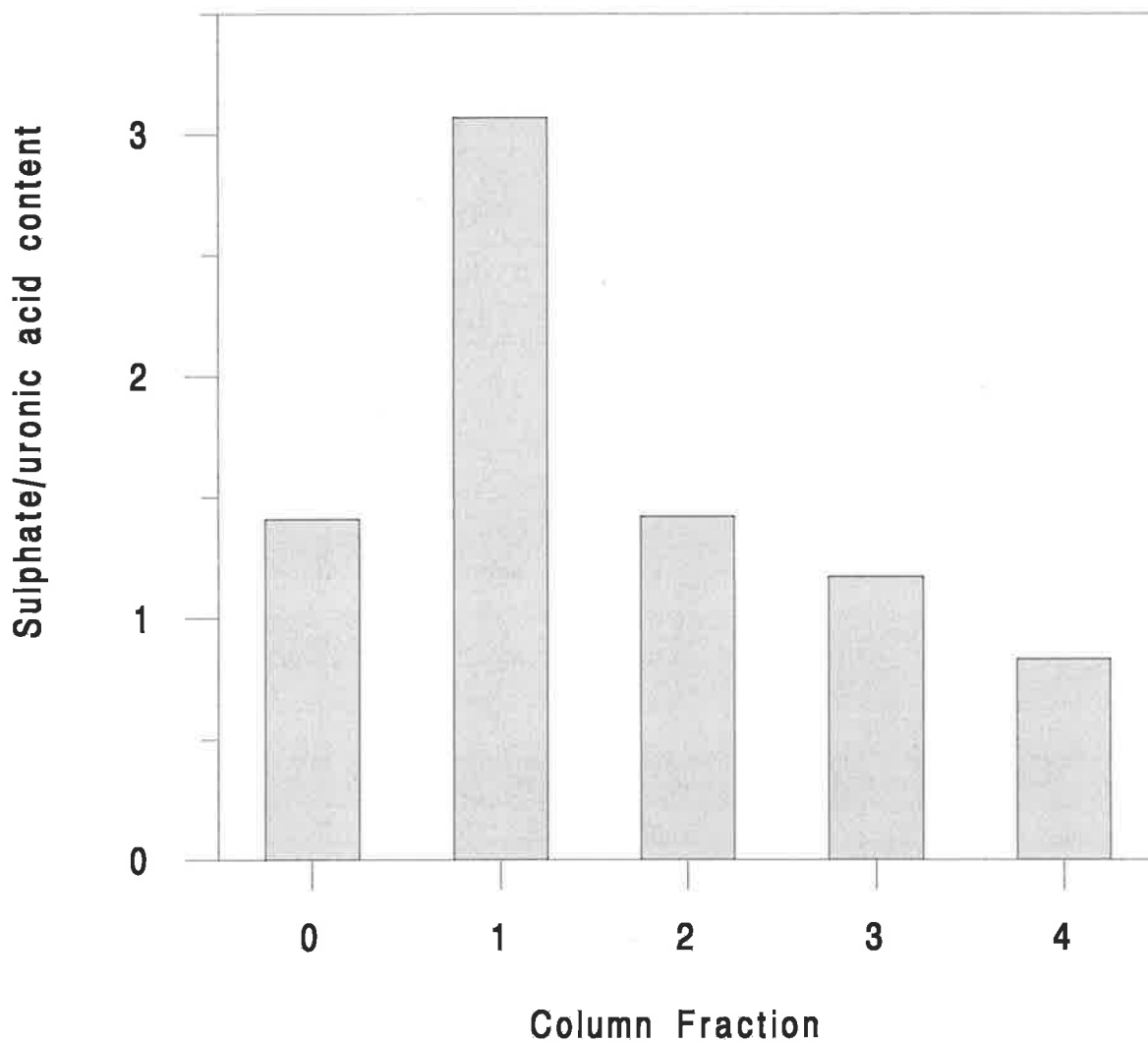


Figure 6.4a: Total sulphate content of unfractionated heparin (0) and heparin fractions (1-4) separated by HLE-Sepharose chromatography as shown in Figure 6.1. Sulphate and uronic acid assays are as described in Sections 2.4.6 and 2.4.5 respectively.

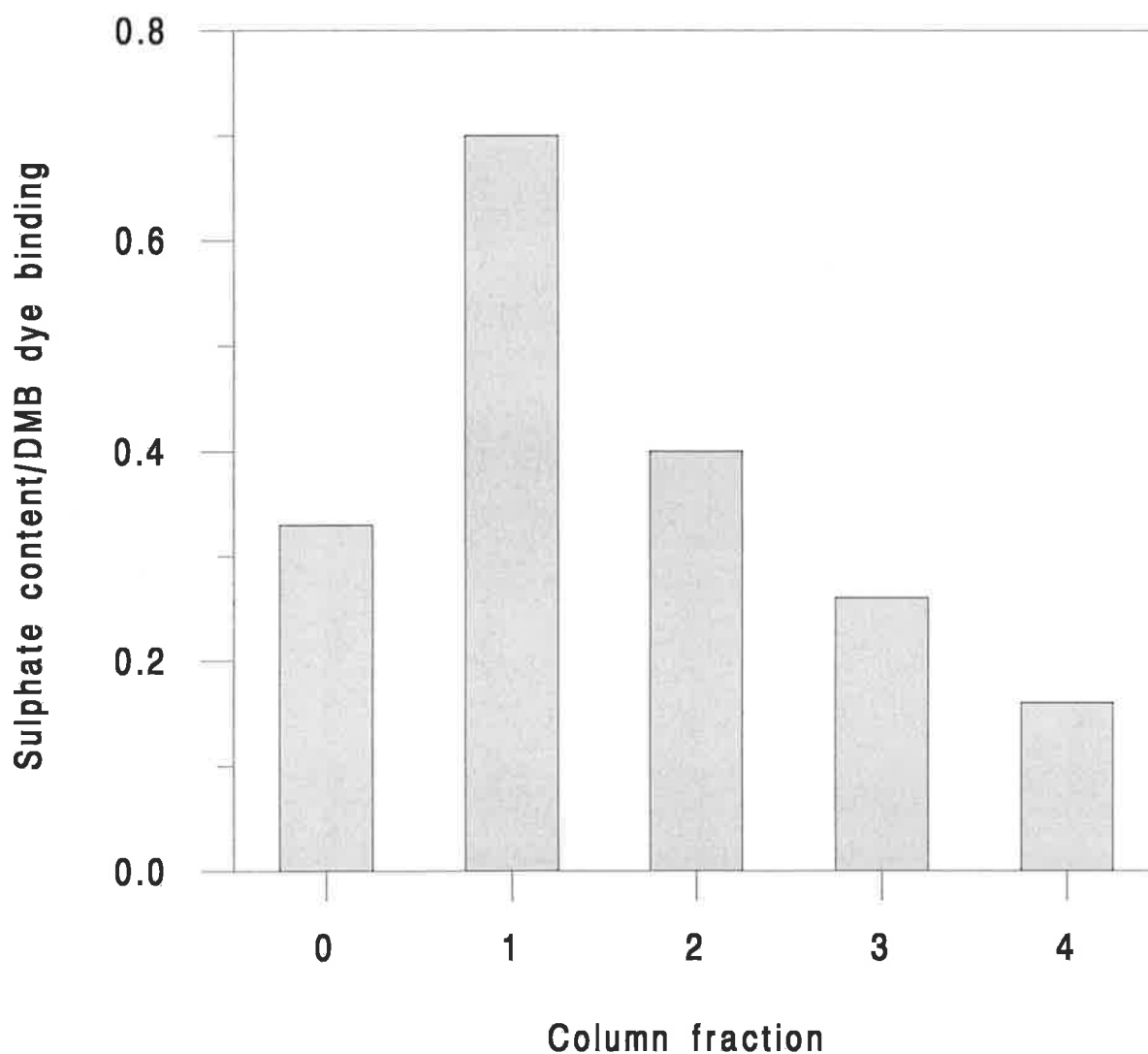


Figure 6.4b: Total sulphate content of unfractionated heparin and heparin fractions (1-4) separated by HLE-Sepharose chromatography as shown in Figure 6.1. Sulphate and DMB dye assays are as described in Sections 2.4.6 and 2.4.7.

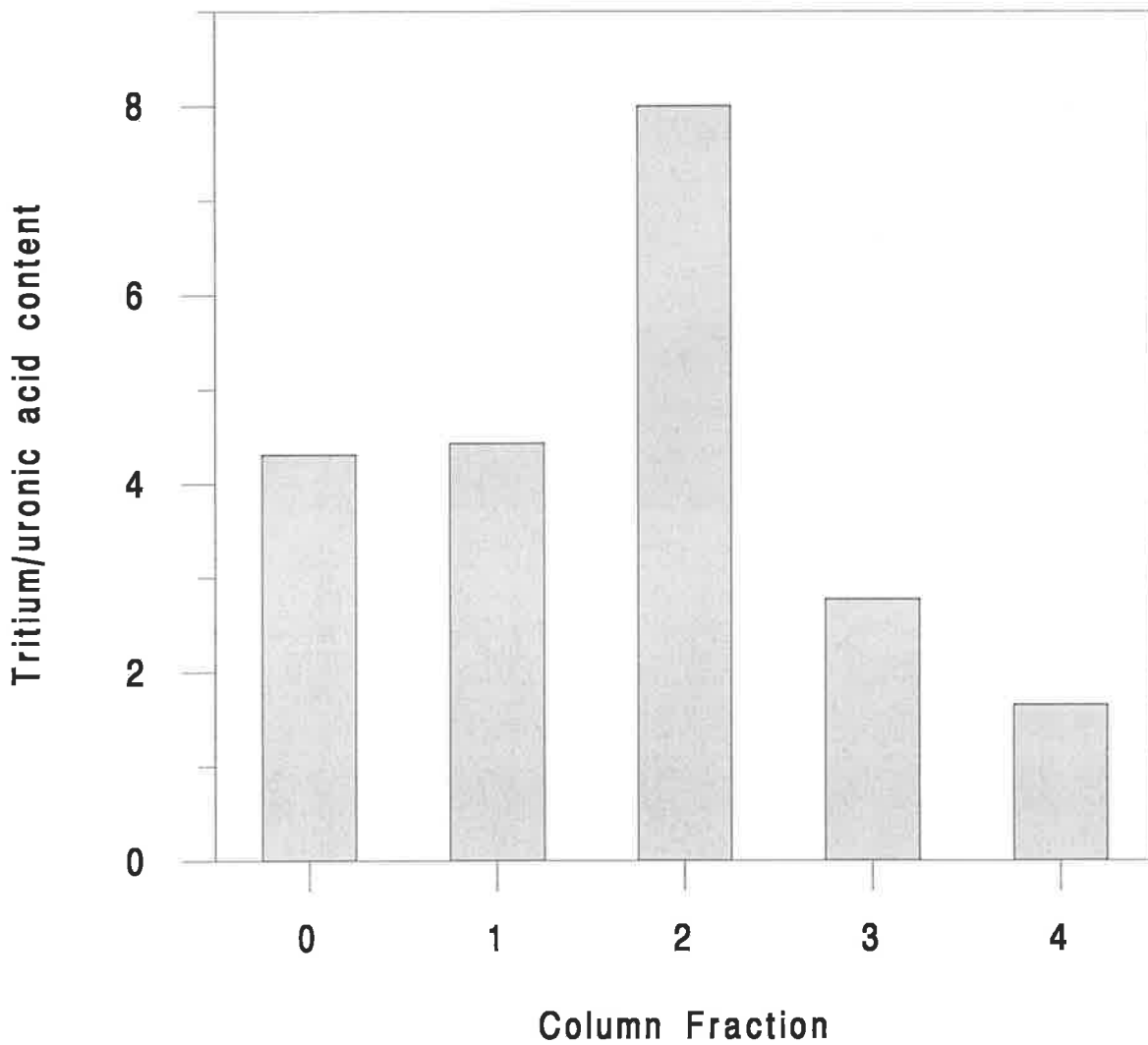


Figure 6.5a: Tritium content of 'end labelled' unfractionated heparin (0) and heparin fractions (1-4) separated by HLE-Sepharose chromatography as shown in Figure 6.1. Tritium labelling and uronic acid assays are as described in Sections 2.3.3 and 2.4.5.

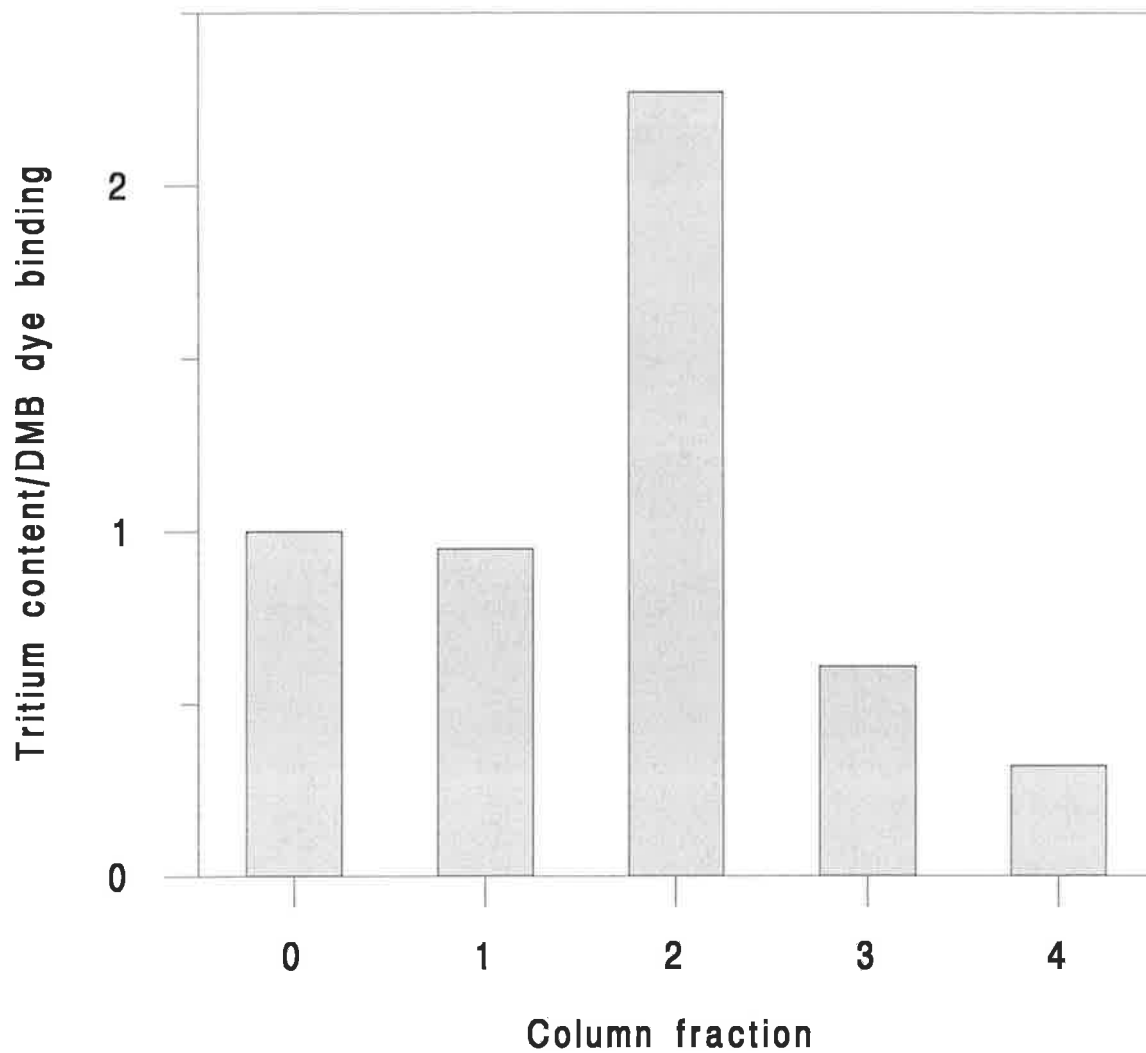


Figure 6.5b: Tritium content of 'end labelled" unfractionated heparin (0) and heparin fractions (1-4) separated by HLE-Sepharose chromatography as shown in Figure 6.1. Tritium labelling and DMB dye assays are as described in Sections 2.3.3 and 2.4.7.

weight. In conclusion, fraction 4 was shown to have a higher mean molecular weight than unfractionated heparin and the other heparin fractions. This is consistent with higher molecular weight fractions having, in general, a greater inhibitory activity towards HLE as discussed previously in chapter 4.

6.3 AFFINITY CHROMATOGRAPHY OF HEPARIN ON ANTITHROMBIN-SEPHAROSE.

The aim of this part of the study was to examine if there was any relationship between the well characterised binding site on heparin for antithrombin and high affinity binding site(s) for HLE. Heparin was separated on an antithrombin-Sepharose column into two fractions, one with no affinity for antithrombin and the remaining fraction (approx. 30%) which contains the sulphated pentasaccharide sequence responsible for the high affinity binding to antithrombin (Figure 6.6). To ensure that no high affinity heparin remained in the unbound fraction because of column overloading, the column was recycled until no heparin remained bound to the column. Overall about 70% of the unfractionated heparin was devoid of affinity for the Sepharose-bound antithrombin III which is consistent with other studies (eg. Andersson et al., 1976). No significant difference was seen between the two fractions in their ability to inhibit HLE activity (HLE inhibition/uronic acid: Low affinity fraction = 4.2 compared to High affinity fraction = 4.6). This demonstrates that the antithrombin binding sequence in heparin has no preferential binding affinity for HLE and would appear to rule out the possibility that this unique sequence might also have an additional role in binding HLE.

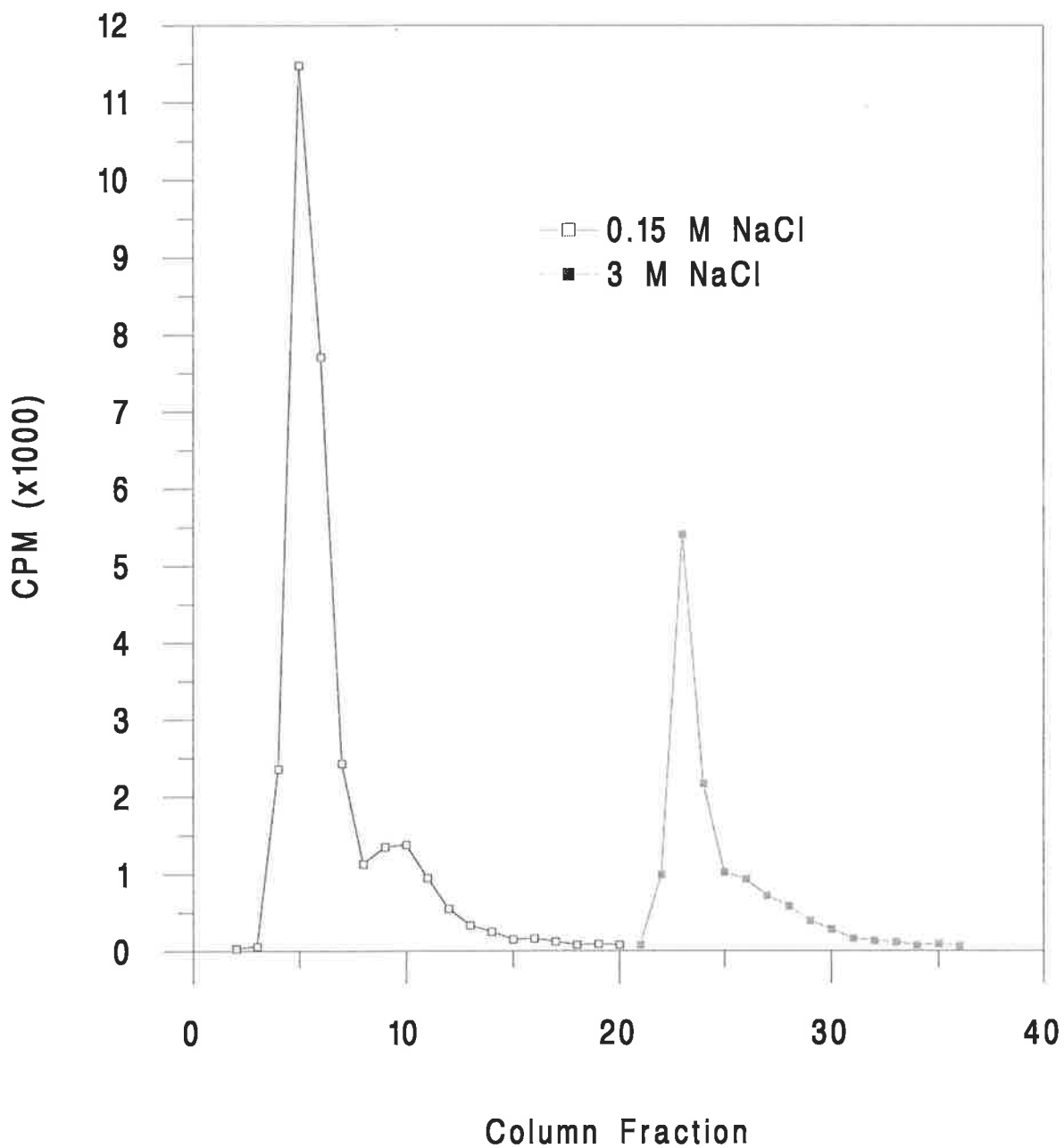


Figure 6.6: Affinity chromatography of heparin on antithrombin III-Sepharose. Preparation of gel and chromatography procedure in Sections 2.3.4 and 2.3.10 respectively.

6.4 ISOLATION OF HLE BINDING FRACTIONS FROM HEPARINASE DIGESTION OF HLE BOUND HEPARIN.

6.4.1 HEPARIN BOUND TO FREE HLE

In an initial series of experiments, heparin bound to HLE was digested with heparinases, following the protocol outlined in Figure 6.7 and described in Section 2.3.11.2. Briefly, at the end of the digestion period, unbound heparin fragments were removed by dialysis, leaving the remaining heparin tightly bound to HLE together with the heparinases. An apoprotin-Sepharose column was used to isolate the bound heparin fractions from HLE; a typical separation is shown in Figure 6.8. The bound heparin fractions were eluted off with 1M NaCl, then the pH was lowered to 5.0 to recover the HLE. While good separations were obtained, variable recoveries were found for heparin fractions and for HLE. This variability was found to occur at the dialysis step. It is most likely to be due to the surface absorption of the cationic HLE and the variable molecular weight cut-off of the dialysis tubing; particularly when dialysing linear molecules such as heparin. Thus further experiments were concentrated on using HLE bound to EAH-Sepharose (see below) which allowed more opportunity to control experimental conditions with good recovery of heparin fractions and the ability to recycle the HLE-EAH-Sepharose for further experiments.

6.4.2 HEPARIN BOUND TO HLE-EAH-SEPHAROSE

The digestion with heparinases of heparin bound to HLE-EAH-Sepharose, is shown diagrammatically in Figure 6.9 and is described in Section 2.3.11.1. Briefly, at the end of the digestion period, unbound heparin fragments and heparinases were eluted from the gel with buffer. The remaining heparin tightly bound to HLE, was eluted off the gel with 2M NaCl. The isolated

Figure 6.7: Outline of procedure for the digestion of heparin bound to HLE with heparin lyases

heparin + HLE

↓ *0.1 M NaCl, 0.005 M PO₄, pH 7.15*

heparin-HLE

↓ *heparin lyase I & II, 24h at 30^o C*

heparin oligos.-HLE + unbound heparin oligos.

↓ *apoprotin-Sepharose*

Apoprotin-Sepharose-HLE-heparin oligos. → unbound heparin oligos.

↓ *1 M NaCl*

Apoprotin-Sepharose-HLE → heparin oligos.

↓ *pH 5, 1 M NaCl*

↓

Apoprotin-Sepharose + HLE

*Biogel P2 desalting
12-22% PAGE*

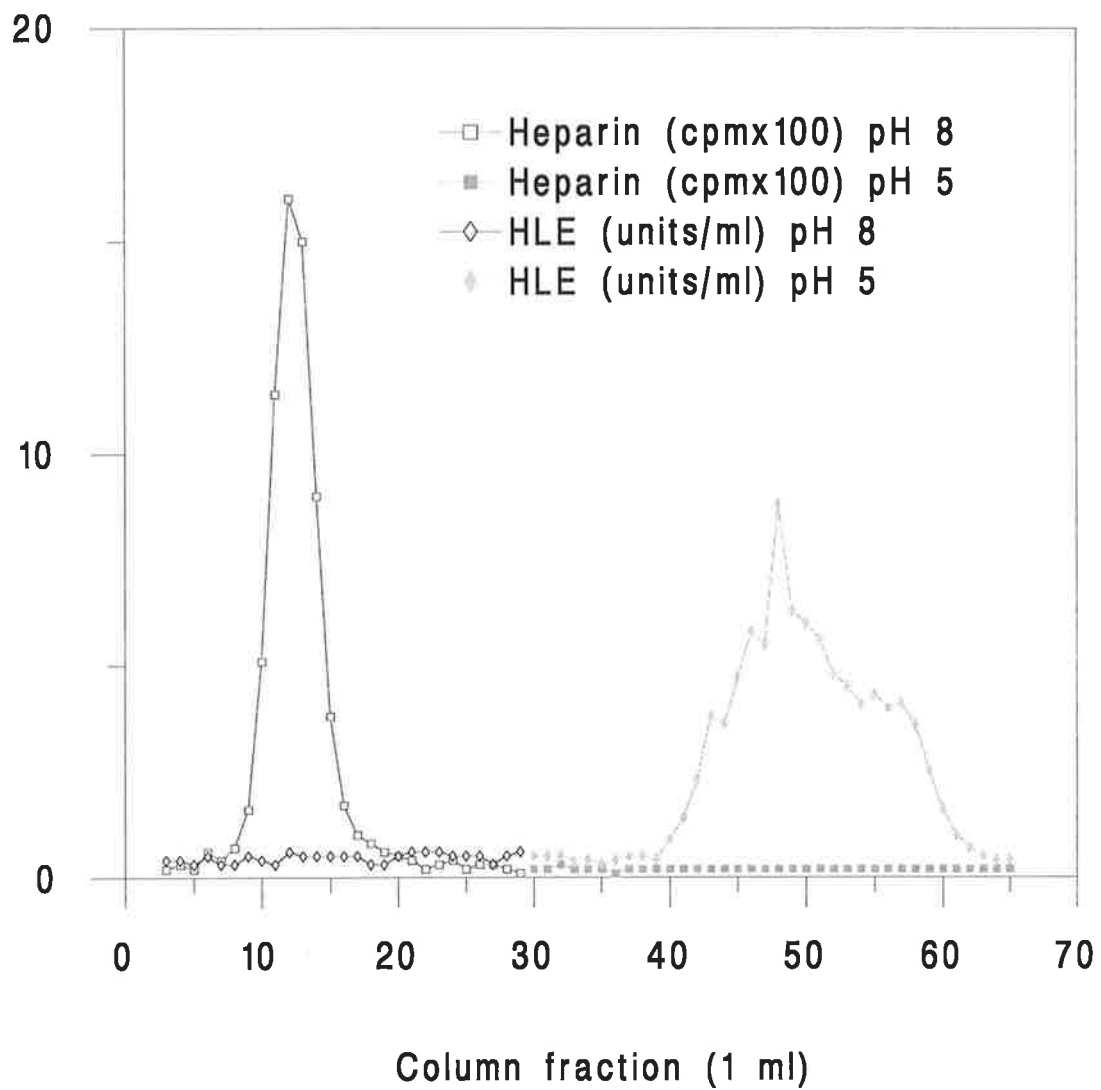


Figure 6.8: Affinity chromatography separation of heparin from HLE with apoprotin-Sepharose. The chromatography procedure and HLE assay are as described in Sections 2.3.11.2 and 2.4.2.3 respectively.

Figure 6.9: Outline of procedure for the digestion of heparin bound to HLE-Sephacrose with heparin lyases

heparin + HLE-Sephacrose

↓ *0.1 M NaCl, 0.005 M PO₄, pH 7.15*

heparin-HLE-Sephacrose

↓ *heparin lyase I & II, 24h at 30° C*

heparin oligos.-HLE-Sephacrose → **unbound heparin oligos.**

↓ *2 M NaCl*

HLE-Sephacrose

→ **heparin oligos.**

↓

*Biogel P2 desalting
12-22% PAGE*

Figure 6.10: Gradient PAGE (12-22%) of heparinase digested heparin fractions bound to HLE-Sepharose. Lane 1, tetrasaccharide, hexasaccharide and octasaccharide markers prepared from heparin (uronic acid content, 10 μg); lane 2, unfractionated heparin (4 μg); lane 3 and 4, digested unbound heparin (4 and 8 μg respectively); lane 5 and 6, residual bound heparin after digestion (5 and 12 μg respectively); lane 7, as for lane 1. HLE-Sepharose preparation, heparinase digestion of heparin, gel preparation and electrophoresis were as described in Sections 2.3.2, 2.3.11 and 2.3.12.

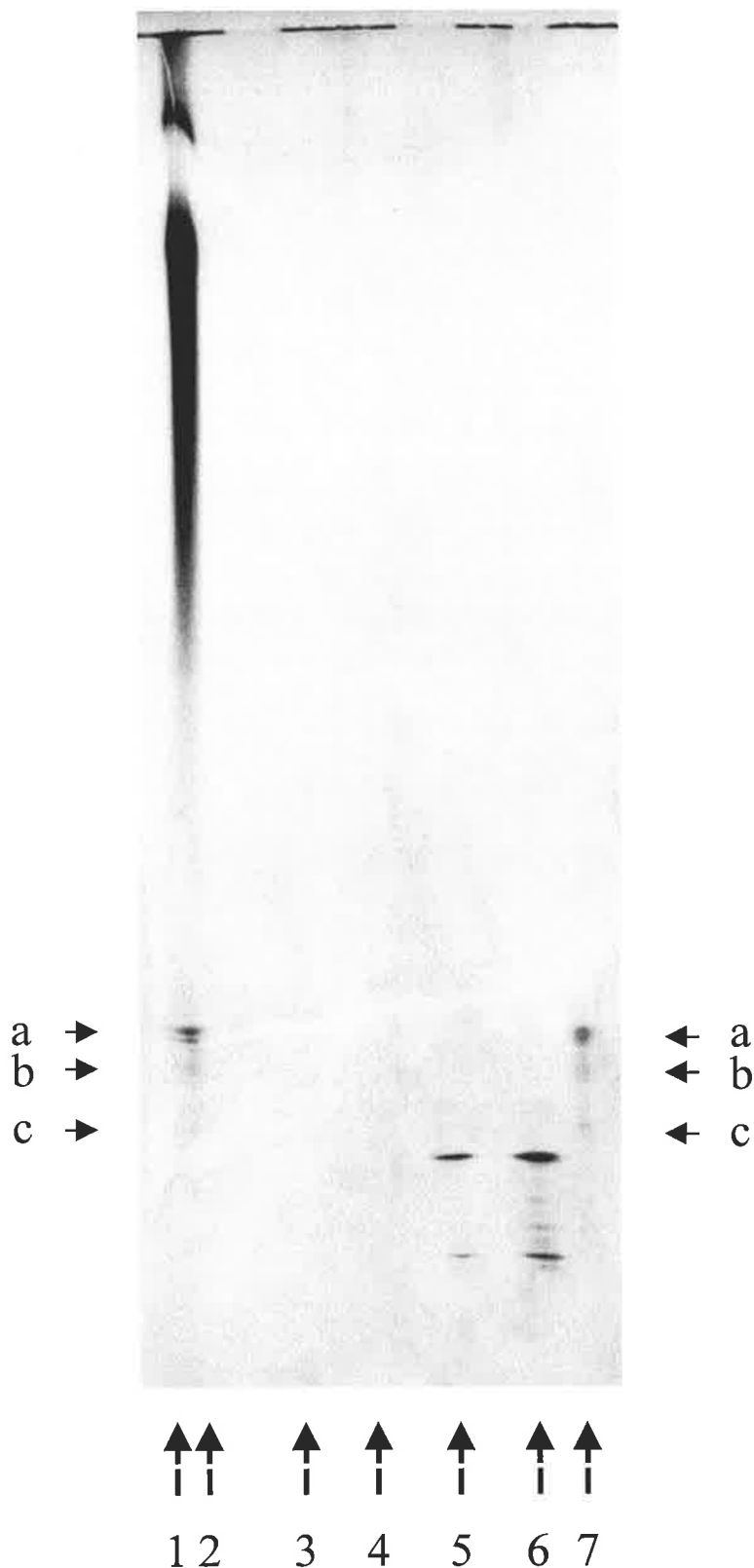
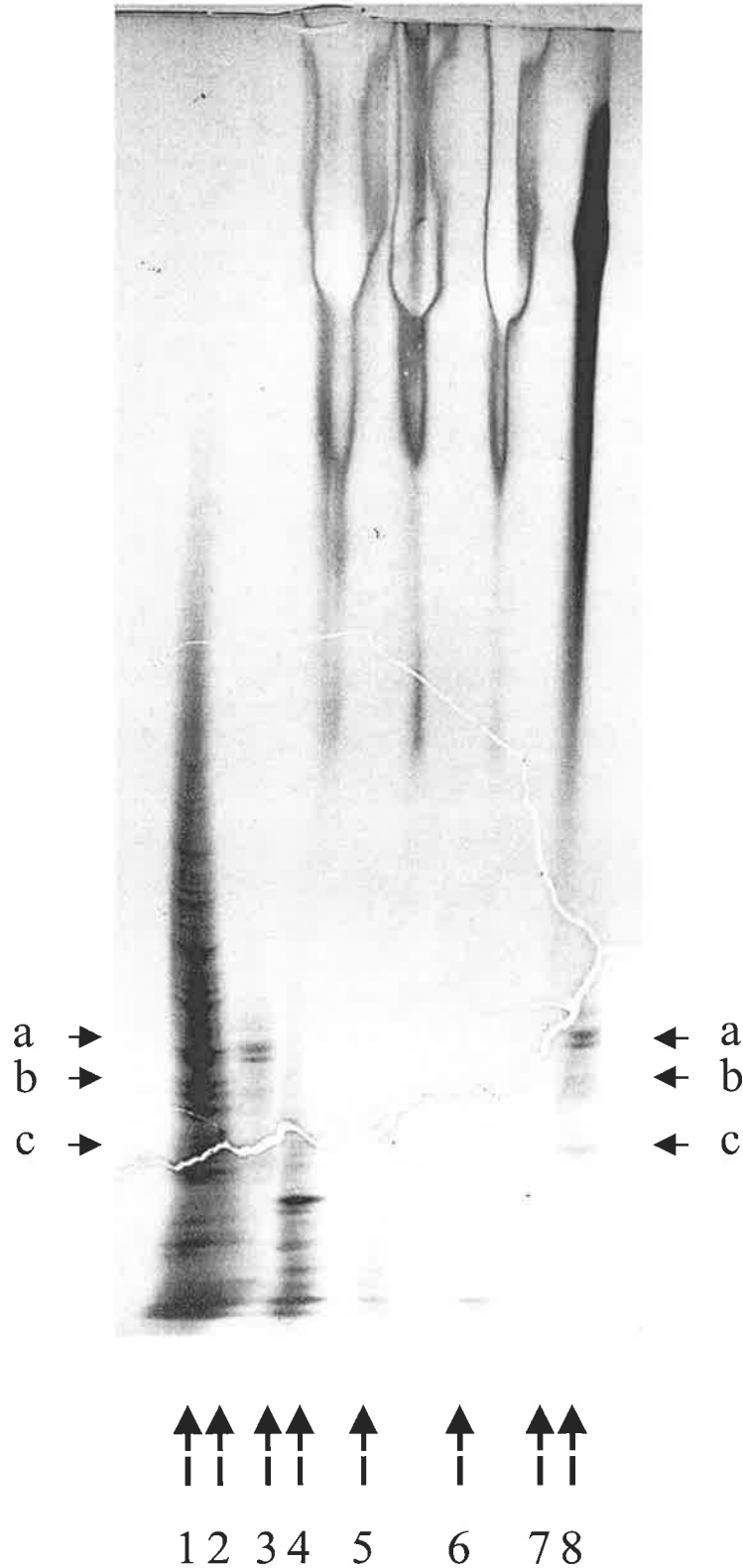


Figure 6.11: Gradient PAGE (12-22%) of heparinase digested heparin fractions bound to HLE-Sepharose. Lane 1, low molecular weight heparin (Opocrin 381/1 *M*, 2000, 10 μ g); lane 2, low molecular weight heparin (Opocrin 381/2 *M*, 850-1000, 10 μ g); lane 3, tetrasaccharide, hexasaccharide and octasaccharide markers prepared from heparin (uronic acid content, 10 μ g); lane 4, residual bound heparin after digestion (10 μ g); lane 5 and 6, digested unbound heparin (10 and 15 μ g respectively); lane 7, unfractionated heparin (4 μ g); lane 8, as for lane 1. Procedure is as for Figure 6.10.



heparin fractions that were bound to HLE after heparinase digestion were then examined with gradient PAGE. The presence of one major and several minor bands of heparin oligosaccharides are shown in the bound heparin fractions from two typical experiments in Figures 6.10 (lanes 5 and 6) and 6.11 (lane 4). This pattern of bands was not seen in heparinase digests of unbound heparin. The chain length of these bands appear to be less than a tetrasaccharide when compared to the tetrasaccharide, hexasaccharide and octasaccharide markers run on the gel, although this may be dependant on their charge. For example, a more highly charged oligosaccharide would run faster and appear to have a slightly lower molecular weight. A pooled fraction of these bands was found to inhibit HLE although not to the degree as seen with intact heparin. For example when the fraction's ability to inhibit HLE activity is expressed as a ratio of uronic acid content a value of 0.23 to 0.51 was found compared to the value seen typically for unfractionated heparin of 4.2 and 0.04 to 0.09 which was seen for the digested unbound heparin control. This finding is consistent with the observations, by myself and others, that very short chain length sulphated oligosaccharides are poor inhibitors of HLE.

6.5 DISCUSSION

Brij 35, was found to reduce the ability of heparin to inhibit elastolytic activity of HLE (see Section 5.3.3 and Table 5.1). It was added to the column buffers to reduce the degree of heparin - HLE interaction, thus allowing heparin to be completely eluted from a HLE-EAH-Sepharose affinity column with a moderate salt concentration of less than 1.5 M NaCl. Brij 35 has also been reported to reduce the inhibitory activity of a number of hydrophobic inhibitors towards HLE (Cook and Ternai, 1988a). The ability of non-ionic surfactants, such as Brij 35, and non-ionic solvents, such as acetonitrile (Frommherz et al., 1991), to lower the binding affinity of heparin towards HLE suggests the involvement of hydrophobic bonds in this binding. It has been proposed that hydrophobic inhibitors of HLE may interact with hydrophobic amino acid side chains of HLE in

the extended substrate binding pocket of HLE (Tyagi and Simon, 1990). Brij 35 binding to one of these nonpolar domains on HLE could sterically hinder the proposed essentially electrostatic interactions between the sulphate groups on sulphated GAGs and some of the positively charged guanidinium groups on HLE (Bode et al., 1989; Navia et al.; 1989). The flexibility of the carbohydrate backbone allows GAGs to closely fit electropositive proteins particularly in the case of highly sulphated GAGs such as heparin where the tight ionic binding is less likely to be affected by steric hindrance than less highly sulphated GAGs. This is supported by the fact that sulphated GAGs which have a high affinity for HLE (eg. heparin) generally are less affected by Brij 35 than those with a lower affinity (eg. chondroitin sulphate) as demonstrated in Table 5.1.

With a 0.05-2 M NaCl gradient, heparin was fractionated into low and high affinity fractions for HLE. A similar result was found for heparan sulphate, which is consistent with fact that it has a similar inhibitory profile to heparin in its inhibition towards HLE (Figure 4.10 and Walsh et al., 1991a). The degree of binding affinity, in general, is consistent with higher enzyme inhibitory activity (Figures 6.1 and 6.3). The NaCl interference in the inhibition of HLE activity by heparin occurs at a concentration well before NaCl starts to displace heparin from HLE (see Section 4.5.1.4 and Figure 4.15; Figure 6.1) which suggests that heparin is less tightly bound to areas important for catalytic activity than other areas on HLE.

[³H]- end labelled heparin (Figure 6.5) and HPLC retention times suggested that fraction 4, the most tightly bound fraction, has a longer average chain length compared to the other fractions and unfractionated heparin. It also suggests that longer chain lengths may contain more, high affinity binding sites and thus are more effective at binding to HLE and inhibiting elastolytic activity. This is consistent with earlier studies (Redini et al., 1988a; Walsh et al., 1991a; Fransson and Johansson, 1981; Drag and Petersen, 1994) that, in general, high molecular weight fractions of heparin and other sulphated GAGs are better inhibitors of HLE than low molecular weight fractions.

A well characterised binding sequence on heparin is the pentasaccharide sequence for antithrombin III binding. My data demonstrate that heparin fractions, with and without the antithrombin III binding site, show approximately equal binding to HLE indicating that the proposed high affinity sequences for HLE are distinct from the antithrombin III binding sequence. Whether they are similar to the antithrombin III binding site awaits detailed structural studies of the heparin saccharide sequences bound to HLE.

As demonstrated in a number of studies, the binding between the anionic heparin polymer and the cationic HLE molecule is essentially ionic in nature. However, for maximum binding of the flexible heparin chain, the positioning of sulphate groups along the heparin may be more important than the degree of sulphation. My data demonstrating a relative fall in the total sulphate content of heparin chains with increased high affinity binding to HLE (Fig. 6.4) would suggest that the distribution and spacing of the sulphate groups along the heparin chain may be important for maximum high affinity binding to HLE, implying the occurrence of high affinity binding sequences along the heparin chain. The finding supports the concept, as discussed by Casu (1990), that only a few suitably spaced anionic groups (sulphate) along the heparin chain could achieve a binding to clusters of basic groups (arginine residues) on a protein (HLE) as strong as that obtained with oversulphated polysaccharides. This specific type of binding is not readily apparent, probably because it is swamped by the overall crowding of sulphate groups along the heparin chain randomly binding to cationic groups on HLE. This specificity of binding of heparin to proteins where some sequences are preferred to others, has been reported for the interaction of relatively low-sulphated heparan sulphate species with thrombin and low-density lipoproteins (Fransson and Johansson, 1981; Fransson and Havsmark, 1982).

Preliminary gradient PAGE studies of heparin fractions, obtained from the remaining tightly bound heparin to HLE after heparinase digestion, demonstrate one major and several minor bands of heparin oligosaccharides. These are likely to contain specific binding sequences for HLE of remaining tightly bound fractions to HLE. The number of bands may reflect the likely multiple binding sites on heparin for HLE although this may not be truly representative because of possible steric hindrance or blockage of heparin binding to HLE by the attachment of the EAH spacer arm of Sepharose to HLE. A pooled fraction of these bands was found to inhibit HLE although not to the degree as seen with intact heparin which is not surprising considering their short chain length. However, what is surprising is that such short chain lengths (approx. tetrasaccharide and less) should inhibit HLE at all which could indicate binding at critical sites on HLE that impairs its catalytic activity. The relative inhibition of HLE by each band and their relative importance in the binding of HLE awaits further studies to characterise and sequence these fractions.

A study by Yamada et al. (1999), which was published after this study was completed, has reported the isolation of a number of octasaccharides from the low-sulphated region of porcine intestinal heparin after treatment with heparinase. Whether these fractions demonstrate any higher affinity binding or contain specific binding sites for HLE has at this stage not been investigated.

The isolation in this study of heparin chains with a high specificity for binding to HLE together with the demonstration of heparin oligosaccharides which tightly bind to HLE, suggests the potential for the development of HLE inhibitors for the treatment of HLE mediated inflammatory diseases such as pulmonary emphysema. Heparin has been used commercially for many years, specifically as an anticoagulant, so that the clinical introduction of a heparin derivative should be simpler than for a totally new therapy.

CHAPTER SEVEN

CONCLUDING REMARKS

7.1 SYNOPSIS AND CONCLUSIONS OF THIS THESIS

7.1.1 FUNCTIONAL α_1 -PROTEASE INHIBITOR CONCENTRATIONS IN SERUM AND BRONCHOALVEOLAR LAVAGE FLUID

The evidence from the literature suggests that α_1 -PI is the major antiprotease reacting with proteases that constitute the protease-antiprotease hypothesis for the aetiology of emphysema. The initial part of this thesis work was directed to establishing assays to determine not only whether α_1 -PI is the major antiprotease but whether it is fully functional as an inhibitor towards HLE. α_1 -PI was measured in the serum, plasma and BAL from emphysema patients and compared with those from normal volunteer adult nonsmokers and smokers, children and patients with an acute lung disease, ARDS. This work resulted in the establishment of rapid automated assays for PPE and HLE inhibition as well as an immunochemical assay for α_1 -PI. Improved reference ranges for future population studies were also determined. One of the initial aims at the outset of the project was to confirm the key finding from two reports (Gadek et al., 1979; Janoff et al.; 1979) that α_1 -PI was inactivated in the BAL of smokers compared to nonsmokers, which was suggested to explain why smokers develop emphysema while nonsmokers did not. Earlier findings (Walsh et al., 1992) were confirmed in the present study, in which no difference in functional α_1 -PI activity was demonstrated in the two groups. Other workers (Stone et al., 1983; Boudier et al.; 1983; Stockley and Afford, 1984; Abboud et al., 1985) have reported similar findings. While this is the consensus view in the literature, it may not be the case in all smokers, as only 10-20% of smokers develop clinically evident emphysema. In the present study only 4 nonsmokers and 4 smokers were available for the BAL study, partly because of the difficulty in recruiting normal volunteers for the BAL procedure and meeting ethics approval requirements. Larger groups will need to be studied if significant

differences are to be detected. There is also the problem that small localised differences in α_1 -PI functional ability, such as at the phagocytic-cell alveolar junction, cannot be detected reliably in the lung washings obtained with the present BAL sampling methods. Direct sampling of alveolar lining fluid from specific areas of the lung combined with more sensitive assays may be needed to resolve this issue.

Of all the subjects studied, only patients with ARDS had significant amounts of functionally inactive α_1 -PI present in the serum and BAL, although this was compensated for by the increase in the total amount of α_1 -PI present. These results are consistent with other studies of ARDS. However the inactivation of α_1 -PI that is seen in the ARDS may not be as readily detectable in emphysema due to the lesser degree of inactivation of α_1 -PI that is likely to occur in a chronic disease such as emphysema compared to the much greater degree that is seen in an acute disease such as the ARDS. The detection of inactivated α_1 -PI is also compounded by the fact that the small rates of change of disease in emphysema often occur at a more local level compared to the large rates of change of disease seen in the ARDS, which involves the whole lung. Nevertheless, an understanding of the mechanism of α_1 -PI inactivation that occurs in ARDS is likely to contribute to an understanding of the mechanism and the detection of α_1 -PI inactivation occurring in emphysema.

In this study, α_1 -PI was found to be responsible for approximately 50% of the anti-HLE activity in BAL from normal nonsmokers, smokers and ARDS. This finding suggests the presence of other inhibitors of HLE in the lower respiratory tract and also that other inhibitors of HLE may have a more important role in the lower respiratory tract, in the development of emphysema, than has been previously suggested. In some early studies, α_1 -PI was thought to account for all of the inhibitory activity in the lower respiratory tract whereas later studies have reported more than

80% (Wewers et al., 1987; Wewers, 1989) and 50% (Afford et al., 1988) α_1 -PI inhibition of HLE. The findings in this study are consistent with other recent studies, which have demonstrated the presence of HLE inhibitors other than α_1 -PI in the lower respiratory tract. In this study as much as 50% inhibition of HLE may be due to the presence of HLE inhibitors, other than α_1 -PI. This suggests that an important next step should be an investigation to determine if these other inhibitors have a role in the development of emphysema. However, because of the difficulty in obtaining volunteers and ethics approval for further BAL studies, the investigation of HLE inhibitors in BAL fluid was not carried further. The major thrust of the project was changed to an *in vitro* study of HLE inhibitors and the investigation of the interesting finding that sulphated GAGs are potent inhibitors of HLE.

7.1.2 ELASTASE INHIBITORS

Because of its broad substrate specificity, HLE can bind to and degrade many protein substrates. In this study HLE activity was found to be inhibited not only by the established antiproteases but also, to varying degrees, by other protein and non-protein compounds, such as albumin, elastin peptides, lung surfactant and heparin. Such is the binding affinity of HLE for protein that it was found that even a nonspecific protein such as albumin can inhibit HLE activity if enough is present. This suggests the possibility that HLE activity in the lung may be regulated not only by the specific antiproteases, such as α_1 -PI, but also by non-specific proteins in the alveolar lining fluid, as well as by the inhibitory effect of lung surfactant. These other protein and non-protein compounds may serve to minimise proteolytic damage to structural proteins, such as elastin. Elastin peptides are also effective inhibitors of HLE and may perform a regulatory role in minimising HLE activity at local sites of elastinolysis. The finding that HLE is strongly inhibited by heparin and heparan sulphate is significant as both components are located on cell surfaces

and are components of proteoglycans in cell and basement membranes, where they may play a regulatory role in HLE activity at the alveolar surface. Sulphated GAG's may also have a more direct role in the regulation of HLE activity as sulphated GAG's, such as heparan sulphate, are synthesised and secreted by neutrophils (Bartold et al, 1989). The role of sulphated GAGs, such as heparin and heparan sulphate, in regulating HLE activity is also suggested in animal model studies of emphysema. For example, Lucey et al. (1991) showed that HLE caused considerably less severe lesions in animal lungs than those induced by PPE with comparable *in vitro* elastolytic activity. On the basis that sulphated GAGs inhibit HLE, but not PPE, *in vitro*, an explanation for the difference in elastolytic activity is suggested by the inhibitory effect of sulphated proteoglycans in the lung towards HLE.

Sulphated GAGs were found to be rapid inhibitors of HLE, in that complete inhibition occurred in less than the minimum time (10 seconds) studied. The essentially ionic nature of the binding was demonstrated by the finding that 0.35 M NaCl and above would completely block the inhibition of HLE activity by heparin. The degree of inhibition obtained was dependent on the type of GAG. For example, the more highly sulphated GAGs, such as heparin and heparan sulphate, were the most effective inhibitors. The importance of chain length was shown by using various low molecular weight heparins where preparations with a M_r of 4000-6000 inhibited equally as well as heparin (average M_r 15000) but preparations of M_r <2000 were not as effective. The effectiveness of a particular sulphated GAG as an inhibitor of HLE also varies with the type of substrate used. For example, whereas heparin was the more effective inhibitor of HLE using a soluble chromogenic substrate, heparan sulphate was more effective with insoluble lung elastin as substrate.

In vitro assay systems often have surfactants, usually non-ionic, added to minimise non-specific interactions such as adsorption of reactants to container surfaces. In this study, as reported

elsewhere, HLE was activated significantly by non-ionic surfactants. HLE activation was confounded by the fact that non-ionic surfactants also interfere with the inhibitory effect of some inhibitors of HLE activity. Non-ionic surfactants did not affect protein inhibitors of HLE, with the exception of α_2 -M, where the relatively poor inhibitory activity was reduced by almost half. The most significant effects were found using non-ionic surfactants with sulphated GAGs. The extent of the effect depended on the order of addition of reagents as well as the type of GAG and the substrate used. In contrast, pulmonary surfactant was found not to interfere with HLE inhibitors. The modifying effects of non-ionic surfactants may be significant in interpreting HLE activity data from *in vitro* assay systems using non-ionic surfactants. In particular, the HLE inhibitory capacity of endogenous and exogenous inhibitors should be assessed in the presence of pulmonary and other surfactants to establish that they are fully functional in the presence of surfactants.

The most significant finding from this part of the study was the effectiveness of heparin as an inhibitor of HLE. While heparin did not completely inhibit HLE activity (approx. 25% residual HLE activity remains) it was more effective as a partial inhibitor of HLE than the primary endogenous inhibitor, α_1 -PI, and ICI 200355, a synthetic inhibitor designed specifically to be a potent inhibitor of HLE. This finding lends support to the proposal that heparin as a component of cell surfaces and tissue in the lung may have some regulatory role towards HLE activity. On the basis of the above findings, further studies of heparin inhibition of HLE activity were undertaken to examine whether there was any specificity in the binding of heparin to HLE.

7.1.3 EVIDENCE FOR SPECIFICITY OF INTERACTION OF HEPARIN WITH HLE

Because of its polyanionic nature, heparin has been reported in the literature to bind to a large number of proteins essentially by relatively nonspecific electrostatic interactions. However in this study, evidence is presented for some specificity of binding of heparin to HLE, a finding which has not been reported elsewhere. Significant results from this part of the study are that heparin can be fractionated into low and high affinity binding fractions for HLE. Not surprisingly, the degree of binding of these fractions for HLE correlated with the degree of inhibitory activity of the fractions towards HLE activity. Heparin was fractionated into four major peaks using a salt gradient in the presence of a non-ionic surfactant on a HLE-Sepharose column. A similar elution pattern was obtained using heparan sulphate on the same column. The degree of binding was found to correlate approximately with the average saccharide chain length of the heparin fractions. This finding is consistent with studies of low molecular weight heparin preparations in this thesis and elsewhere where inhibition of heparin activity approximately correlates with the molecular weight of the heparin preparations.

Most studies indicate that increased sulphation of GAGs results in a higher affinity binding for cationic proteins such as HLE. Heparin is one of the most highly sulphated GAGs and one of the strongest polyanions in the body, resulting in strong binding to HLE. The surprising finding from this study was that the degree of binding and inhibition of the isolated heparin fractions towards HLE did not correlate with sulphate content but rather with a relative decrease in sulphate content. This demonstrates that the binding does not depend on maximal sulphate content but rather suggests some specificity in the arrangement of the sulphate groups on heparin such as has been shown for specific binding sites on heparin for certain proteins, such as antithrombin III. This study also demonstrates that binding sites on heparin with a high affinity for HLE appear to be independent of the antithrombin III binding site. It has been suggested that the flexible

backbone of iduronic acid containing GAGs, such as heparin, allows tighter binding of the GAG to other surfaces. On the basis of the results obtained in this study, it is proposed that maximal binding can only theoretically be achieved if the sulphate spacing on the backbone is not too crowded but set at intervals defined by the cationic residues on HLE so as allow maximal binding of the flexible backbone of heparin to HLE. Some evidence for higher affinity binding in undersulphated GAGs is suggested in another study by the finding that there is a preferred binding of relatively low-sulphated heparan sulphate species with thrombin and low-density lipoproteins (Fransson and Johansson, 1981; Fransson and Havsmark, 1982).

Gradient PAGE electrophoresis has revealed the presence of one major and several minor bands of heparin oligosaccharides in the heparin fraction that remains bound to HLE after exhaustive heparinase digestion. The number of bands may reflect the likely multiple binding sites on heparin for HLE. Although the saccharide chain length of the heparin oligosaccharides bands appear to be less than a tetrasaccharide, a pool of these oligosaccharide fractions was able to inhibit HLE, albeit much less than did unfractionated intact heparin. It is likely that the heparinase digested fractions that remain bound to HLE contain specific binding sequences to HLE. A knowledge of these specific sequences should allow the synthesis of other specific oligosaccharide derived inhibitors of HLE and could lead to development of even more effective inhibitors of HLE-mediated inflammatory diseases.

7.2 FUTURE STUDIES

The most significant findings from the studies reported in this thesis are those concerning sulphated GAG inhibition of HLE activity. For example, heparan sulphate appears to be a better inhibitor of HLE using insoluble lung elastin as substrate than is heparin, which is the reverse of

what is found with insoluble substrates used in this study. A more extensive study using sulphated proteoglycans, particularly those containing heparin and heparan sulphate, may give some insight as to whether heparin and heparan sulphate have a regulatory role towards HLE as components of cell and basement membranes at the lung surface.

It was demonstrated that some heparin chains in unfractionated commercial heparin have a higher affinity than others for binding to HLE. This high affinity binding was found to be independent of the relative total sulphate content of the heparin fraction. Heparinase digestion of heparin bound to HLE revealed the presence of heparin oligosaccharides with a high affinity binding for HLE. The isolation of heparin, with high-affinity fractions for HLE, as described in this thesis offers the potential to provide for a new therapy that could be used clinically to supplement the naturally occurring antiproteases in the treatment of HLE-mediated inflammatory diseases such as pulmonary emphysema. If the specific binding saccharide sequences are known it will allow the synthesis of other specific HLE inhibitors incorporating these sequences, which could lead to development of even more effective inhibitors.

Heparin has been used commercially for many years, specifically as an anticoagulant. The profile of its side effects is well documented so that clinical introduction of any heparin derivative should be simpler than for a totally new therapy using a completely synthetic HLE inhibitor. In addition to its inhibition of HLE activity in inflammation, heparin has also been shown to interrupt diverse cellular events in inflammatory disease processes and thus a heparin derivative may have wider applications as a "natural drug" to treat a range of inflammatory diseases. As the antithrombin III site appears to be separate from the HLE binding sequences it is possible that derivatives could be synthesised without anticoagulant activity or to have dual roles, as both anti-inflammatory and antithrombotic agents.

Further development of the heparin fractions prepared by heparinase digestion, as described in this thesis, has not proceeded beyond the preliminary stage because of funding and time constraints. However, I believe that the exciting findings from this project should be developed further. If I were to continue the project, the plan of investigation for future studies would be as follows. It is essential that further refinement of heparin fraction isolation procedures be undertaken to improve recoveries, as well as scaling up the procedure, so that sufficient material can be recovered for detailed characterisation and sequencing. In addition to the isolation of heparin oligosaccharide fractions after exhaustive heparinase digestion, heparin fractions should also be obtained after partial heparinase digestion. A timed study of heparinase digestion of heparin bound to HLE would give much information on lesser affinity binding sites on heparin for HLE. The heparin oligosaccharide fractions obtained after partial heparinase digestion would be of longer saccharide chain length and most likely would be better inhibitors of HLE activity as suggested by low molecular weight heparin studies in this thesis. The sequencing of the series of heparin oligosaccharide fractions obtained from a timed study of heparinase digestion of heparin bound to HLE would, in conjunction with the known sequence of HLE, allow a binding profile of heparin towards HLE to be built up. This timed study should reveal optimum heparin binding sequences for HLE to be obtained allowing the development of more effective derivatives. This stage of the work should include the lodging of patent applications on the isolated and synthesised sequences. On completion of satisfactory *in vitro* studies, an investigation of the effectiveness of the inhibitors in animal model studies of emphysema and other HLE associated inflammatory diseases should be undertaken. If the results from animal model studies are successful, the most promising oligosaccharide preparations could then be considered for evaluation as anti-inflammatory agents in clinical trials. The evaluation of these preparations in preventing the development of emphysema in patients at high risk of developing the disease or in slowing the progression of emphysema in patients with the early stages of the

disease should only commence if all the previous evaluations were successful because of the long trial period required.

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