INVESTIGATION OF THE PEPTIDES PRODUCED FROM HUMAN ELASTIN BY DIGESTION WITH NEUTROPHIL ELASTASE AND WITH CATHEPSIN G

by

SHARON HANNAH BSc.
(HERIOT-WATT)

Thesis presented for the degree
of
Doctor of Philosopy
University of Edinburgh

April 1992

DECLARATION

I declare that this Thesis was composed by myself and that the experimental studies reported are my own. None of the work included in this Thesis has been submitted for any other degree or professional qualification.

ACKNOWLEDGEMENTS

The work presented in this thesis was financially supported by the Faculty of Medicine.

In addition to the people acknowledged in the course of this thesis, I would like to extend my appreciation to Dr. A.P. Ryle for his supervision, and my thanks to Dr. J. Haywood and Dr. S.R. Johnstone for their time and patience when I was using the Gilson LC system. I am indebted to my colleagues, particularly Dr. J-M Sallenave, for a great deal of good advice. My gratitude is extended to Mrs. A. Mason for typing this thesis.

Finally, I would like to thank Myles C.B. Marshall, for teaching me perseverance.

ABSTRACT

Emphysema is a degenerative lung disease which, it is suggested, results probably from repeated periods of proteinase:antiproteinase imbalance during which excess of enzyme attacks the extracellular matrix. Of the many enzymes produced by inflammatory cells human neutrophil elastase (HNE) is thought to be the major offending enzyme. It attacks elastin, which is responsible for the elastic recoil of the lung. If emphysema was simply a result of the destruction of elastin by HNE then degradation products of elastin would inevitably be present, at least transiently, in the serum of patients. The aim of this project was to separate and characterise the soluble peptides resulting from the digestion of elastin with HNE and/or human neutrophil cathepsin G (HNCG), another neutrophilic enzyme, which is primarily bactericidal and, to determine if any of the peptides were characteristic of digestion by one enzyme or combination of enzymes.

HNE and HNCG were isolated from purulent sputum, and elastin was isolated by two methods from post-mortem lungs. The digestion of the elastin by the enzymes was followed by measuring the amino groups liberated during the course of the digestion. A method was developed for the measurement of the insoluble as well as the soluble products of digestion. Initially, the amounts of soluble and insoluble products were similar, but the amount of soluble products soon exceeded the amount of insoluble products. The soluble products of digestion were separated by reverse-phase chromatography. The peptides separated into two groups (A

and B), regardless of which enzyme was involved in the initial digestion. Both groups were heterogeneous mixtures of peptides. Filtration experiments and amino acid analysis showed that the groups of peptides differed in size and composition.

HNE digested elastin to a greater extent than did HNCG. However, the peptide profiles produced when elastin was digested with HNE or with HNCG did not differ obviously; consistent, specific peptides produced exclusively as the result of the action of a particular enzyme could not be identified.

ABBREVIATIONS, CONVENTIONS AND BUFFERS

a) Abbreviations

α_1Achy	alpha-1-antichymotrypsin
α-1-PI	alpha-1-proteinase inhibitor
BCA	bicinchoninic acid
BSA	bovine serum albumin
DCPIP	2,6-dichlorophenolindophenol
DMSO	dimethyl sulphoxide
DN'ase	deoxyribonuclease
EDTA	ethylene diamine tetra acetic acid
HNCG	human neutrophil cathepsin G
HNE	human neutrophil elastase
NAD^+	nicotinamide adenine dinucleotide
PPE	porcine pancreatic elastase
PTC-amino acid	phenylthiocarbamayl-amino acid
STANA	succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide
Suc-Ala-Ala-Pro-Phe-pNA	succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-p-nitroanilide
TFA	trifluoroacetic acid
TNBS	2,4,6-trinitrobenzene sulphonic acid
Tnp-peptide	2,4,6-trinitrophenylated peptide
U	A unit of enzyme activity defined as the activity which produces one µmol of product per minute

b) Conventions- Amino acid nomenclature

The standard three letter abbreviations are used for all the natural amino acids of the proteins and Des and Ide for the cross-links desmosine and isodesmosine respectively throughout the thesis.

c) Buffer compositions

(i) 50mM sodium acetate buffer pH 4.5

Acetic acid was added to sodium acetate such that the final concentrations of the acid and base were 31.75mM and 18.25mM respectively.

(ii) 0.1M sodium phosphate buffer, pH 8.0

A solution (approximately 90ml) containing 0.01moles of disodium hydrogen orthophosphate was adjusted to pH 8.0 at room temperature with HCl. The volume was then made up to 100ml with distilled water so that the solution was 0.1M with respect to phosphate.

(iii) 50mM Tris-HCl, pH 8.0

A solution (approximately 480ml) containing 25mmoles of Tris was adjusted to pH 8.0 at room temperature with HCl. The volume was then made up to 500ml with distilled water giving a solution with a concentration of 50mM with respect to Tris.

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CHAPTER 1

INTRODUCTION

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INTRODUCTION

Chronic obstructive pulmonary diseases (COPD) may be divided into bronchitis and emphysema. Emphysema is defined as "an anatomical alteration of the lung characterised by an abnormal enlargement of the air spaces distal to the terminal, non-respiratory bronchiole, accompanied by destructive changes in the alveolar walls" (Meneely et al, (1962) *Am. Rev. Respir. Dis.* **8 5**, 762-768).

In the early 1960's two major discoveries opened a new avenue of research in the study of the pathogenesis of emphysema. First, Laurell and Eriksson (1963) suggested that some emphysema is a consequence of a genetic deficiency of alpha-1-proteinase (α-1-PI) (further elaboration of this work was presented by Erikkson, 1965). In 1966 Kueppers and Bern showed that α-1-PI inhibits the proteolytic enzymes of neutrophils and Ohlsson (1971) showed that α-1-PI has a high affinity for human neutrophil elastase (HNE). Secondly, Gross et al (1964) demonstrated that emphysematous-like lesions could be produced by instilling papain into the trachea of hamsters. The same type of lesions could be produced in response to an instillation of elastase (Kaplan et al, 1973). Interestingly, Janoff (1985a) stated that papain has been shown to possess elastolytic activity when used in a crude form. These two discoveries were linked

together as the proteinase:antiproteinase theory of emphysema. The theory suggests that a deficiency of active inhibitors leads to an excess of proteinases in the lung. These proteinases may attack the extracellular matrix proteins of the lung and produce emphysema.

However the majority of patients who develop emphysema do not have a genetic deficiency of α-1-PI. The genetic deficiency of α-1-PI is the result of a homozygous recessive inheritance which produces the Pi_{zz} phenotype. Lieberman (1969) and Hutchison et al (1971) found that only 10% and 11% respectively of patients with emphysema were Pi_{zz} individuals, so approximately 90% of patients develop emphysema as a result of conditions other than a genetic deficiency of circulating inhibitor. These patients are usually smokers. The link between cigarette smoke and emphysema gave rise to a second theory for the pathogenesis of emphysema known as the oxidant:antioxidant theory. This theory suggests that excess endogeneous or exogeneous oxidants can oxidatively inactivate the inhibitors of the lung. This produces an increased proteinase burden in the tissue which may lead to tissue destruction. However, the theory does not explain why the majority of cigarette smokers do not develop emphysema. Cohen (1991) summarised the present state of emphysema research by declaring that, "All of these hypotheses remain unproven. We have fallen into somewhat of a trap in that we have performed an enormous number of experiments to show what may happen and very few experiments to show what does happen".

Before any experiments can be designed to investigate a scientific problem, it is useful to consider the results presented by previous

investigations. This introduction will be divided into 4 sections. Each section will examine a particular topic of importance in the study of the destruction of a protein of the extracellular matrix during emphysema. First, the cellular origins of HNE and the role of neutrophils in phagocytosis will be discussed and the circumstances which may cause the cells to release excess enzymes will be considered. Secondly, the classical mechanism of HNE and another neutrophilic enzyme, cathepsin G (HNCG) and the natural inhibitory mechanisms used by the lung to control these enzymes will be discussed. Excess HNE has the potential to digest proteins of the extracellular matrix. Elastin is one component of the extracellular matrix which is known to be digested by HNE (Janoff and Scherer, 1968). So the third section will review the biology of elastin. Finally, the evidence implicating elastin digestion in the pathogenesis of emphysema, and the potential use of degradation products will be discussed. This section will state the aims of the work presented in this Thesis.

1.1 The role of neutrophils in the destruction of the lung

The human body contains many types of cells which protect it against infection. A significant role is played by inflammatory cells such as monocytes, macrophages and polymorphonuclear leukocytes, which are all capable of producing destructive enzymes. One type of polymorphonuclear leukocyte of some importance in defence and tissue turnover is the neutrophil. Neutrophils are granular cells which contain many enzymes including proteolytic enzymes such as neutrophil elastase which is found in the azurophil granules and hydrolytic enzymes such as lysozyme which is

found in the specific granules. They also contain oxidative enzymes such as the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex and myeloperoxidase.

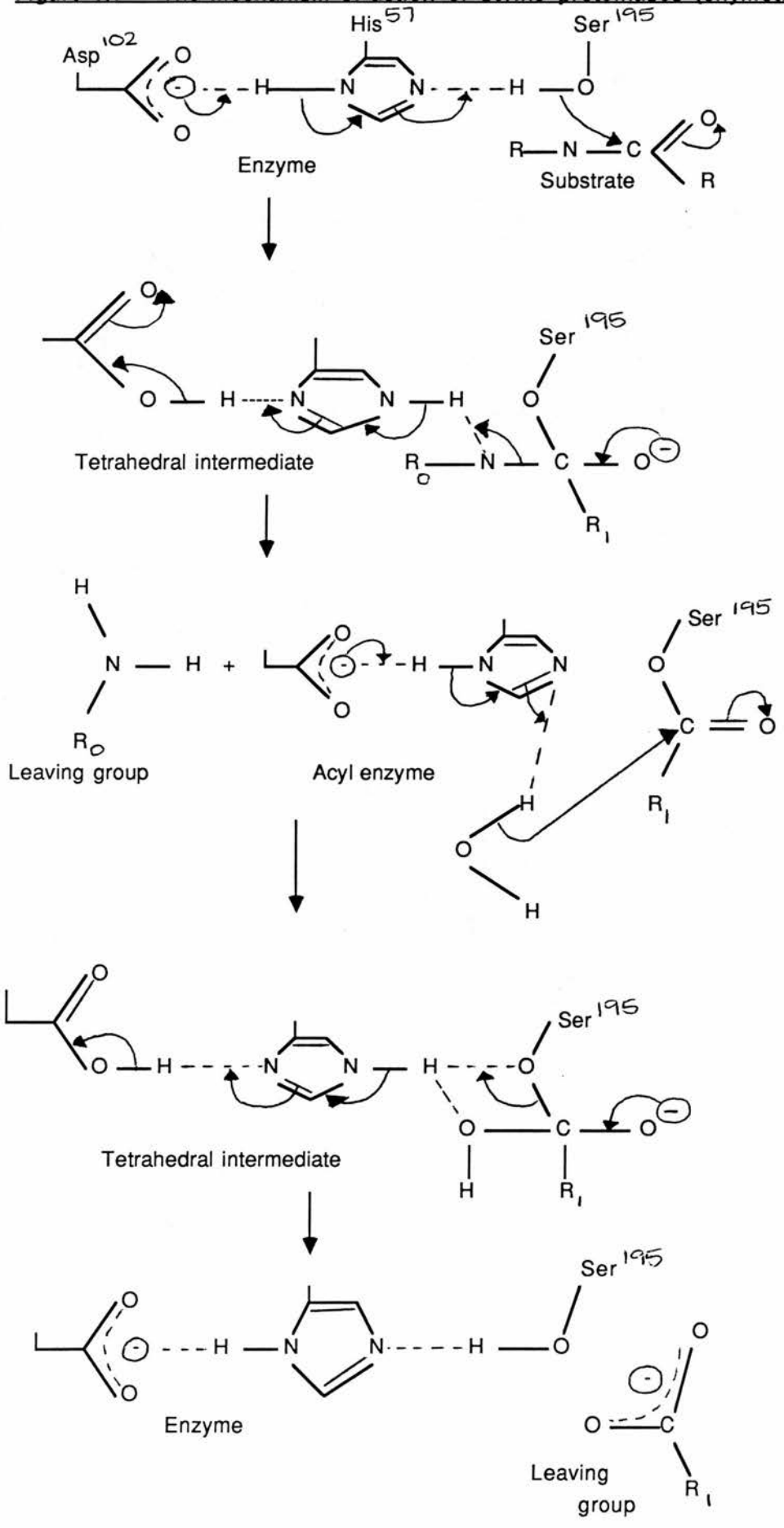
The phagocytic mechanism used by neutrophils to protect the lung during infection have been extensively reviewed by Ryan and Majno (1977) and Haslett and Warren (1990). Briefly, when bacteria are inhaled the complement system becomes activated and complement factors such as C3b and C5a are produced. The invading bacteria become coated with opsonins such as the complement factor C3b and neutrophils are chemotactically attracted to the site of infection in response to the presence of complement factor C5a. The opsonins coating the bacterium induce the contact between the neutrophil and the bacterium and they bind together via the C3b receptors on the neutrophil membrane. After contact the bacterium is ingested into a phagosome in the neutrophil. The azurophil granules merge with the phagosome (Malech and Gallin, 1987), and the enzymes in the granule (HNE, HNCG and proteinase-3) destroy the bacterium. The bactericidal actions of HNCG have been documented by Odeberg and Olsson (1976). The ability of HNE and HNCG to digest the proteins of *E. coli* have been documented by Blondin et al (1978). The oxidants of the neutrophil such as hydrogen peroxide, hypochlorous acid and superoxide radicals are also involved in the destruction of bacteria during phagocytosis (Weiss, 1989). After the bacterium has been destroyed the remaining oxidants are removed by enzymes such as catalase or antioxidant such as glutathione, and the proteolytic enzymes are controlled by inhibitors with which the enzymes form a complex. HNE:α-1-PI complexes may be

removed by macrophages (Campbell et al, 1979). Some leakage of the proteolytic enzymes may occur on the death of the neutrophil (Weissmann et al, 1980), but any leakage can be controlled by inhibitors.

So, the enzymes and oxidants used during phagocytosis to destroy invading pathogens or in tissue turn-over are controlled by inhibitors and antioxidants. However, the phagocytic mechanisms may be activated in response to an irritant. Niewoeher et al. (1974) demonstrated that polymorphonuclear leukocytes accumulate in the airspaces and tissues of the respiratory bronchioles of cigarette smokers, and Janoff (1983) concluded that the products of cigarette smoke can activate polymorphonuclear leukocytes *in vitro* causing them to release oxygen-derived free radicals. The excess of radicals will cause an imbalance between the oxidants and antioxidants which can potentially cause disease. The excess oxidants have the potential to oxidatively inactivate inhibitors (Johnson and Travis, 1978; see Section 1.2) to produce an increase of the proteinase burden of the lung. Similarly, if a patient has a genetic deficiency of an inhibitor it will not be possible to establish a proteinase:antiproteinase balance after phagocytosis, so free active enzyme may remain in the tissue where they could potentially cause the destruction of the extracellular matrix.

There are many enzymes derived from inflammatory cells which could reach excessive levels during a proteinase:antiproteinase imbalance. The enzymes which have been implicated with emphysema are the elastases (reviewed by Janoff, 1985b). Macrophages (Werb and Gordon, 1975) and monocytes (Senior et al, 1982; Campbell et al, 1989) are both capable of secreting elastases, but the enzyme which has attracted most attention as a

Figure 1.1 The mechanism of action of serine proteinases (chymotrypsin numbering)



causative agent in emphysema is HNE.

1.2 Characterisation of human neutrophil elastase and human neutrophil cathepsin G

HNE (EC 3.4.21.37) is located in the azurophil granule of neutrophils at a concentration of 3pg/cell (Ohlsson and Olsson, 1974). The other proteolytic, azurophilic granular contents are HNCG (EC 3.4.21.20) and proteinase-3. The three enzymes are endoproteinases which are classified as serine proteinases along with porcine pancreatic elastase, trypsin and chymotrypsin.

Serine proteinases have a characteristic active site triad, consisting of histidine, serine and aspartic acid, which is located in a crevice between two antiparallel β-barrel cylindrical domains (Bode et al, 1989). The substrate of the enzyme is accommodated in the crevice, and proteolysis proceeds at alkaline pH as shown in Figure 1.1.

Although the enzymes share the same catalytic mechanism and have a great deal of sequence homology they differ in their substrate requirements. Traditionally, the residue at the carbonyl side of the peptide bond cleaved by an enzyme is referred to as the P_1 residue of the substrate and the residue on the amino side of the bond is the P_1' residue (Schechter and Berger, 1967). The different substrate requirements of the various serine proteinases are dictated by subtle differences in the sequence of the active site of the enzyme, which leads to a requirement for substrates with different P_1 residues. For example, the active site pocket of chymotrypsin contains non-polar residues so it binds bulky non-polar residues including

aromatic residues. In the case of trypsin, an aspartic acid residue replaces a serine of chymotrypsin to give a negatively charged active site which attracts basic residues such as lysine and arginine.

Zimmerman and Ashe (1977), Nakajima et al (1979) and Tanaka et al (1985) studied the subsite specificity of HNE and HNCG. Zimmerman and Ashe (1977) measured the k_{cat}/K_m of various substrates which differed at the P_1 position. They concluded that HNE had a preference for valine in the P_1 position, but also cleaved adjacent to alanine and to a lesser extent isoleucine. Nakajima et al (1979) and Tanaka et al (1985) expanded this study and examined the extended substrate requirements of HNE and HNCG. The substrate requirements of proteinase-3, the third serine proteinase of the azurophil granules were studied by Rao et al (1991). The best substrates for the three enzymes were concluded to be:-

			Cleavage site			
			|			
	P_4	P_3	P_2	P_1	P_1'	P_2'
HNE:	Ala	Ala	Pro	Val	Thr	Ala
HNCG:	Ala	Ala	Pro	Phe	Ala	Ala
Proteinase-3:	Ala	Ala	Pro	Val		

It may be concluded that HNE has a requirement for small hydrophobic amino acids in the P_1 position of its substrate. Interestingly, HNE is capable of accommodating alanine-isoleucine-proline-methionine in its binding site and cleaves at the carbonyl side of methionine (Travis and

Salvesen, 1983). This is significant because this tetrapeptide corresponds to the reactive site sequence of α-1-PI (Johnson and Travis, 1978; Nakajima et al, 1979). Proteinase-3 displays a great deal of similarity to HNE. It also requires a small aliphatic amino acid in the P_1 position of its substrate, and is inhibited by α-1-PI. However, it has an absolute requirement for proline in the P_2 position (Rao et al, 1991) and so is unable to cleave succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide (STANA) which is commonly used to measure the activity of HNE.

HNCG has a requirement for bulky or aromatic residues in the P_1 position; it is more inclined to cleave substrates with leucine or methionine in the P_1 position than tryptophan or tyrosine in the P_1 position (Tanaka et al, 1985). The natural inhibitor of HNCG is α-1-antichymotrypsin (α_1 Achy) which has a leucine in the P_1 position of the reactive site sequence so the mechanism of the reaction between α_1Achy and HNCG is probably similar to that between α-1-PI and HNE.

In general, the catalytic action of serine proteinases may be irreversibly inhibited by reacting the active site serine with organic fluorophosphates such as di-isopropylphosphorofluoridate. A more selective inhibition of the serine proteinases may be achieved by using synthetic chloromethylketones which act to alkylate the histidine of the active site triad.

Inhibitors are used to control the activity of enzymes in the body. Serum contains a number of inhibitors of serine proteinases which are

termed serpins. These include α-1-PI which inhibits trypsin, HNE and HNCG and α_1Achy which inhibits chymotrypsin and HNCG.

Usually, 90% of the circulating inhibitory capacity against HNE is provided by α-1-PI; the other 10% of the serum inhibitory capacity against serine proteinases is provided by $\alpha_2$macroglobulin. α-1-PI is a 52kDa glycoprotein which has an active site methionine (Met^{258}) positioned in a reactive site loop near the C-terminus. The inhibitor binds with a 1:1 stoichiometry to the active site of HNE to form an inactive enzyme inhibitor complex which cannot be broken by denaturing agents (Travis and Salvesen, 1983). The inhibitor in the complex does not appear to be digested by the HNE (Carrell and Owen, 1985). Longstaffe and Gaffney (1991) recently presented evidence to suggest that a covalent bond is not formed between the serpin $\alpha_2$antiplasmin and the serine proteinase plasmin during inhibition, so a pseudoirreversible inhibition occurs. The conformations which may be adopted by the complex have recently been discussed by Mast et al (1991). The complex is removed from the circulation by other inflammatory cells (see page 4) via receptor-mediated mechanisms. Native inhibitor and proteolytically cleaved inhibitor are not cleared from the circulation (Mast et al. 1991).

In assigning a role for α-1-PI in the control of proteinases within the lung tissue it is assumed that the inhibitor is capable of moving from the serum to the tissues. So, a second level of control may exist within the tissue itself. This control may be provided by low molecular weight inhibitors

such as mucus proteinase inhibitor (Hochstrasser et al, 1972; Ohlsson and Tegner, 1977) and elastase-specific inhibitor (Hochstrasser et al, 1981; Sallenave and Ryle, 1991). Sallenave et al. (1992) recently showed that elastase-specific inhibitor from sputum has sequence homology with elafin, an inhibitor isolated from the skin of patients with psoriasis, which is an inflammatory condition (Wiedow et al, 1990). So, low molecular weight inhibitors produced in different tissues during inflammation appear to be related. The importance of such low molecular weight inhibitors should not be understated, especially in light of the work presented by Morrison et al, (1990) which showed that mucus proteinase inhibitor, but not α-1-PI, is capable of inhibiting HNE which has been adsorbed onto elastin. However, the k_{ass} of HNE with α-1-PI is 65 $\mu M^{-1}.s^{-1}$ (Beatty et al, 1980) but the values for the k_{ass} of HNE with elastase specific inhibitor and mucus proteinase inhibitor are 16.7$\mu M^{-1}.s^{-1}$ (Sallenave and Ryle, 1991) and 6.4$\mu M^{-1}.s^{-1}$ (Boudier and Bieth, 1989) respectively. So, even though the low molecular weight inhibitors are undoubtably of some importance, α-1-PI is considered to be the major physiological inhibitor of HNE when judged on the basis of these association constants.

Since α-1-PI is the major physiological inhibitor of HNE the presence of insufficient active α-1-PI is the most logical cause of an imbalance between HNE and the antiproteinases of the lung. In a minority of cases the inhibitor may be inactivated during a bacterial infection. For example, *Staphylococcus aureus* has been implicated in the production of

elastinolytic damage to tissue during Staphylococcal pneumonia (Nowak and Miedzobrodski, 1991), possibly as a result of the production by the bacterium of a serine proteinase which is capable of inactivating α-1-PI. However, the generally accepted reason for a deficiency of active α-1-PI is a genetic inability to produce normal α-1-PI. The most characteristic deficiency is the consequence of the inheritance of a homozygous Pi_{zz} phenotype instead of the normal Pi_{mm} phenotype. The α-1-PI synthesised by Pi_{zz} individuals appears to be abnormally glycosylated, and is retained in inclusion bodies within their hepatocytes (Eriksson and Larsson, 1975). As a result of this retention the serum level of α-1-PI in Pi_{zz} subjects is less than 10% of the normal. Although the α-1-PI which does appear in the serum has normal inhibitory activity. The homozygous Pi_{zz} subjects were predisposed to the development of emphysema (Laurell and Eriksson, 1963); but heterozygous Pi_{mz} subjects are no more susceptible to developing emphysema than normal subjects (Eriksson, 1965; Lieberman, 1969; Hutchison et al, 1971).

Patients with this genetic deficiency of α-1-PI will tend to develop emphysema by the age of 50 (Hutchison et al, 1971). In a recent familial study, Apprill et al (1990) concluded that α-1-PI deficiency alone does not automatically condemn the subject to an emphysematous state. The study found that only one of three Pi_{zz} siblings developed severe emphysema and this subject was a heavy smoker; so the risk of developing emphysema may be increased if the Pi_{zz} individual is a smoker.

The majority of emphysematous patients do not have a genetic deficiency of α-1-PI (see page 2). These patients are usually heavy cigarette smokers who develop emphysema in their 8th decade. The link between smoking and emphysema suggested that the proteinase:antiproteinase imbalance is related to an oxidant:antioxidant imbalance in which excessive oxidants oxidise the antiproteinase. This is supported by experiments which showed that the active site methionine of α-1-PI may be chemically oxidised giving a sulphoxide derivative (Johnson and Travis, 1979) which associates 2000 times more slowly with HNE than does normal α-1-PI (Beatty et al, 1980). α-1-PI may be oxidised enzymically by the myeloperoxidase system (Matheson et al, 1982), which has implications *in vivo*. Mucus proteinase inhibitor also has an active site methionine which can potentially be oxidised (Janoff et al, 1980).

The mechanisms surrounding the production of a proteinase:antiproteinase imbalance and the oxidant:antioxidant imbalance are the subject of some debate, but it is universally accepted that uncontrolled proteinase within the lung will have the ability to cause extensive damage to the structural proteins of the extracellular matrix. This damage could be associated with the development of emphysema.

1.3 The destruction of elastin and the extracellular matrix

The extracellular matrix of the lung consists of insoluble structural macromolecules including collagen, elastin, proteoglycans and glycoproteins. HNE is capable of digesting collagen type III (Mainardi et al,1980), fibronectin (McDonald and Kelley, 1980) and elastin (Janoff and Scherer, 1968).

Elastin is the insoluble protein component of elastic fibres found in all tissues which is responsible for the elastic recoil of lungs and the major blood vessels (Rosenbloom, 1987). The elastic fibres in the lung are intimately associated with collagenous fibres. Elastic fibres encircle each alveolar duct and the mouth of the alveoli (Pierce and Ebert, 1965). Damage to the elastic fibres was first associated with emphysema in 1907 when Orsos observed fraying and rupture of elastic fibres in areas of alveolar destruction. Elastic fibres are morphologically arranged as discrete fibres or fenestrated sheets (Cleary and Cliff, 1978). The fibres consist of a microfibrillar component made up of glycoproteins such as the microfibril associated glycoprotein (Gibson et al, 1986) and fibrillin (Sakai et al, 1986), which acts as a scaffolding on which the protein, elastin, is deposited.

Elastin is synthesised as the soluble precursor, tropoelastin by smooth muscle cells (Ross, 1971; Thyberg et al, 1979), fibroblasts (Fahrenbach et al, 1966) and chondroblasts (Quintarelli et al, 1979). Tropoelastin is encoded by a 40 kilobase single copy gene which is located on chromosome 2 (Emmanuel et al, 1985). The DNA encoding tropoelastin has an intron:exon ratio of 15:1 (Indik et al, 1989), and has 34 exons

containing 2,358 translated nucleotides (Rosenbloom et al, 1991). At least 6 of the exons may be alternatively spliced (Indik et al, 1987), which gives rise to 3.5 kilobase primary transcripts which have differing sequences (Indik et al, 1989).

The sequence of human foetal aortic tropoelastin was determined by Indik et al (1987) who examined cDNA clones. Tropoelastin is approximately 70 kDa and consists of approximately 780 amino acids. The hydrophobic and hydrophilic domains of the protein are encoded by different exons (Indik et al, 1987) so the amino acids of tropoelastin are arranged as alternating hydrophobic and hydrophilic domains. Tropoelastin contains a large proportion of small amino acids. Glycine and alanine account for 29% and 22% respectively of the composition of tropoelastin, so the mean residue weight of the amino acids in tropoelastin is only 85.

After translation, molecules of tropoelastin are packaged into vesicles and secreted into the extracellular matrix (Rosenbloom, 1987) where the tropoelastin is deposited onto the microfibril. Recent evidence suggests the that deposition of the tropoelastin may involve a receptor-mediated mechanism. Fibroblasts synthesise a 120kDa receptor complex which is found on the cell surface and has a 67kDa receptor subunit (reviewed by Robert et al, 1989). The subunit has a protein-binding site which recognises the Val-Gly-Val-Ala-Pro-Gly hexapeptide unit of elastin (Wrenn et al, 1988) and a carbohydrate binding site (Hinek et al, 1988). It has been proposed by Mecham (1991) that tropoelastin may bind to the receptor during secretion from the fibroblast via the protein binding site. The receptor eventually binds to the microfibril glycoproteins via the carbohydrate binding

Figure 1.2 The formation of Desmosine

(Adapted from Piez, 1968 and Sandberg et al, 1981)

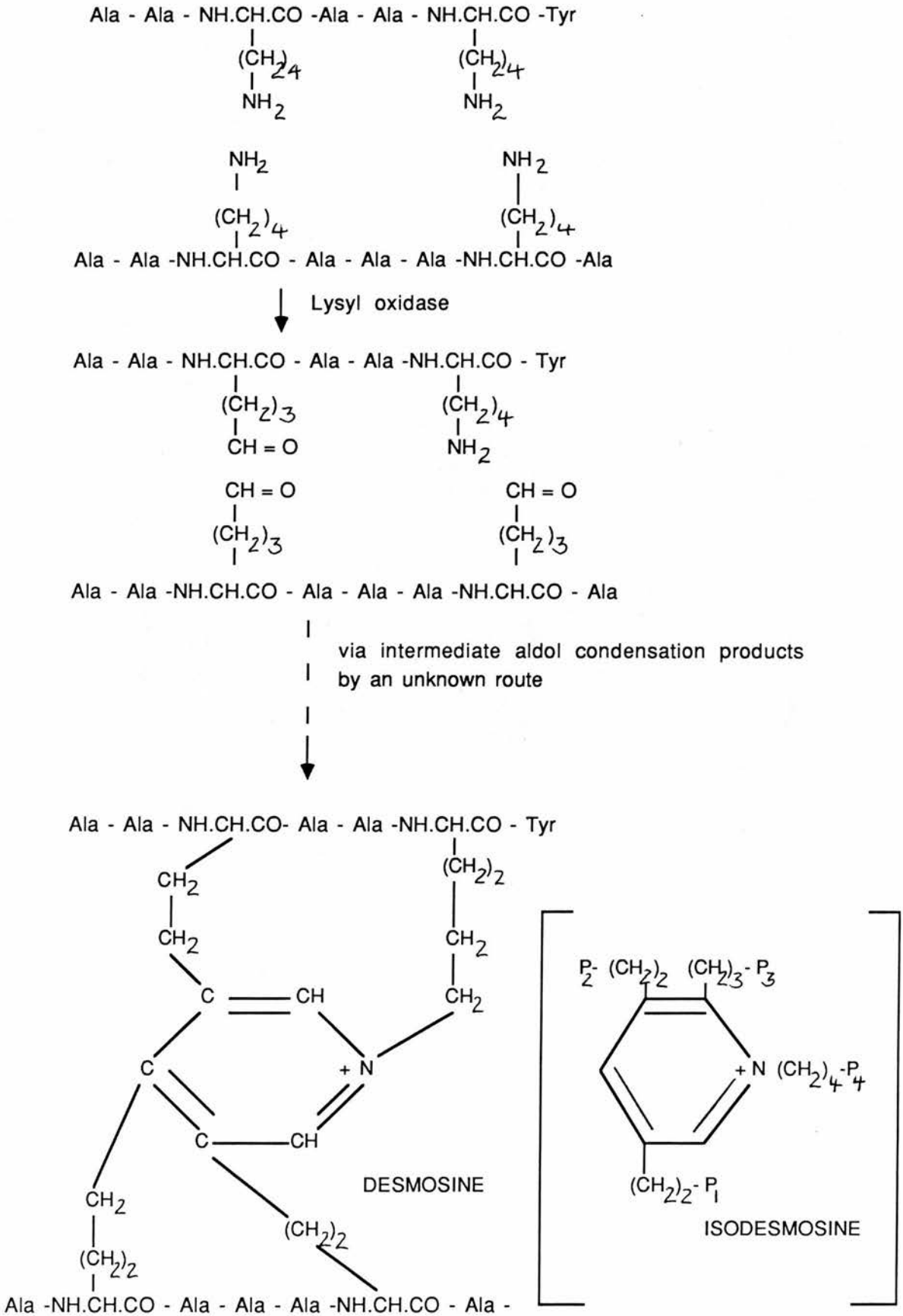
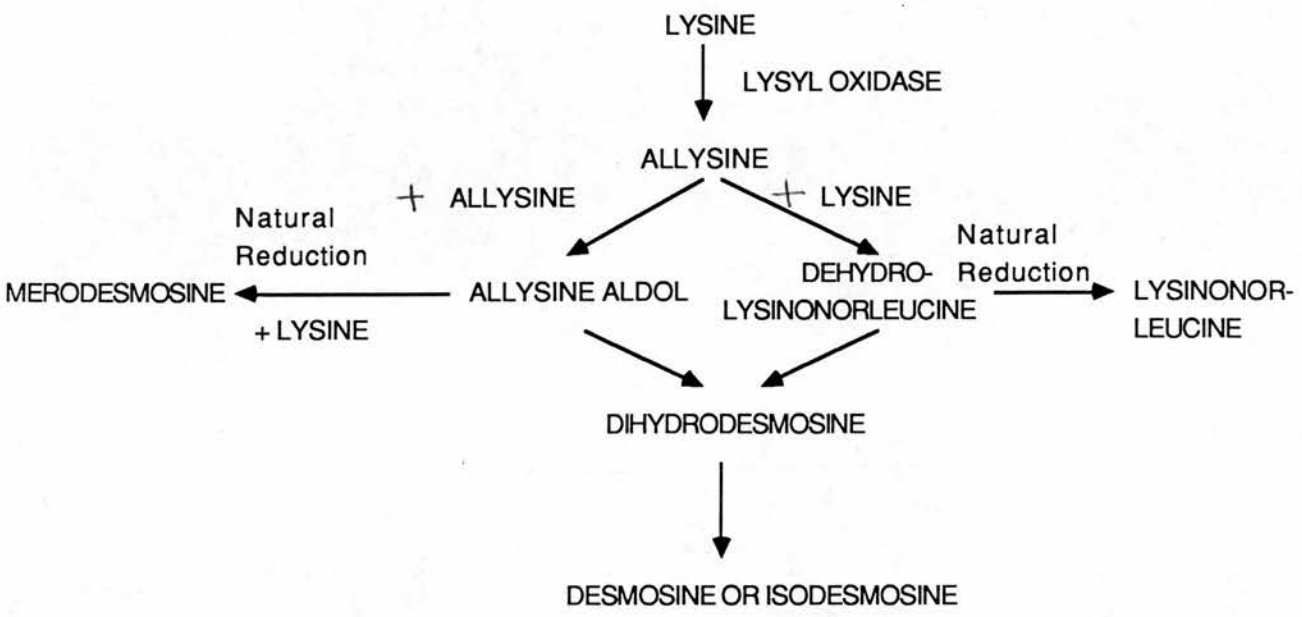


Figure 1.3. The possible routes of spontaneous condensation of the aldol condensation products which lead to cross-link formation



This depicts the various ways in which the cross-links may be formed from residues of allysine (Eyre et al, 1984).

site, which decreases the affinity between the tropoelastin and the protein binding site. So, the tropoelastin is then transferred onto the microfibril. At the same time the receptor is released from its transmembrane anchor. Molecules of tropoelastin are capable of associating together hydrophobically to form coacervates (detailed further in Section 6.3.1); this characteristic may be of particular importance in aligning the tropoelastin onto the microfibril at the end of secretion events (Cox et al, 1974; Narayanan et al, 1978). After secretion into the extracellular matrix and deposition on the microfibril the soluble tropoelastin must be converted to insoluble elastin by the formation of cross-links.

The hydrophilic domains of tropoelastin are rich in polyalanine regions which contain doublets of lysine residues. These lysine residues may be oxidatively deaminated at the 6-amino group to give the corresponding adipic semialdehyde residue (allysine). The oxidation is catalysed by lysyl oxidase (EC 1.4.3.13), a copper-dependent enzyme secreted by fibroblasts (Siegel, 1979). Three modified lysine residues and the 6-amino group of a fourth lysine are capable of condensing together to give tetrafunctional cross-links (Figure 1.2) called desmosine and isodesmosine. Desmosine and the isomer, isodesmosine are unique to elastin, and are primarily responsible for the insoluble nature of elastin. However, the route of cross-link formation is uncertain and the various possibilities may be represented as detailed in Figure 1.3. Eyre et al (1984) stated that only 8-10 of the lysine residues from a molecule of tropoelastin will be accommodated in the desmosine and isodesmosine cross-links, and up to 15 will exist as cross-link intermediates, including the bifunctional

cross-link lysinonorleucine (Franzblau et al, 1969). Richmond (1990) has presented evidence to suggest that elastin may form lysinonorleucine cross-link with the glycoproteins of the microfibril.

The hydrophobic domains of elastin contain a number of small peptide sequences which are repeated several times in succession. These peptide units are stabilised by hydrophobic interactions. The combination of the tetrafunctional cross-links and hydrophobic interactions make elastin extremely insoluble. This insolubility may be exploited when elastin is purified. Mature, cross-linked elastin is more resistant to hydrolysis by sodium hydroxide treatment at 90°C for 45 minutes than collagens which will be dissolved by this treatment (Lansing et al, 1952). Ross and Bornstein (1969) considered that this treatment was harsh and probably caused some damage to the elastin, so they developed a method of isolation which involved removing the contaminating proteins from the elastin with denaturants and enzymes which were incapable of digesting elastin (the purification of elastin is discussed further in Section 3.2).

Although elastin is very stable, it is not immune to attack by enzymes. Elastin is a natural substrate for HNE, porcine pancreatic elastase and to a lesser extent HNCG because it is very rich in valine, proline, glycine and alanine (13%, 13%, 29% and 22% respectively; see Section 1.2). So elastin degradation is a potential consequence of a proteinase:antiproteinase imbalance towards excessive active HNE.

1.4 The evidence implicating elastin destruction by HNE in the pathogenesis of emphysema

A number of pieces of evidence suggested that elastin destruction may occur during the development of emphysema. Animal experiments suggested that elastic tissue was damaged when elastases were instilled into the trachea of the animal (Kaplan et al, 1973). The repaired fibres were shown to be abnormal (Fierer et al, 1976), and emphysema was exacerbated if the animals were rendered deficient in lysyl oxidase (Kuhn and Starcher, 1976). This suggested that proteins which were substrates for lysyl oxidase i.e., elastin and collagens, are damaged in the lung disease. Further evidence implicating elastin destruction in emphysema was the observation that the lungs of typical emphysematous patients displayed a loss of elastic recoil (Karlinsky and Snider, 1978), and Senior et al (1977) observed that treatment with HNE caused a decrease of lung elastin content. However, the only experiments to directly correlate HNE and elastin destruction in emphysema were performed by Damiano et al (1986). They used immunocytochemical techniques to observe the presence of HNE on elastin in areas of lung with emphysema. However, Fox et al (1988) were unable to re-produce these experimental results.

If elastin damage is associated with the pathogenesis of emphysema then it may be suggested that degradative products must appear in the body fluids of emphysematous patients. This suggestion was confirmed by Stone et al (1991), who used reverse-phase chromatography to confirm the presence of elevated levels of desmosine in the urine of hamsters with elastase-induced emphysema, and by a number of immunological

investigations (Harel et al, 1980; Gunja-Smith, 1985; Kucich et al, 1985; Pai et al, 1985; Laurent et al, 1988). The immunological investigations suggested that patients with emphysema had elevated levels of elastin-derived peptides in their urine (Harel et al, 1980) and plasma (Kucich et al, 1985). Kucich et al (1985) took their studies a step further and isolated the urinary elastin-derived peptides from COPD patients and non-smokers. They discovered that 90% of the peptides in the urine of COPD patients were between 4kDa-20kDa, and 10% were approximately 1kDa, and the urine of the non-smokers did not contain any of the smaller peptides.

The antibodies used in the immunological investigations were raised against a mixture of soluble elastin-derived peptides. In general, the antibodies raised against elastin map to two epitopes on the elastin (Mecham and Lange, 1982). The first group of antibodies maps to the alanine-rich cross-link regions, and the second group of antibodies recognises the hydrophobic elastic regions, which are subject to sequence variation. The two groups of antibodies do not cross-react. Kucich et al (1981) presented evidence to suggest that antibodies raised against peptides obtained by oxalic acid digestion of elastin cross-reacted poorly with peptides obtained by elastase digestion.

Since peptides produced from elastin by different digestion methods contain different epitopes it may be suggested that some elastin-derived peptides are specific for a particular type of chemical or enzymic digestion, so the identification of a peptide produced specifically as a result of digestion with HNE rather than with HNCG or elastinolytic enzymes from other cells, such as macrophages, would be useful in confirming that elastin

degradation in emphysema was a result of digestion by HNE.

The aim of this project was to separate and characterise the soluble lung elastin-derived peptides produced by digestion with HNE and/or HNCG; and identify any specific peptide(s) which were consistently liberated by the digestion with only one enzyme or combination of enzymes. The approach taken to achieve this aim may be divided into 4 main areas. First, the enzymes and the elastin were isolated (detailed in Chapter 3). Secondly, a method for measuring soluble peptides was adapted, and a method for measuring total products of digestion regardless of whether or not they were soluble was developed (detailed Chapters 4 and 5). Thirdly the digestion patterns produced when elastin was digested with HNE and/or HNCG were investigated (detailed in Chapters 6,7 and 8). Finally, the strategies taken to separate and characterise the soluble peptides resulting from the digestion of the elastin with the enzymes are detailed in Chapter 9.

In the course of this work two unexpected problems arose which required further investigation. First, the development of a method for measuring total amino groups revealed that the reaction between sulphite and 2,6-dichlorophenolindophenol (DCPIP) had an unexpected stoichiometry (detailed in Chapter 5 and the Appendix). Secondly, the amino termini of the peptides resulting from the digestion of lung elastin with HNE appeared to become resistant to detection after 24 hours of digestion. This is investigated in Chapter 6.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 ISOLATION OF ENZYMES

Human neutrophil elastase (HNE) and human neutrophil cathepsin G (HNCG) were isolated from purulent sputum according to the method of Martodam et al (1979), based on the method of Twumasi and Liener (1977), and detailed further in Section 3.1.2. The sputum was made available by Dr. A. Greening, Western General Hospital, Edinburgh.

2.1.1 Determination of HNE activity against succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide

The substrate (STANA) was purchased from Scientific Marketing Associates.

The activity of HNE was determined by a method similar to the method of Heck et al (1985).

STANA (20mM in DMSO,10µl) was mixed with 0.99ml of 50mM Tris-HCl/1.0M NaCl, pH 8.0, and equilibrated to 37°C. Aliquots of the chromatographic fractions (50µl) or 10µl of pure enzyme solution were added to the assay mixture and incubated at 37°C for exactly 30 minutes in the case of the chromatographic fractions or exactly 10 minutes for the pure enzyme solution; the rate of reaction was constant over these time intervals. The final substrate concentrations in the assay mixtures were 0.190mM and

0.198mM when 50µl or 10µl respectively of sample were assayed. The rate of change of concentration of 4-nitroaniline was determined from the rate of increase of absorbance at 410nm. The molar absorbance of 4-nitroaniline at 410nm was taken as 8800 $l.mol^{-1}.cm^{-1}$. (Nakajima et al, 1979). A unit of enzyme activity was defined as the activity required to produce 1µmol of 4-nitroaniline per minute (as defined by IUPAC/IUB).

2.1.2 Determination of HNCG activity against succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-p-nitroanilide

HNCG activity was determined exactly as the assay of HNE except that 10µl of 0.1M succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-pNA; purchased from Sigma) in DMSO, was used as the substrate. This gave a final substrate concentration of 0.95mM or 0.99mM when the activity of 50µl or 10µl respectively of sample was measured (DelMar et al, 1979; Nakajima et al, 1979).

2.2 ISOLATION OF ELASTIN

Human post-mortem tissue was supplied by Dr. D. Lamb, Department of Pathology, University of Edinburgh. Because of safety regulations regarding the handling of human tissue, it was necessary for the Department of Pathology to fix the lungs and aorta in either ethanol or acetone for 3 days prior to use.

2.2.1 Isolation of lung elastin

After the removal of the trachea and the major bronchi the tissue was treated according to the degradative and non-degradative methods of Reilly and Travis (1980) which were based on the methods of Lansing et al, (1952) and Ross and Bornstein (1969) respectively.

2.2.2 Isolation of aortic elastin

Aortic elastin was isolated according to the method of Paz et al, (1976).

2.2.3 Amino acid analysis

Elastin was hydrolysed at 110°C for 22 hours in 6M HCl under argon. The hydrolysates were dried under vacuum in the presence of sodium hydroxide, then dissolved in 0.025% potassium EDTA solution (according to Applied Biosystems guidelines). Amino acid analysis on the hydrolysate was performed by Mr A. D. Cronshaw of the Wel-Met Protein Characterisation Service, University of Edinburgh on Applied Biosystems equipment, according to the manufacturer's instructions.

2.3 DIGESTION OF ELASTIN BY ENZYMES

2.3.1 Digestion procedure

Elastin suspensions (3mg/ml in 0.1M sodium phosphate buffer/0.15M NaCl, pH 7.4) were treated with HNE and/or HNCG (5, 10 or 15mU/ml of suspension) at 37°C in a shaking water bath, and aliquots were removed

periodically. Treatment with 10mU/ml corresponds to an enzyme to substrate (w/w) ratio of of 1:100 or 1:130 for HNE or HNCG respectively.

2.3.2 Determination of peptide production using assays for protein

Soluble peptides were isolated from the insoluble material by centrifugation for 10 minutes in an MSE microfuge. The quantity of soluble peptides was measured as protein, by three methods.

a) Bradford method

Bradford reagent was prepared according to the method of Bradford (1976). Reagent (2.5ml) was added to 0.5ml of supernatant, and the absorbance at 595nm was measured against a reagent blank within an hour. The quantity of peptides was estimated by comparing the absorbance to a standard curve prepared with bovine serum albumin (BSA, purchased from Sigma).

b) Bicinchoninic acid (BCA) method

The BCA method (Pierce) is based on the method of Smith et al (1985).

Duplicate digestion supernatants (10µl) were pipetted into a 96-well microtitre plate (Falcon) with a capacity of 300µl per well. Reagent (200µl), prepared according to the manufacturer's recommendations, was added to each well. The plate was incubated at room temperature for 2 hours, after which the absorbance at 570nm was measured in a Dynatech Microplate

Reader (Model MR600). The quantity of peptides was estimated by comparison to a standard curve of BSA, samples of which were included on each plate.

c) Biuret method

Duplicate digestion supernatants (50µl) were treated with 200µl of biuret reagent, prepared in accordance with the method of Gornall et al (1949), in a 96-well microtitre plate (Falcon). After incubation at room temperature for 30 minutes the absorbance at 570nm was measured in a Dynatech Microplate Reader (Model MR600). The amount of peptides was estimated by comparison to a standard curve of BSA, samples of which were included on each plate.

2.3.3 Determination of soluble peptides

2,4,6-Trinitrobenzene sulphonic acid (TNBS), otherwise known as picrylsulphonic acid, was purchased from Sigma.

The digestion sample was centrifuged in an MSE microfuge for 5 minutes at high speed. Amino groups in the supernatant were measured by a method based on the procedure of Satake et al (1960) as modified in Sigma protocol P2297.

Triplicate aliquots of the supernatant (30µl-200µl) were made to a volume of 0.5ml and added to 0.5ml of 0.1M sodium phosphate buffer, pH 8.0. 0.1% TNBS (0.5ml) was added to the solutions, which were mixed and incubated at 37°C for 2 hours. After incubation the absorbance at 420nm

was measured in a Pye Unicam SP6-550 uv/vis spectrophotometer against a reagent blank. The quantity of free amino groups produced per milligram of elastin was calculated by relating the above absorbance to a standard curve prepared with glycine (0-0.25μmol).

2.3.4 Development of a method for the determination of total peptides

A method for the determination of total peptides was developed and is detailed in Chapter 5. Triplicate aliquots (30μl-200μl) of the digestion suspension were made up to a volume of 0.5ml and added to 0.5ml of 0.1M sodium phosphate buffer, pH 8.0; 1ml of 0.2mM 2,6-dichlorophenolindophenol (DCPIP, purchased from BDH) in 0.05M phosphate buffer, pH 8.0 containing 0.05% TNBS was added, and the solutions were incubated at 37°C for 2 hours. After incubation, the absorbance of DCPIP was measured at 605nm against water. The quantity of free amino groups per milligram of elastin was calculated by relating the loss of absorbance at 605nm, as compared to a reagent blank (absorbance at 605nm approximately 1.75), to a standard curve of amount of glycine (0-0.25μmol) against decrease of absorbance of DCPIP at 605nm.

2.3.5 Determination of insoluble peptides

The quantity of free amino groups per milligram of elastin due to insoluble peptides was calculated as the difference between the increase in the amount of total peptides equivalent to glycine per milligram of elastin

and the increase in the amount of soluble peptides equivalent to glycine per milligram of elastin.

2.4 CHROMATOGRAPHIC SEPARATION OF THE SOLUBLE PRODUCTS OF DIGESTION

2.4.1 Molecular exclusion chromatography

Molecular exclusion chromatography was performed using a 7.8mm x 30cm Anagel-TSK G3000 SWXL column (Anachem) or a 25ml Superose-12 column (Pharmacia) in conjunction with a Gilson liquid chromatography system.

Soluble peptides (0.2ml), separated from the insoluble material by high speed centrifugation in an MSE microfuge for 10 minutes, were fractionated using 50mM sodium phosphate buffer/0.15M NaCl pH 7.4 at a flow rate of 1ml/min for 40 minutes (pressure not exceeding 4 MPa). The absorbance of the eluate was monitored at either 220nm or 270nm and fractions (1 minute, 1ml) were collected.

2.4.2 Reverse-phase chromatography using a PepRPC column

Reverse-phase chromatography was performed using a C_2/C_{18} PepRPC HR 5/5 column (0.5cm x 5cm; Pharmacia) in conjunction with a Gilson liquid chromatography system, at a pressure not exceeding 4 MPa.

Digestion samples (200µg-400µg as determined by BCA assay) were collected after 24 or 48 hours of digestion and concentrated or diluted to

0.5ml if necessary. The samples were centrifuged for 10 minutes at high speed in an MSE microfuge, and the supernatant was loaded onto the column which had been equilibrated with 2% aqueous acetonitrile (Far UV HPLC grade, FSA)/0.1% trifluoroacetic acid (TFA, HPLC grade, FSA). All solvents were filtered through 0.45µm nitrocellulose membranes (47mm diameter; Whatman) and degassed by sonication (20 minutes in a sonic bath) prior to use. The peptides were separated using a gradient of 2%-100% acetonitrile/0.1% TFA at a flow rate of 0.5ml/min (initial pressure of 1.5MPa) over 220 minutes. The absorbance of the eluate was monitored at 220nm, unless otherwise stated, and fractions (2 minutes, 1ml) were collected from 1 minute after injection, in a 96-well fraction collection rack (Beckman). The path length of the flow cell was 1cm.

2.4.3 Reverse-phase chromatography using an Aquapore RP-300 column

Single peptide peaks eluted from the PepRPC HR 5/5 column between 25%-30% acetonitrile/0.1% TFA were concentrated by evaporation at 37°C to approximately 50µl in a Gyrovap centrifugal evaporator (Howe). The concentrated samples were diluted to 500µl with 1% TFA, and further purified by Mr S. D. Peacock of the Wel-Met Protein Characterisation Service. Briefly, aliquots (100µl - 200µl) were loaded onto a C_{18} Aquapore RP-300 column (30 x 2.1mm, Brownlee, ABI) using a 130A Separation System (ABI).

The peptides were eluted from the column with a gradient of 0%-70%

acetonitrile/0.1% - 0.08% TFA over 45 minutes at a flow rate of 200µl/min (initial pressure 2.0MPa). The absorbance of the eluate was monitored at 220nm and recorded at a sensitivity of 0.1 absorbance units full-scale. Individual peaks were collected manually in Eppendorf tubes, when the absorbance shown on the chart recorder increased above 0.03 absorbance units.

Aliquots (100µl-300µl) of the peptide peaks were concentrated by evaporation to approximately 50µl then hydrolysed as detailed in Section 2.2.3.

CHAPTERS 3-9

RESULTS

CHAPTER 3

ISOLATION OF THE ENZYMES AND PREPARATION OF THE SUBSTRATE

CHAPTER 3

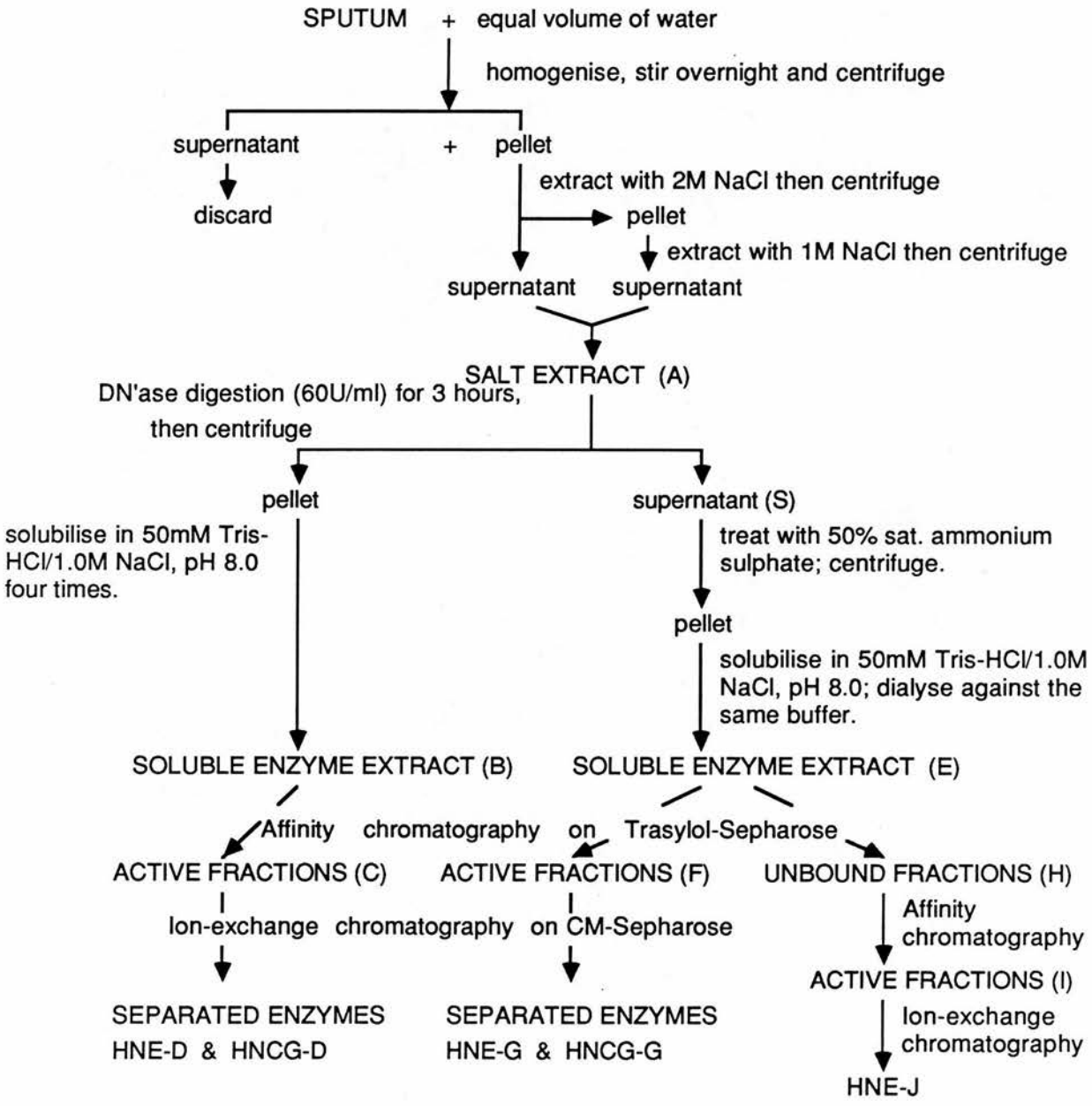
ISOLATION OF THE ENZYMES AND PREPARATION OF THE SUBSTRATE

3.1 ISOLATION OF HNE AND HNCG

3.1.1 Source of the enzymes

HNE and HNCG may be isolated from three sources of granulocytic cells; whole blood (Baugh & Travis, 1976); neutrophils obtained by leukophoresis of normal donors or myeloid leukaemia patients (Heck et al, 1985); or purulent sputum (Twumasi & Liener, 1977). The advantages of using sputum are that it is readily available in large quantities from hospitalised bronchitics whereas leukocytes, particularly from myeloid leukaemia patients, are difficult to obtain; secondly, purulent sputum provides higher yields of enzymes (w/w) as compared to the yields from whole blood (w/v) (Twumasi & Liener, 1977). However, it should be noted that the purulence of the sputum is an important factor since clear mucoid sputum does not contain significant amounts of elastase (Stockley et al, 1984). This is presumably because elastase is produced by inflammatory cells and the purulence is a feature of extreme inflammation of the airways (Ryan & Majno, 1977). Since the degree of purulence between different sputa will vary, it follows that the yield of enzymes will vary accordingly so there is little point in comparing the yields from different pools of sputa.

Figure 3.1 Purification scheme of the isolation of the enzymes from sputum



See text for experimental details. In the interests of simplicity within the text, the enzymes isolated from the DN'ase digestion pellet will be referred to as HNE-D and HNCG-D; the enzymes isolated from the subsequent ammonium sulphate precipitation of the salt extract will be referred to as HNE-G and HNCG-G; and the HNE isolated from the unbound material will be referred to as HNE-J.

3.1.2 Isolation of the enzymes

The enzymes were isolated on four occasions from pooled sputa provided by bronchitic patients (0.5l-1.5l, depending upon availability) by a method based on the procedures detailed by Twumasi and Liener (1977) and Martodam et al (1979) (Figure 3.1).

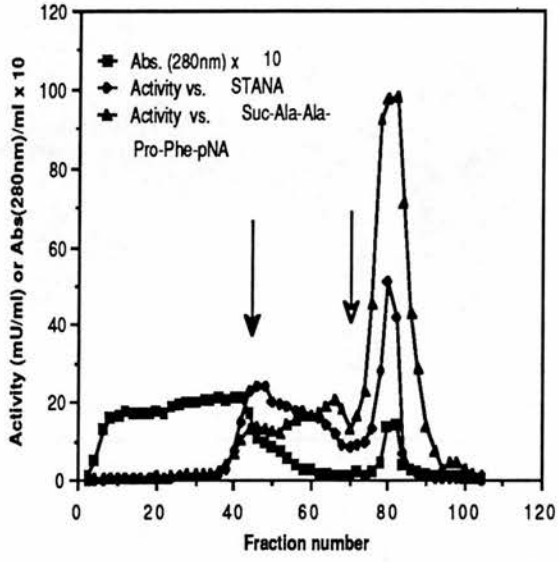
(a) Preparation of the salt extract

Pooled sputa were homogenised with an equal volume of distilled water in a Waring blender and stirred overnight at 4°C. The solution was centrifuged at 30,000g for 20 minutes at 4°C in a J2-21 centrifuge (Beckman) with a JA-14 rotor. The supernatant was discarded. The enzymes were solubilised from the pellet by suspending and homogenising the pellet in an equal volume of 2M NaCl. The pellet suspension was centrifuged as before, producing a supernatant which was retained, and a pellet which was homogenised in 1M NaCl, stirred at 4°C overnight and centrifuged. The supernatant was added to the 2M NaCl treatment supernatant, and the pellet was discarded. The pooled supernatants were dialysed against 50mM Tris-HCl/0.035M $MgCl_2$, pH7.4 for 24 hours at 4°C to give the salt extract (A).

(b) Deoxyribonuclease digestion of the salt extract

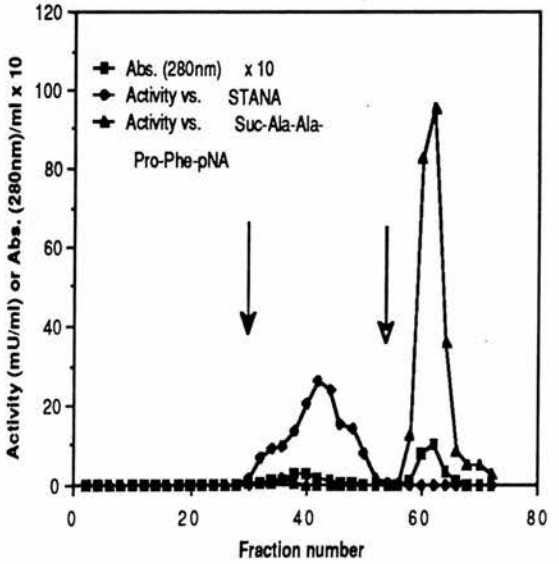
The salt extract (A) was treated with deoxyribonuclease (DN'ase, Sigma, 60U/ml) at 25°C for 3 hours; the nuclease digested DNA to release the enzyme, as a precipitate, from nucleoprotein complexes (Lieberman &

Figure 3.2a Affinity chromatography on Trasylol-Sepharose of the solubilised pellet resulting from DN'ase digestion of the salt extract



Fraction B (400ml) was applied to a Trasylol-Sepharose column (2.5cm x 10cm, void volume 50ml) equilibrated with 50mM Tris-HCl/1.0M NaCl, pH8.0. After washing (↓) with the same buffer the eluent was changed (↓) to 50mM sodium acetate buffer/1.0M NaCl, pH4.5. Fractions (10ml) were collected.

Figure 3.2b Ion-exchange chromatography of the active material from Trasylol-Sepharose (Figure 3.2a)



Fraction C (230ml) was applied to a CM-Sepharose column (2cm x 25cm, void volume 80ml) equilibrated with 50mM sodium acetate buffer/0.1M NaCl, pH4.5. After washing with the same buffer the ionic strength was increased to 0.45M NaCl (↓) to elute HNE, and then to 1.0M NaCl (↓) to elute HNCG. Fractions (10ml) were collected.

Kurnick, 1962). The precipitated enzyme was collected by centrifugation (30,000g for 20 minutes) as a pellet. The enzymes in the pellet were solubilised by three extractions with 50mM Tris-HCl/1.0M NaCl, pH 8.0. The three supernatants were pooled and the remaining pellet was suspended in 50mM Tris-HCl/1.0M NaCl, pH 8.0 and sonicated at room temperature for 20 minutes in a sonic bath (Kerry). The supernatant obtained by centrifugation, was added to the supernatants from the 3 previous extractions to give a pooled enzyme solution (B), and the pellet was discarded.

(c) Separation of the enzymes from other proteins in the pooled enzyme solution (B) by affinity chromatography

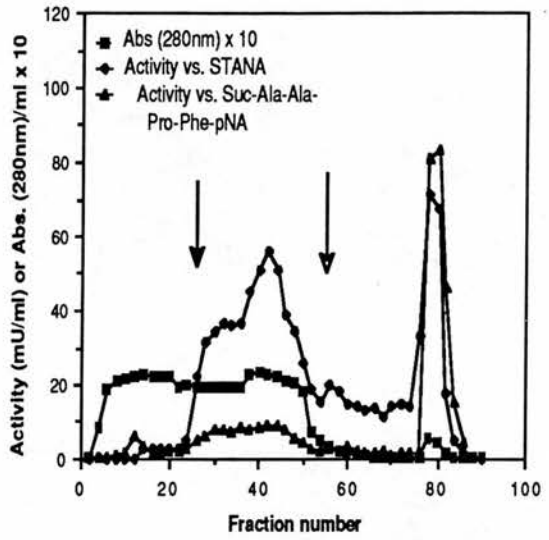
The pooled enzyme solution (B) was applied to a Trasylol-Sepharose column (2.5cm x 10cm, prepared by Mrs. S. Libor) equilibrated with 50mM Tris-HCl/1.0M NaCl, pH 8.0. Weakly bound material was washed off the column with the Tris buffer and when the absorbance at 280nm of the eluate had decreased to 0.03, the serine proteinases were eluted from the column by decreasing the pH with 50mM sodium acetate buffer/1.0M NaCl, pH 4.5 (Figure 3.2a).

Enzymically active material was pooled (C) and dialysed against 50mM sodium acetate buffer/0.1M NaCl, pH 4.5 for 24 hours at 4°C.

(d) Separation of the two enzymes by ion-exchange chromatography

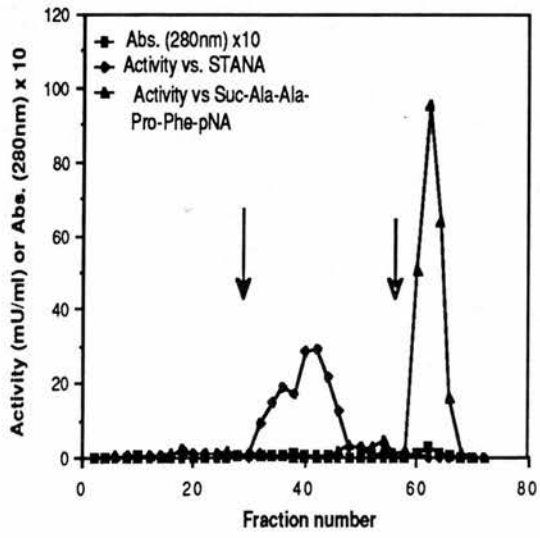
Dialysed enzymes were applied to a CM-Sepharose column (2cm x 25cm) equilibrated with 50mM sodium acetate buffer/0.1M NaCl, pH 4.5.

Figure 3.3a Affinity chromatography on Trasylol-Sepharose of the solubilised ammonium sulphate precipitate obtained from the salt extract (E)



Fraction E (400ml) was applied to a Trasylol-Sepharose column (2.5cm x 10cm, void volume 50ml) equilibrated with 50mM Tris-HCl/1.0M NaCl, pH8.0. Unbound fractions (↓ to ↓) were collected; after which the eluant was changed to 50mM sodium acetate buffer/1.0M NaCl, pH4.5 and the bound fractions (10ml each) were collected.

Figure 3.3b Ion-exchange chromatography of the eluant from Trasylol-Sepharose (figure 3.3a)



Fraction F (230ml) was applied to a CM-Sepharose column (2cm x 25cm, void volume 80ml) equilibrated with 50mM sodium acetate buffer/0.1M NaCl, pH4.5. After washing with the same buffer the ionic strength was increased to 0.45M NaCl (↓) to elute HNE, and then to 1.0M NaCl (↓) to elute HNCG. Fractions (10ml) were collected.

The enzymes (D) were eluted from the ion-exchange column by stepwise increases of the ionic strength of the sodium acetate buffer; HNE was eluted with 50mM sodium acetate buffer/0.45M NaCl, pH 4.5 and HNCG was eluted with 50mM sodium acetate buffer/1.0M NaCl, pH 4.5 (Figure 3.2b), after which they were concentrated by ultrafiltration, dialysed against 1mM acetic acid overnight and freeze dried.

(e) Ammonium sulphate treatment of the remaining salt extract supernatant

The salt extract supernatant (S) remaining after the removal of the enzyme pellet, resulting from DN'ase digestion, still contained considerable activity against STANA and Suc-Ala-Ala-Pro-Phe-pNA. Attempts were made to isolate further enzyme from the salt extract supernatant by 50% saturation with ammonium sulphate. The resultant precipitate was collected by centrifugation and the supernatant was discarded. The pellet was solubilised in 50mM Tris-HCl/1.0M NaCl, pH 8.0, then dialysed overnight against the Tris buffer at 4°C to remove the ammonium sulphate. The dialysed enzyme solution (E) was applied to the Trasylol-Sepharose column, equilibrated with 50mM Tris-HCl/1.0M NaCl, pH 8.0. Some of the weakly bound material was active against STANA and was collected (H) separately from the active material which bound to Trasylol-Sepharose and was eluted (F) as previously detailed (Figure 3.3a). The serine proteinases were then separated from each other (G) by ion-exchange chromatography (Figure 3.3b).

Table 3.1 Purification of HNE and HNCG

Purification product	Total protein (A[280]xml)	Total activity (Units)	Specific activity (U/A[280]xml)	Recovery (%) of step	Recovery (%) overall	Overall purification
A. Salt extract	**35000** *35000*	**76.1** *43.3*	**0.0022** *0.0012*		**100** *100*	**1** *1*
B. Soluble extract of the DN'ase pellet	**1316** *1316*	**18.4** *22.0*	**0.014** *0.017*	**24** *51*	**24** *51*	**6.5** *14*
C. Fractions 72-105 from Trasylol-Sepharose (fig 3.2a)	**95** *95*	**3.4** *8.4*	**0.036** *0.088*	**18.5** *38.0*	**4.5** *19.0*	**16.6** *71*
D. Fractions 29-52 (HNE) and 56-72 (HNCG) from CM-Sepharose (fig 3.2b)	**21** *21*	**3.1** *4.9*	**0.148** *0.231*	**91.2** *58.1*	**4** *11*	**68.2** *272*
E. Soluble extract of the ammonium sulphate precipitate	**3056** *3056*	**16.8** *7.6*	**0.0055** *0.0025*	**22** *17*	**22** *17*	**2.5** *2*
F. Fractions 76-88 from Trasylol-Sepharose (fig 3.3a)	**36.1** *36.1*	**3.9** *4.7*	**0.109** *0.130*	**23** *65*	**5** *11*	**50.2** *105*
G. Fractions 30-50 (HNE) and 59-69 (HNCG) from CM-Sepharose (fig 3.3b)	**9.0** *10.5*	**3.2** *4.5*	**0.354** *0.434*	**80** *91*	**4** *10*	**163** *350*
H. Unbound fractions from Trasylol-Sepharose (21-60) (fig 3.3a)	**640** *640*	**11.9** *2.3*	**0.019** *0.004*		**15.6** *5.3*	**8.6** *3.3*
I. Fractions bound to Trasylol-Sepharose on 2nd passage	**22.2** *22.2*	**5.8** *0.04*	**0.262** *0.002*	**49** *2*	**7.6** *0.1*	**119** *1.7*
J. HNE active fractions from CM-Sepharose	**14.4** *Negligible*	**5.6**	**0.385**	**95**	**7.5**	**220**

The total recovery of pure enzyme from 1.5l of pooled purulent sputa, expressed as the sum of products D, G and J, were 16% and 21% for HNE (bold figures) and HNCG (italic figures) respectively.

(f) Isolation of HNE from the unbound material (H)

The activity against STANA demonstrated by the unbound material (H) may simply indicate the presence of an enzyme other than HNE which is capable of hydrolysing STANA but is incapable of binding to Trasylol-Sepharose, for example, the metalloproteinase monocyte-derived macrophage elastase. However, Baugh and Travis (1976) noted that HNCG binds more tightly than HNE to Trasylol-Sepharose. Therefore it is possible that the unbound material contained HNE which was excluded from the Trasylol-Sepharose column because HNCG had occupied the majority of the binding sites. The unbound material (H) was re-applied to the Trasylol-Sepharose column. Any material which still did not bind was discarded and assumed to contain enzyme(s) other than HNE. The bound material (I) was eluted with 50mM sodium acetate buffer/1.0M NaCl, pH 4.5 and then separated (J) by ion-exchange chromatography, as previously detailed.

An example of the resultant purification scheme, when the enzymes were isolated on a single occasion from 1.5l of pooled sputa, is shown in Table 3.1. The enzymes were isolated on four occasions; comparison of the results led to a number of general conclusions.

1. The recovery of HNCG after DN'ase digestion of the salt extract and the subsequent solubilisation of the enzyme pellet is twice that of HNE; but is only 50%.

2. After DN'ase treatment and the isolation of the resulting enzyme pellet, enzymic activity is still detected in the remaining salt extract

Table 3.2 The cross-contamination of each preparation of pure enzyme with the second enzyme

a. Contamination of HNE with HNCG

Enzyme preparation	Specific activity against Suc-Ala-Ala-Pro-Phe -pNA U/[A(280nm).ml]	Specific activity as % of the specific activity of the purest HNCG 0.434U/[A(280nm).ml]
HNE-D	0.00008	0.02
HNE-G	0.0022	0.46
HNE-J	0.0066	1.52

b. Contamination of HNCG with HNE

Enzyme preparation	Specific activity against STANA U/[A(280nm).ml]	Specific activity as % of the specific activity of the purest HNE 0.385U/[A(280nm).ml]
HNCG-D	0.0067	1.74
HNCG-G	0.0133	3.45

The cross-contamination between the two enzymes, when the specific activity of the contaminating enzyme was expressed as a percentage of the specific activity of the purest sample of that enzyme, was minimal and did not exceed 4%.

supernatant. Some of this residual activity can be precipitated by 50% saturation with ammonium sulphate. The inclusion of this step was of particular use in recovering further HNE.

3. Trasylol-Sepharose affinity chromatography produced low recoveries of HNE (18%-25% of the activity loaded onto the column was recovered). The recovery of HNCG was significantly higher (38%-65%).

4. When material which was active against STANA, but appeared incapable of binding to Trasylol-Sepharose, was applied to the affinity column on a second occasion, some of the material was capable of binding to the adsorbent. This may be a consequence of preferential binding of HNCG during the first passage through the column together with an observed decline in column capacity with time.

5. The specific activities of the enzymes isolated from the pellet produced by DN'ase digestion of the salt extract were lower than the specific activities of the enzymes isolated from the subsequent ammonium sulphate precipitate.

6. The cross-contamination between the enzymes (Table 3.2) was minimal.

The use of DN'ase digestion of the salt extract was introduced by Lieberman and Kurnick (1962) who suggested it was necessary for the

release of chymotrypsin-like proteinase (HNCG) from nucleoprotein complexes. In practice this procedure led to the recovery of a greater proportion of the HNCG activity than the HNE activity as an enzyme precipitate. A proportion of the remaining activity could be isolated from the salt extract supernatant by precipitation with ammonium sulphate. However, such precipitation will not specifically precipitate HNE and HNCG; but they may be isolated from any contaminating proteins by affinity chromatography on Trasylol-Sepharose, which binds serine proteinases. When the eluate from Trasylol-Sepharose was further separated by CM-Sepharose, all the protein eluted in the enzymically active peaks, as seen in Figure 3.2b, confirming that only enzymically active proteins were bound by Trasylol-Sepharose.

Although the enzymes eluted from Trasylol-Sepharose had increased specific activities, the recoveries, particularly of HNE, were poor. A possible explanation was that the capacity of the column diminishes with use as a result of irreversible binding of enzyme or of enzymic degradation of the Trasylol. Alternatively the capacity of the column may decrease as a result of a non-specific effect, such as the irreversible occupation of the Sepharose beads with any mucus or acid-polysaccharides co-precipitating with the enzymes from the salt extract. In practice it was found that a column which bound 95mg of enzyme at first use was subsequently only capable of binding 36mg of enzymes. Unbound material was usually active against STANA, probably because HNCG preferentially binds to the ever-decreasing available sites on the column causing the exclusion of HNE. So, unbound material was re-loaded onto the Trasylol-Sepharose column in an

Table 3.3 Comparative analysis of the preparations of HNE and HNCG with published data

Enzyme	Preparations						Published data	
	D	G	J	D	G	J	Heck (a)	T & L (b)
	U/A(280nm/ml)			U/mg(c)			U/mg	U/mg
HNE	0.148	0.354	0.385	0.150	0.360	0.390	0.180	0.382
HNCG	0.331	0.434	_	0.348	0.654	_	_	_

a) Activity as quoted by Heck et al (1985)

b) Calculated from the activity (0.645U/mg) quoted by Twumasi and Liener (1977) as described in the text.

c) Protein mass estimated by the method of Bradford (1976).

attempt to isolate further HNE.

However, even when an extra affinity chromatography step and an ammonium sulphate precipitation were included in the purification scheme, the final recoveries of HNE and HNCG activities were only 16% and 21% respectively. These values compare well with the recoveries of 11% and 16% of HNE and HNCG activities from the salt extract when using leukocytes as the initial material (Heck et al, 1985); but Twumasi and Liener (1977) report recoveries from the salt extract of sputum, donated by cystic fibrosis patients, of 54% and 63% for HNE and HNCG activities respectively. This sputum has been shown by Goldstein and Doring (1986) to be rich in HNE and HNCG activities, so cystic fibrosis sputa provide a greater initial concentration of enzymes and may be a preferable source.

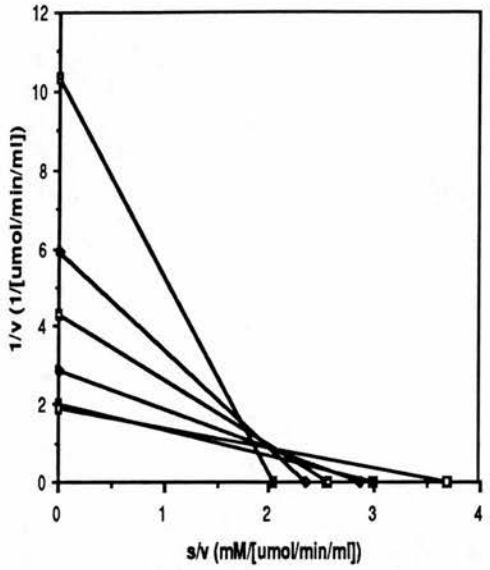
In general, the quantity of enzymes isolated from sputum is variable; and a factor of greater importance which should be considered is the quality of the enzymes.

3.1.3 Comparison of the enzyme preparations to those of Heck et al (1985) and Twumasi and Liener (1977)

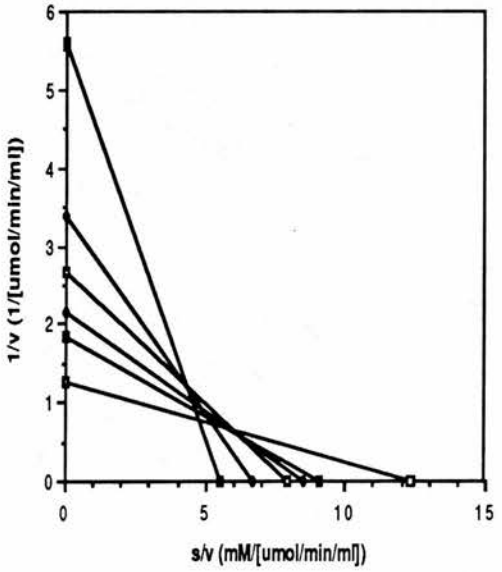
The comparative results presented in Table 3.3 show that the final preparations of HNE eluted from CM-Sepharose had specific activities between 0.15-0.39U/mg. HNE-D had the lowest specific activity; but compared well with the specific activity calculated by Heck et al (1985) who obtained a preparation with a specific activity of 0.103U/mg after ion-exchange chromatography, rising to 0.180U/mg when further purified by molecular exclusion chromatography.

Figure 3.4 **Determination of the Km values for HNE and HNCG under stated conditions using a non-parametric method (Cornish-Bowden and Eisenthal, 1978)**

a) **Km of HNE against STANA in 50mM Tris-HCl/1.0M NaCl, pH8.0, and 1% DMSO at 37°C**



b) **Km of HNCG against Suc-Ala-Ala-Pro-Phe-pNA in 50mM Tris-HCl/1.0M NaCl, pH8.0, and 1% DMSO at 37°C**



The K_m and V_{max} values under the stated conditions were 2.33mM and 1.25µmol/min/ml respectively for the activity of HNE (0.408mg/ml) measured against STANA; and 6.0mM and 1.18µmol/min/ml respectively for the activity of HNCG (0.515mg/ml) measured against Suc-Ala-Ala- Pro-Phe-pNA.

In comparing these data to those of Twumasi and Liener (1977) we must notice that they measured HNE activity in 0.2M Tris-HCl/1.0M NaCl, pH 8.0 and 25°C with a STANA concentration of 1mM. Therefore, the specific activity obtained by Twumasi and Liener was converted to the value which would have been expected when hydrolysing 0.198mM STANA in 50mM Tris-HCl/1.0M NaCl, pH 8.0 and 1% DMSO at 37°C by using the Michaelis-Menten equation, given that the K_m of HNE with STANA in 50mM Tris-HCl/1.0M NaCl, pH 8.0 and 1% DMSO at 37°C was calculated as 2.33mM (Figure 3.4a) and the velocity of the reaction of a preparation of HNE with 0.198mM STANA was found during the course of this work to be 1.93 times faster at 37°C when compared to the rate at 25°C. The calculated value for the specific activity of the HNE preparation isolated by Twumasi and Liener (1977) was 0.382U/mg, which is close to the specific activities of HNE-G and HNE-J. Twumasi and Liener appeared to measure enzyme activity against STANA in the absence of any solvent such as DMSO; so the calculated value for the specific activity of this preparation of HNE against STANA, in 50mM Tris-HCl/1.0M NaCl, pH 8.0 and 1% DMSO at 37°C, is approximate as it neglects the effect the solvent may have on the activity of the enzyme. Consideration of the effect of solvents on elastases is very important as 1% N-methyl-pyrrolidone causes an increase of both the K_m and k_{cat} of PPE (Bieth et al, 1974); and dimethylformamide exerts the same effect on HNE (Lestienne and Bieth, 1980). In both these cases the value of k_{cat}/K_m remained constant regardless of the amount of solvent present. So, a more informative comparison between the HNE preparation isolated by Twumasi

and Liener and that isolated during this investigation would be provided by the values of k_{cat}/K_m of the two enzyme preparations. At 37°C and pH 8.0, HNE-G (0.408mg/ml; molecular weight approximately 28000) had a V_{max} of 1.25U/ml. Given that:-

$$k_{cat} = V_{max}/[e_o]$$

then k_{cat} has a value of $1.43s^{-1}$. So, the value of k_{cat}/K_m of HNE-G at 37°C and pH 8.0 is $0.614mM^{-1}.s^{-1}$, since the K_m of HNE at 37°C and pH 8.0 is 2.33mM (Figure 3.4a). It has already been stated that the velocity of STANA (0.198mM) hydrolysis at 37°C was found by experimentation to be 1.93 times greater than that at 25°C, so it is possible to calculate the value of k_{cat} at 37°C and pH 8.0 of the Twumasi and Liener preparation of HNE by using the quoted values of k_{cat} and K_m at 25°C and pH 8.0 ($0.45s^{-1}$ and 1.82mM respectively) and the K_m of HNE at 37°C and pH 8.0 (2.33mM) as follows:-

$$\frac{v_{37°C}}{v_{25°C}} = \frac{kcat_{37°C}}{kcat_{25°C}} \times \frac{Km_{25°C} + s}{Km_{37°C} + s}$$

So, at 0.198mM STANA

$$1.93 = \frac{kcat_{37°C}}{kcat_{25°C}} \times \frac{1.82 + 0.198}{2.33 + 0.198}$$

And the k_{cat} at 37°C of the HNE isolated by Twumasi and Liener is:-

$$k_{cat\ 37°C} = \frac{1.93}{0.798} \times 0.45 = 1.09s^{-1}$$

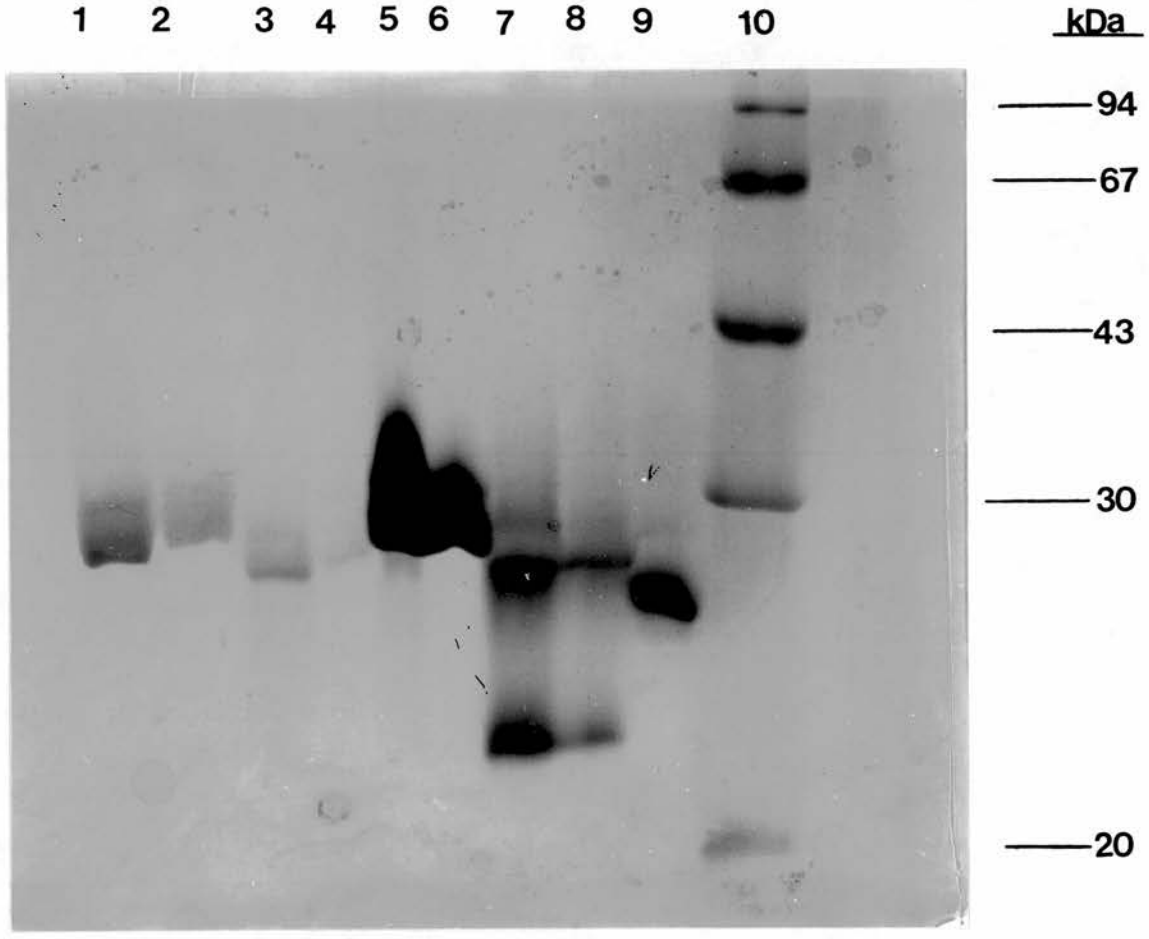
So, at 37°C

$$k_{cat}/K_m = 0.468\ mM^{-1}.\ s^{-1}.$$

Therefore, HNE-G has a k_{cat}/K_m value which is similar to that of the HNE isolated by Twumasi and Liener, so it may be concluded that after taking into account the differences of substrate concentration, temperature and solvent used in measuring the activity of the two enzyme preparations, HNE-G is of a similar quality to the enzyme isolated by Twumasi and Liener.

The specific activities of the HNCG preparations cannot be compared to those isolated by Martodam et al (1979) and Heck et al (1985) because in each case a different synthetic substrate was used to measure enzymic activity. The substrate used by Martodam et al (1979) was N-benzoyl-DL-phenylalanine-β-naphthylamide and N-benzyloxycarbonyl-L-tyrosine-p-nitrophenyl ester was used by Heck et al (1985). For the purposes of any future comparison of HNCG activity, the K_m and V_{max} of HNCG (0.515mg/ml) in 50mM Tris-HCl/1.0M NaCl, pH 8.0 and 1% DMSO at 37°C were determined and found to be 6mM and 1.18U/ml (Figure 3.4b). Assuming the molecular weight of HNCG is 30 000 and that the enzyme isolated was fully active, the k_{cat} for the above reaction was calculated as $1.15s^{-1}$.

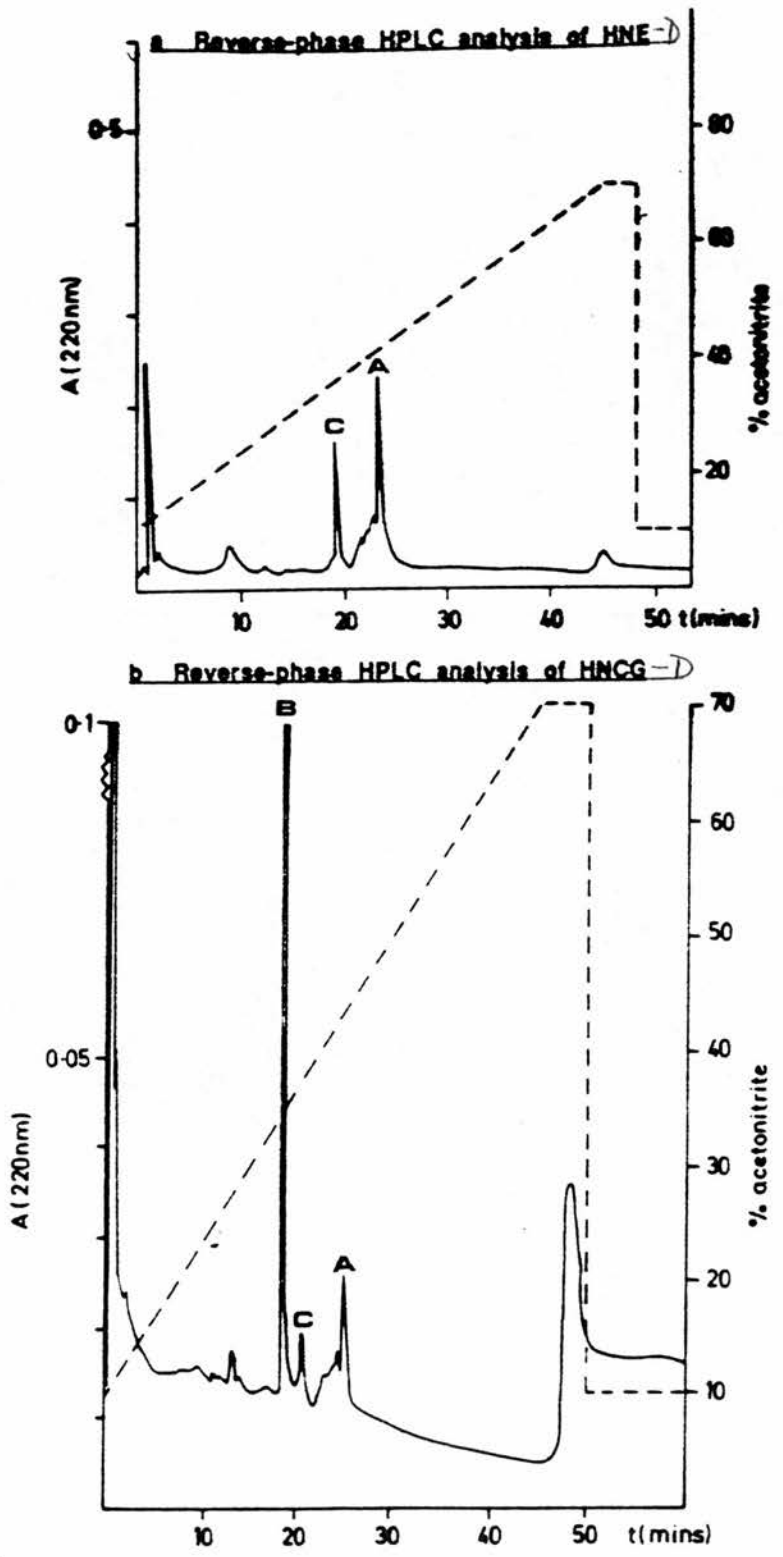
Figure 3.5 SDS-PAGE of samples of the enzyme preparations using a 15% polyacrylamide gel (Laemmli, 1970)



Examination of the enzymes by SDS-PAGE at pH 8.8 using a 15% polyacrylamide separating gel and a 4.5% stacking gel (total dimensions 0.15cm x 16cm x 20cm), run at 70V and 15mA for 16 hours and stained with Coomassie blue.

Tracks: 1 & 2, 14µg & 7µg HNCG-G (0.434U/mg); 3 & 4 , 14µg & 7µg HNE-G (0.354U/mg); 5 & 6, 50µg & 25µg HNCG-D (0.231U/mg); 7 & 8, 50µg & 25µg HNE-D (0.148U/mg);9 irrelevant sample; 10 molecular weight markers

Figure 3.6 Reverse-phase analysis of HNE and HNCG



a) HNE (approximately 200pmol of a preparation with a specific activity of 0.210U/mg) and b) HNCG (approximately 200pmol of a preparation with a specific activity of 0.345U/mg) were examined by reverse-phase chromatography using an Aquapore RP-300 column (Brownlee, ABI) eluted with a gradient of 10-70% acetonitrile/0.1% TFA over 45 minutes (flow rate 200 µl/min). Peak A from chromatograph (a) and peak B from chromatograph (b) were collected manually and N-terminal sequence analysis confirmed them to be HNE and HNCG respectively.

3.1.4. Investigation of the difference between the specific activity of HNE-D and HNE-G

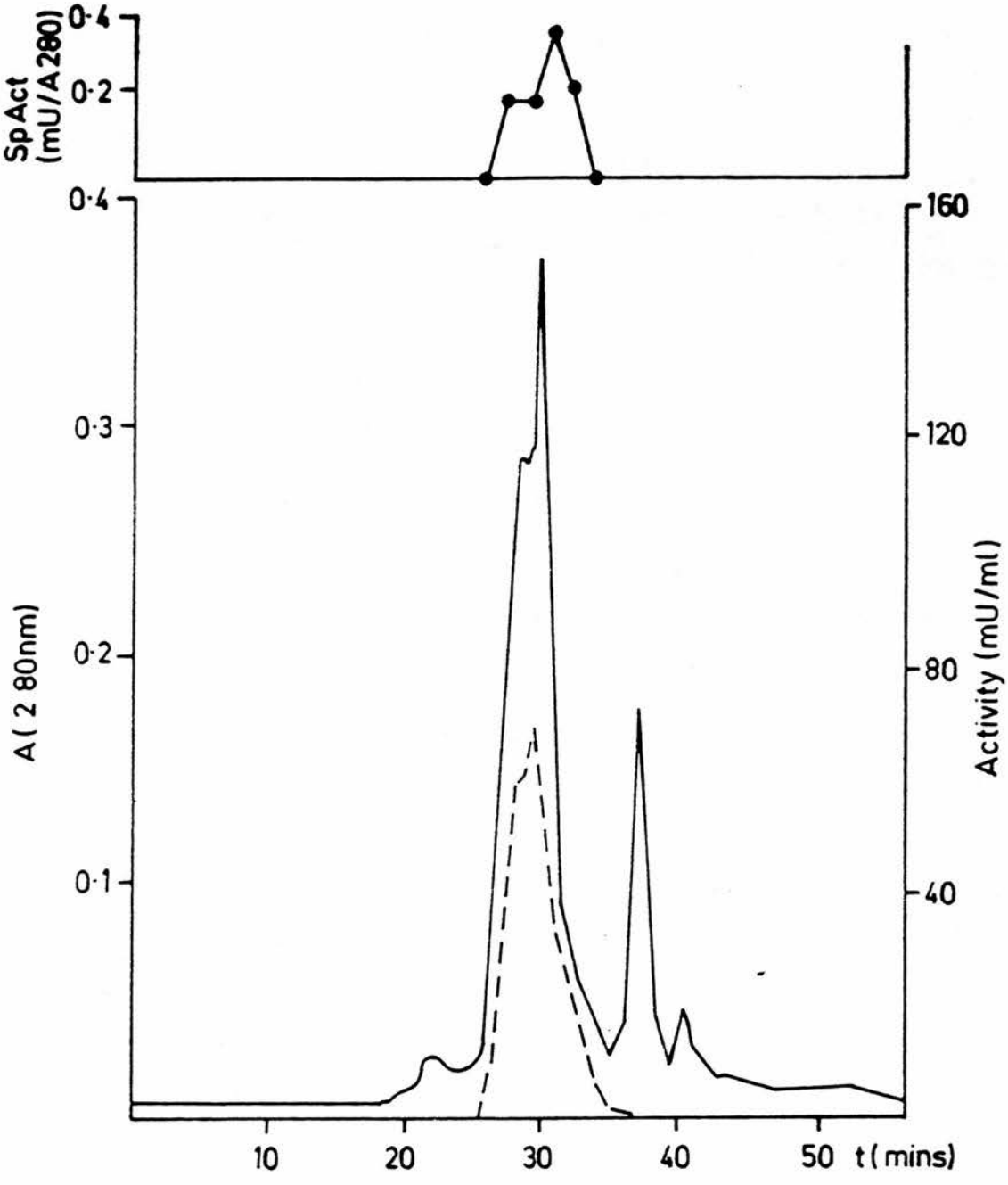
The specific activity of HNE-D was always less than the specific activity of HNE-G. However, each value agreed with one published value (Heck et al, 1985 and Twumasi and Liener, 1977 respectively). Preparations were separated by SDS-PAGE (Figure 3.5) and examined for any obvious differences between the preparations.

The molecular weight of the HNCG and the major HNE preparations were approximately 27-30kDa, although the HNE preparations appeared slightly smaller than the HNCG preparations. These values are similar to those obtained by Martodam et al (1979) and others for the HNE isoforms and for HNCG. A noticeable difference between HNE-D and HNE-G is the presence of a species of approximately 22kDa in the former preparation. This is too small to be HNE, HNCG or the third serine proteinase found in azurophil granules, known as proteinase-3, which is at least 27kDa (Kao et al, 1988; Wilde et al, 1990) but it does explain the lower specific activity of HNE-D. Samples of HNE-D and HNCG-D were analysed by reverse-phase chromatography to assess the extent of contamination by other species.

3.1.5 Reverse-phase chromatographic separation of the enzymes and N-terminal sequence analysis

Samples of the HNE-D and the HNCG-D were examined by reverse-phase chromatography on an Aquapore RP-300 column (Brownlee, ABI) (Figures 3.6a and 3.6b). The peaks presumed to correspond to HNE and HNCG were collected manually and the N-terminal sequences were

Figure 3.7 Molecular exclusion chromatography of HNE-D



HNE (0.30U in 200µl) was examined by molecular exclusion chromatography using a 25ml Superose-12 column (Pharmacia) eluted with 50mM sodium phosphate buffer/0.2M NaCl, pH 7.2 at a flow rate of 0.5ml/min (pressure=1.5MPa). The absorbance of the eluate was detected at 280nm (————). Fractions (1ml, 2 minutes) were collected and their activity against 0.198mM-STANA was measured (– – – –). The specific activity of the fractions (————) is shown in the upper trace.

determined by Miss L. A. Kerr of the Wel-Met Protein Characterisation Service to be:-

HNE: Ile-Val-Gly-Gly-Arg-Arg-Ala-Arg-Pro-His

and

HNCG: Ile-Ile-Gly-Gly-Arg-Glu-Ser-Arg-Pro

These sequences are identical to those given by Martodam et al (1979); and the HNE N-terminal sequence is identical to that deduced from the gene sequence of HNE (Farley et al, 1989).

The HNE appeared to be 40% contaminated with another species (by comparison of A_{220} values of Figure 3.6a). The specific activity against STANA of the preparation prior to reverse-phase chromatographic separation was 0.210 U/mg of which only 60% of the material (as A_{220}) was HNE. The fact that the contaminating peak (C) has an absorbance at 220nm is not evidence that it is a protein; assuming the contaminant was a protein incapable of hydrolysing STANA, it follows that the specific activity of "pure" HNE would have been 0.350 U/mg. Evidence suggesting the contaminating material was a protein incapable of hydrolysing STANA was obtained when a sample of the HNE was separated by molecular exclusion chromatography (Figure 3.7). The activity against STANA was localised to peaks which eluted at a position corresponding approximately to the molecular weight of the HNE isoforms. The pure "HNE" showed signs of separating into at least two isoforms (as A_{280}). It may be tentatively suggested that the second isoform had a greater specific activity, this may be compared with the work of Heck et al (1985) who separated HNE into 4

isoforms of apparently different specific activities. Their smallest isoform appeared to have the greatest specific activity. In Figure 3.7 a smaller contaminating species seemed to be eluted after 37 minutes, but was incapable of hydrolysing STANA. However, this is not indicative of an inability to hydrolyse elastin.

The ability of equal activities (as determined against STANA) of HNE-D (specific activity 0.148 U/mg) and HNE-G (specific activity 0.354 U/mg) to digest collagenase-treated elastin at pH 7.4 and 37°C (detailed in Section 2.3.1) were compared. Triplicate aliquots were removed after 24 hours of digestion and the soluble peptides were measured as detailed in Section 2.3.3. The amounts of peptides produced by the action of HNE-D/mg of elastin and HNE-G/mg of elastin were 0.890 ± 0.094μmol and 0.915 ± 0.091μmol. Since digestion by HNE-D did not yield a greater amount of peptides than did the digestion by HNE-G, it was concluded that the contaminant in HNE-D was inactive against elastin. In general, preparations with a specific activity of approximately 0.350U/mg, as determined against 0.198mM STANA at pH 8.0 and 37°C in the presence of 1M NaCl, were used for the digestion of elastin.

Table 3.4 The recovery of lung elastin as Lansing elastin and collagenase-treated elastin

	Lung 1	Lung 2	Lung 3
Mass of Lansing elastin (g)	1.12	1.31	2.80
% of original tissue (wet weight)	0.23	0.33	0.70
Mass of collagenase-treated elastin (g)	6.40	10.68	6.42
% of original tissue (wet weight)	1.33	2.67	1.61

The recovery of Lansing lung elastin and collagenase-treated lung elastin was approximately 0.5% and 1.8 % of the original weight of tissue, which may be compared with the recoveries noted by Reilly and Travis (1980) which were 0.05%-0.075% of the original tissue when the elastin was isolated by either method.

3.2 ISOLATION OF ELASTIN

3.2.1 Isolation of lung elastin

Lungs were removed at post-mortem from 70 year olds who had not died as a result of respiratory disease. The tissue was fixed for 3 days in ethanol or acetone, then homogenised and washed several times in 50mM Tris-HCl/0.15M NaCl, pH 8.0, defatted in acetone and air-dried to give an acetone powder. The mass of the dry powder was 7.5%-9% of the mass of wet tissue used.

The acetone powders were divided into two and the elastin was isolated from one half by the method of Lansing et al (1952) and from the other half by the method of Ross and Bornstein (1969) (Table 3.4). The Lansing treatment is often described as "degradative" since it involves solubilising all other proteins by boiling the tissue in 0.1M sodium hydroxide solution. Although this provides a relatively pure preparation of elastin; it is possible that some damage will be inflicted on the elastin. An increase of N-terminal residues is produced by prolonged treatment with the alkali (greater than 45 minutes) indicating that hydrolysis has occurred (Gotte et al, 1963). In comparison, the method of Ross and Bornstein involves more gently solubilising other proteins by treating the acetone powder with denaturants, such as guanidine hydrochloride, and dithiothreitol and the enzyme bacterial collagenase (Sigma). The resultant elastin is termed the collagenase-treated elastin (Reilly and Travis, 1980).

Table 3.5 Amino acid analysis of Lansing elastin

Amino acid	residues/1000 residues					
	Lung 1	Lung 2	Lung 3	Average	Lansing (a)	Indik (b)
Asx	0	23.1	32.1	17.7	7	4
Glx	42.7	36.8	33.5	36.3	22	19.5
Ser	0	15.5	0	5.0	7	20.5
Gly	264.5	292.7	285.9	270.2	335	290
His	0	0	0	0	6	3
Arg	18.7	13.8	0	10.4	6	15
Thr	0	0	0	0	7	17
Ala	235.6	229.5	242.2	226.7	267	217
Pro	127.4	112.1	125.7	117.0	109	130
Tyr	23.4	15.1	25.1	20.4	23	18
Val	144.5	128.0	135.4	130.8	100	127
Met	0	0	0	0	4	1
Cys	0	0	0	0	0	3
Ile	27.5	25.7	21.3	23.9	19	22.5
Leu	82.7	68.7	71.0	71.3	55	45
Phe	33.0	26.8	27.9	28.1	20	20.5
Lys	0	12.3	0	3.9	1	46
Hyp	38.5			37		
Des	1.94			1.87		
Ide	1.58			1.53		

a) The composition of Lansing elastin as quoted by Reilly and Travis (1980).

b) The composition of elastin determined by Indik et al (1987) from the cDNA sequence of human tropoelastin.

Table 3.6 Amino acid analysis of collagenase-treated elastin

Amino acid	residues/1000 residues					
	Lung 1	Lung 2	Lung 3	Average	R & T (a)	Indik (b)
Asx	45.2	72.8	67.2	58.1	12	4
Glx	83.3	86.2	62.4	72.7	26	19.5
Ser	29.3	34.2	33.1	30.3	14	20.5
Gly	277.4	268.6	227.2	242.3	309	290
His	0	0	0	0	2	3
Arg	45.1	41.6	35.2	38.2	8	15
Thr	0	0	22.2	7.0	16	17
Ala	160.5	153.1	152.5	146.1	241	217
Pro	117.6	108.9	93.0	100.1	109	130
Tyr	12.4	13.2	11.4	11.6	22	18
Val	85.4	81.5	94.8	82.0	118	127
Met	0	0	0	0	4	1
Cys	0	0	0	0	0	3
Ile	24.1	23.7	67.6	36.2	21	22.5
Leu	63.3	62.1	74.4	62.6	57	45
Phe	28.0	27.6	31.0	27.2	21	20.5
Lys	28.2	26.5	28.0	25.9	8	46
Hyp	67			63		
Des	0.74			0.70		
Ide	0.58			0.54		

a) Composition of collagenase-treated elastin as quoted by Reilly and Travis (1980).

b) Composition of elastin quoted by Indik et al (1987) from the cDNA sequence of human tropoelastin.

3.2.2 Amino acid analysis of the preparations of lung elastin

The relative purity of insoluble elastin is traditionally assessed by amino acid analysis of an acid hydrolysate of elastin. Samples of all the elastin preparations were acid hydrolysed as detailed in Section 2.2.3. Amino acid analysis was then undertaken on 10µl sample of the hydrolysate (containing 0.2µg of original material) by the Wel-Met Protein Characterisation Service (Tables 3.5 and 3.6). On a separate occasion, the temperature and gradient of the amino acid separation were altered to allow the measurement of hydroxyproline, which would otherwise co-elute with aspartic acid. It was only possible to analyse one pair of lung elastins by this method.

The amounts of desmosine and isodesmosine in the acid hydrolysates were kindly determined by Mr. G. Fiaux using a method adapted from the procedure devised by Yamaguchi et al, (1987). Briefly, the constituents of the acid hydrolysate (10µl) were separated on a C_{18} reverse-phase column. The absorbance of the eluate was detected at 270nm; desmosine and isodesmosine eluted at characteristic times. The amount of each of the cross-links in the sample of hydrolysate was determined from the peak area by consulting a standard curve relating peak area to amount of desmosine or isodesmosine. These values were related to those of the other amino acid's solely by the volumes applied to the analytical columns.

Comparison of the literature figures with the composition of the lung elastins and the composition of the tropoelastin gene sequence show a great deal of variability. This variability can also be noted when comparing

the compositions of lung elastins isolated by the same method and when comparing elastin isolated from the same lung but by different methods. Variability has been noted by several other authors (Paz et al, 1976; Soskel and Sandberg, 1983), and has been suggested to be a consequence of the crude nature of the methods of isolation of lung elastin from lung tissue, which is rich in other structural proteins which are also poorly soluble. A consequence of the inconsistent compositions of the lung elastin is that it is difficult to estimate the extent of possible contamination with collagen or glycoprotein. Therefore, determination of purity of elastin is usually decided on a qualitative basis as a lack of methionine and cysteine, low levels of hydroxyproline (1-2%), and large amounts (20% or more) of alanine and valine (greater than 7.5%) (Soskel and Sandberg, 1983). This method of assessing purity led Soskel and Sandberg (1983) to the conclusion that the Lansing method of isolation yielded a relatively purer elastin than non-degradative methods such as the method of Richmond (1974) which is based on the method of Ross and Bornstein (1969). Comparison of the compositions with the gene sequence of tropoelastin shows that for collagenase-treated elastin, the content of the acidic amino acids and the most frequent amino acids such as glycine, alanine, proline and valine deviate from the gene figures to a greater extent than do those for Lansing elastin. The yield of collagenase-treated elastin was on average four-fold greater than the yield of Lansing elastin from the same tissue (Table 3.4). This suggests, in agreement with Soskel and Sandberg (1983), that elastin isolated by a non-degradative method was less pure than the Lansing elastin.

Table 3.7 Amino acid composition of human skin collagen

Amino acid	residues/1000 residues	
	Skin collagen (a)	Lansing lung elastin (b)
Asx	45	7
Glx	73	22
Ser	36	7
Gly	336	335
His	5	6
Arg	51	6
Thr	17.5	7
Ala	110	267
Pro	128	109
Tyr	3	23
Val	24.4	100
Met	6	4
Cys	-	0
Ile	9.5	19
Leu	24	55
Phe	12	20
Lys	27	1
Hyp	94	12
Hyl	6	-

a) The composition of skin collagen as quoted by Bornstein and Piez (1964).

b) The composition of Lansing lung elastin as quoted by Reilly and Travis (1980).

3.2.3. Determination of collagen contamination in preparations of lung elastin

Contamination of elastin with collagen would give elevated levels of acidic amino acids, lysine, arginine and hydroxyproline, combined with a proportional decrease in the content of alanine and valine (Table 3.7). The levels of acidic amino acids in elastin have been suggested to increase with age (Indik et al, 1989) by virtue of the fact that they are localised within exon 10A, which as a result of alternative splicing, is poorly expressed in foetal aortic elastin but appears to be expressed well in elderly aorta. The variability of these amino acids with age suggests they are of little use in assessing the collagen content of elastin isolated from elderly patients. Contamination by collagen can be specifically assessed by measuring the presence of hydroxyproline. Hydroxyproline accounts of 9%-10% of the composition of collagen but only 1%-2% of the composition of elastin, and is very rare in other proteins. The amounts of collagen and elastin present within a preparation of elastin have been deduced by Stone et al (1987) to conform to the equations:-

$$\text{Collagen } (\mu g) = 0.92\ [\text{nmol Hyp} - 9(\text{nmol Des} + \text{Ide})]$$

and

$$\text{Elastin } (\mu g) = 43 \times \text{nmol (Des + Ide)}$$

These equations assume that the average residue masses in elastin and collagen are 85 and 92 respectively; and the average hydroxyproline (Hyp) and desmosine (Des) plus isodesmosine (Ide) content of elastin are

Table 3.8 Calculation of collagen contamination of the preparations of lung elastin

Elastin preparation	Mass of material hydrolysed (ug)	Mass of collagen (ug)	% of initial mass	Mass of elastin (ug)	% of initial mass
Lansing elastin	0.342	0.006	2	0.145	42
Collagenase-treated elastin	0.240	0.047	20	0.053	22

The concentration of hydroxyproline and desmosine/isodesmosine present in acid hydrolysates of 'pure' lung elastin preparations were used to calculate the theoretical collagen and elastin contents of the preparations (Stone et al, 1987). The resulting values were expressed as a percentage of the mass of elastin which was hydrolysed.

18 residues per 1000 and 2 residues per 1000 respectively.

When the equations were applied to the preparation of Lansing elastin and collagenase-treated elastin (Table 3.8) the latter preparation was calculated to have a 10-fold greater collagen content than the former. Further evidence to suggest collagenase-treated elastin contained a greater proportion of collagen was produced when each preparation was digested with bacterial collagenase (enzyme:substrate ratio 1:75 (w/w); Sigma) at 37°C and pH 7.5 for 5 hours. The quantities of soluble peptide products were estimated by the method detailed in Section 2.3.1., and corresponded to approximately 8% and 3% of the collagenase-treated elastin and Lansing elastin.

The calculated amounts of collagen and elastin only accounted for 44% and 42% of the original mass of Lansing elastin and collagenase-treated elastin respectively which suggests that either the preparations were contaminated with other proteins or other macromolecules, or there were large losses during the acid hydrolysis and subsequent dilutions. The latter suggestion was investigated by acid hydrolysis and amino acid analysis of a known amount of pure β-lactoglobulin, which has a known composition (Braunitzer et al, 1973). The recovery of β-lactoglobulin, as a percentage of the material hydrolysed, was 37%. It was concluded that the low recoveries of the elastin preparations are probably a consequence of losses produced during hydrolysis and sample preparation rather than the presence of other macromolecules.

Table 3.9 Amino acid composition of human aortic elastin

Amino acid	residues/1000 residues			
	Human aortic elastin	Dog aortic elastin (a)	Human tropoelastin (b)	Microfibril (c)
Asx	69	6	4	68
Glx	57	24	19	222
Ser	34	15	20	45
Gly	219	338	283	45
His	0	5	3	34
Arg	24	7	15	56
Thr	19	20	17	40
Ala	182	238	212	49
Pro	91	103	127	106
Tyr	17	24	18	51
Val	110	99	124	69
Met	0	0	1	4
Cys	0	0	3	68
Ile	27	26	22	22
Leu	78	44	41	72
Phe	33	24	20	22
Lys	22	4	45	26
Hyp	14	14	_	_
Des	1.0	7	_	_
Ide	1.3	3	_	_

a) The composition of dog aortic elastin isolated by Paz et al (1976).

b) The composition of tropoelastin from the cDNA sequence (Indik et al, 1987).

c) Bovine microfibrillar component (Indik et al, 1989).

3.2.4. Isolation of aortic elastin

The aorta and lung 2 were removed from the same patient. The aorta was fixed in acetone for 3 days before the elastin was isolated by the method of Paz et al (1976) which involved isolating the elastin from minced aorta by trichloroacetic acid extraction followed by successive treatments with bacterial collagenase, then dithiothreitol with 6M guanidine hydrochloride and finally urea, dithiothreitol and sodium dodecyl sulphate. The mass of aortic elastin recovered from 23.7g of aorta was 2.3g.

After isolation a sample of the elastin was subjected to total acid hydrolysis and the amino acid composition of the aortic elastin, including hydroxyproline, desmosine and isodesmosine, was determined (Table 3.9). The values for the percentage of collagen and elastin in the aortic elastin, calculated from the previously given equations (Stone et al, 1987), were 0% and 38% respectively. The proportions of aspartic acid and glutamic acid in the acid hydrolysate of the aortic elastin were much higher than expected. The microfibril component of elastic fibres is rich in aspartic acid and glutamic acid (Table 3.9; Indik et al, 1989), suggesting that the glycoproteins of the microfibril component of the elastic tissue had not been completely removed from the elastin during the isolation of the protein. Gotte et al (1963) stated that the ease with which the other components of the matrix were removed from the elastin varied from tissue to tissue. He observed that aortic elastin was difficult to separate from the microfibril component of the elastic tissue, which seemed to be interpenetrated within the elastin, and stated that aortic elastin isolated by alkali treatment at 98°C contained some polysaccharide-containing material.

3.2.5 Concluding remarks about the isolation of elastin

The suggestion that the harsh treatment of lung tissue by hot sodium hydroxide to yield pure elastin is a "degradative" procedure (Serafini-Fracassini et al, 1975) was put into context by Gotte et al (1963) who suggested that the damage to the elastin itself was minimal when the treatment was limited to 45 minutes at 98°C with 0.1M sodium hydroxide. However, the amounts of serine and threonine are lower than expected in the case of Lansing elastin and this may be related to the fact that they are labile on alkali hydrolysis. In comparison, the amounts of alanine and valine were as expected. These residues are not easily chemically damaged, and they are the main P_1 residues of substrates of HNE. Concerns about the "degradative" procedure led to the development of "non-degradative" methods which claimed to be less destructive; but the final preparations had a greater degree of contamination by collagen, which complicates any study which is concerned primarily with the products of digestion of pure elastin. A further disadvantage of the "non-degradative" methods is that the treatment with denaturants such as guanidine and urea may change the physical structure of the proteins; damage to elastin is not necessarily avoided by omitting the harsh sodium hydroxide treatment. This was observed by Gosline (1976) who noted that the treatment of elastin with urea causes an increase in the swollen volume of the elastin.

Therefore, none of the methods of isolation is ideal; but in the interests of producing a relatively pure product the methods of Lansing et al (1952) and Paz et al (1976) are most appropriate, although their "degradative" nature should not be ignored. Similarly, although Lansing

lung elastin and Paz aortic elastin are of similar quality, they were isolated by different methods. Although any differences between the preparations encountered in the course of this work could be suggestive of differences between elastins isolated from different organs (a consequence of alternative splicing of the tropoelastin gene; Indik, et al, 1989), it cannot be proven to be the case within the boundaries of this work, because the tissues have been subjected to different treatments, and the aortic elastin may contain some of the microfibril glycoprotein.

CHAPTER 4

MEASUREMENT OF THE SOLUBLE PRODUCTS OF ENZYMIC DIGESTION OF ELASTIN

Figure 4.1 Estimation of soluble elastin-derived peptides as detectible protein determined by standard protein assays

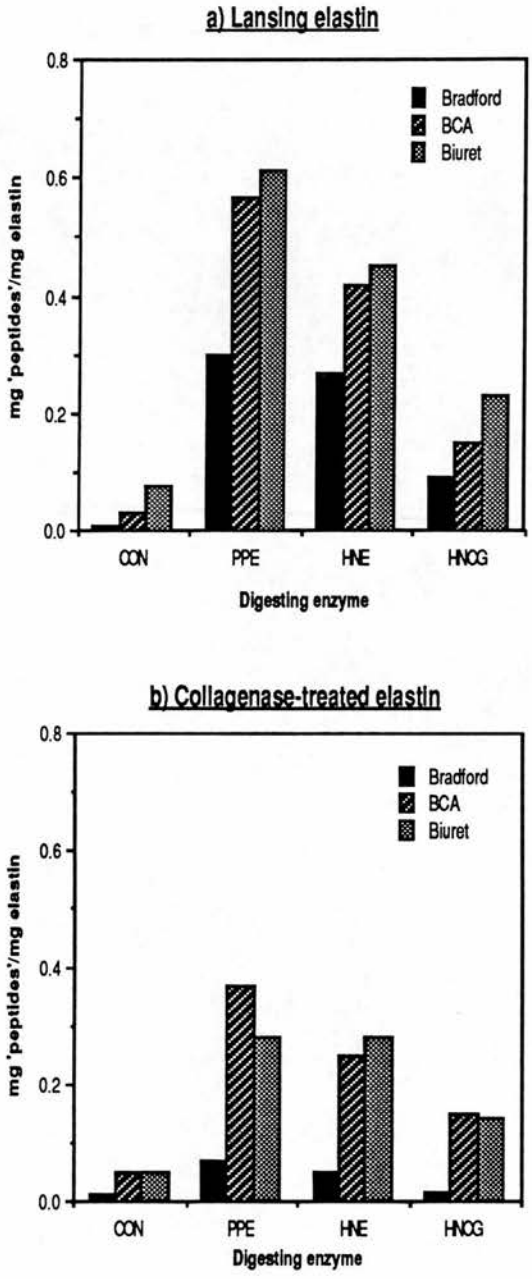
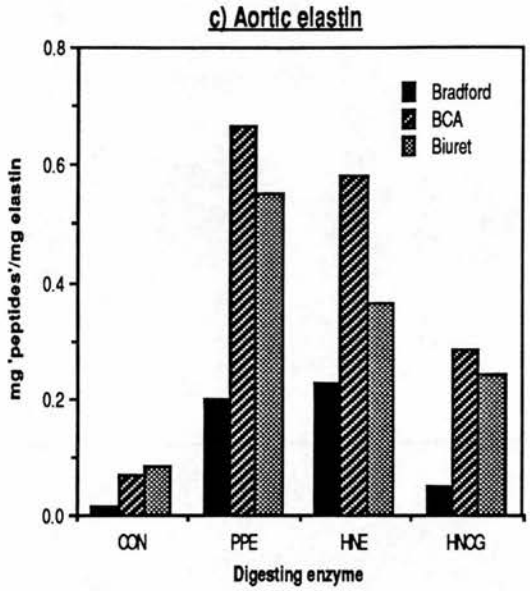


Figure 4.1 Estimation of soluble elastin-derived peptides as detectible protein determined by standard protein assays (continued)



Estimation of the soluble peptides from undigested control suspensions of elastin (CON), and those produced by the enzymic digestion of a) Lansing elastin; b) collagenase-treated elastin and c) aortic elastin with PPE, HNE or HNCG (as detailed in section 4.1.1) by three standard methods of protein estimation (detailed in section 2.3.2).

CHAPTER 4

MEASUREMENT OF THE SOLUBLE PRODUCTS OF ENZYMIC DIGESTION OF ELASTIN

A preliminary investigation was done to find the most suitable means of measuring the extent of digestion of elastin.

4.1.1 Digestion of elastin and isolation of the soluble peptides

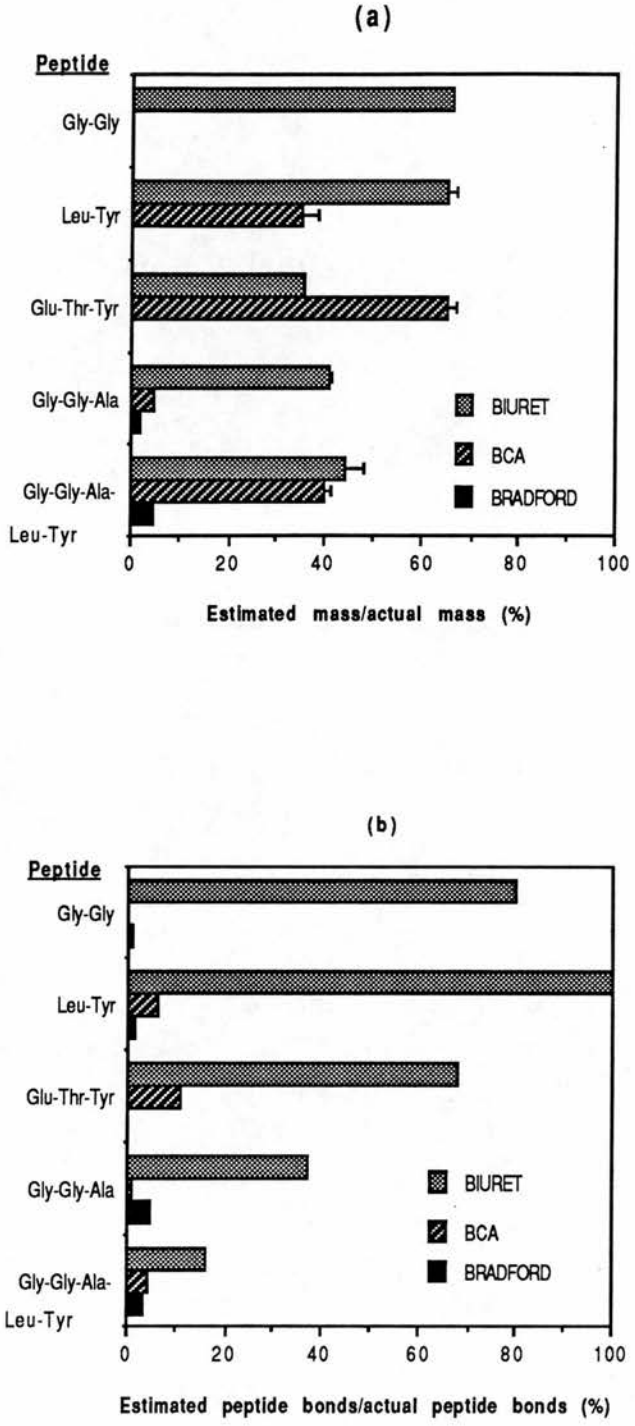
A single suspension of each elastin preparation (3mg/ml in 0.1M sodium phosphate buffer/0.15M NaCl, pH 7.4) was digested for 24 hours at 37°C with either porcine pancreatic elastase (PPE; 2mU/ml, as measured against 0.198mM STANA; corresponding to an enzyme substrate ratio of 1:1000 w/w); HNE (10mU/ml; 1:100 enzyme substrate ratio, w/w) or HNCG (10mU/ml, 1:130 enzyme substrate ratio, w/w). After digestion, the insoluble and soluble peptides were separated by centrifugation in an MSE microfuge at high speed for 10 minutes.

4.1.2 Measurement of the mass of soluble peptides

The mass of the soluble elastin-derived peptides were measured by three standard methods of protein estimation, detailed in Section 2.3.2 (Figures 4.1a, b and c).

Comparisons between the three methods show widely varying

Figure 4.2 Demonstration of the relative abilities of standard protein estimation methods to measure small peptides



Known masses (a) and the number of peptide bonds (b) of four peptides (Gly-Gly, glycyl-glycine; Leu-Tyr, leucyl-tyrosine; Glu-Thr-Tyr, glutamyl-threonyl-tyrosine; Gly-Gly-Ala, glycyl-glycyl-alanine (all from Bachem AG); and Gly-Gly-Ala-Leu-Tyr, glycyl-glycyl-alanyl-leucyl- tyrosine, synthesised by Dr A P Ryle by the condensation of Gly-Gly-Ala with Leu-Tyr) were estimated by the Bradford method (n=3) and by the BCA and biuret methods on three occasions in quadruplicate (n=12). The estimated mass or number of bonds detected by each method was expressed as a percentage of the known mass or number of bonds; thus indicating the relative ability of each method to detect small peptides.

results. The Bradford method, which is dependent on the binding of the dye Coomassie blue to the peptides, appeared ineffective. This is presumably because the dye binds predominantly via arginyl residues (Compton & Jones, 1985) (representing 1% of the composition of elastin), and does not bind to alanine- or glycine- rich sequences which constitute large stretches of the sequence of elastin (Indik et al, 1987). In contrast, the BCA and biuret methods gave significantly higher and quite concordant estimates of the soluble peptides. These methods are both based on the detection of peptide bonds and detect peptides larger than dipeptides. The BCA method is additionally dependent on the presence of particular residues such as tyrosine (Wiechelman et al, 1988). Both methods are suitable for estimating proteins and large peptides which have many peptide bonds and a representative mixture of amino acid residues, but small peptides may be underestimated. This is important when estimating the amount of potentially heterogeneous elastin-derived peptides. The ability of the three methods to detect the mass of small peptides and the peptide bonds within small peptides was tested using some small pure peptides (Figures 4.2a and b). As expected the Bradford method was ineffective in measuring small peptides devoid of arginyl residues. The BCA and biuret methods were relatively more effective in estimating the small peptides. However, the BCA method detected tyrosine-free tripeptide ineffectively and the biuret method appeared able to detect dipeptides, which was rather surprising, but responded less well to peptides with 2-4 peptide bonds. Although the biuret method was quite effective in the detection of peptide bonds, it was concluded that with both the BCA and biuret methods the recoveries of the

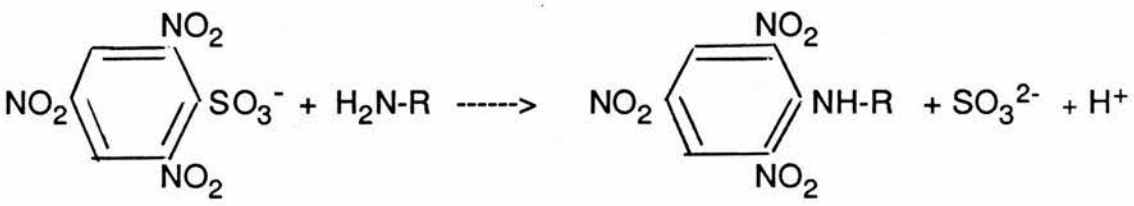
small peptides, in terms of mass, were still significantly lower than 100%, so the smaller peptides in a heterogeneous mixture of elastin-derived peptides may be underestimated.

4.2 Measurement of the free amino groups of the soluble peptides

The estimation of soluble peptides in molar terms is possible by measuring the amino groups present in a mixture of peptides; this assumes that only 2-amino groups and no lysine 6-amino groups are found in the peptides. Since the side groups of the majority of the few lysine residues of elastin are oxidised to the corresponding aldehyde or incorporated into cross-links, their contribution to the estimation of the amino groups of elastin-derived peptides is negligible.

4.2.1. Measurement of free amino groups using TNBS

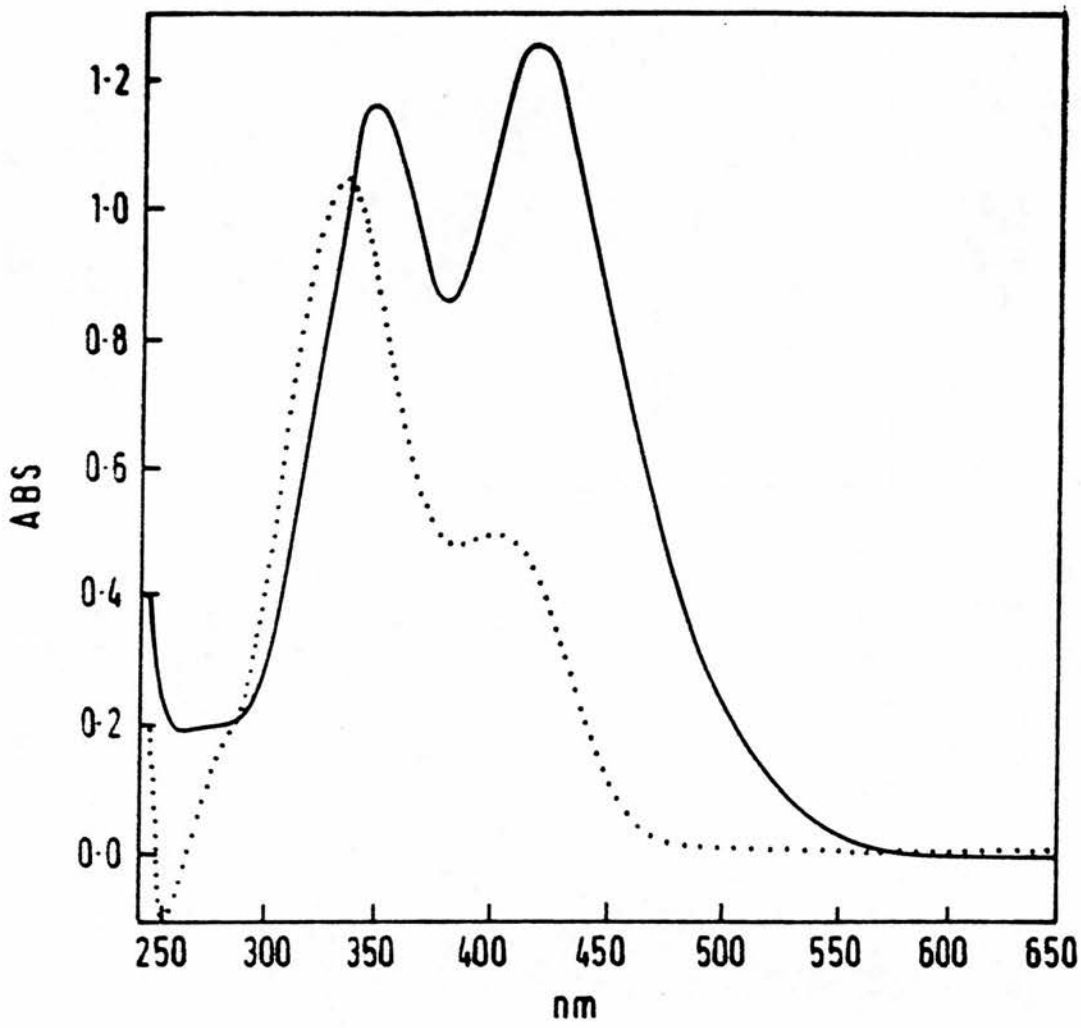
Amino groups of peptides react with TNBS at pH 8.0 to give 2,4,6-trinitrophenylated peptides (Tnp-peptides).



TNBS Tnp-peptide

The reaction may be standardised by incubating a pure amino acid

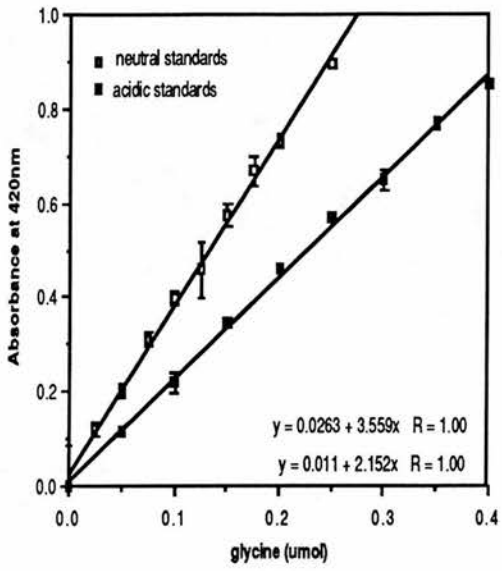
Figure 4.3 The absorption spectra of the reaction product of the TNBS and glycine reaction at pH 8.0, measured in neutral and acid conditions



Duplicate samples of 0.5µmol of glycine (0.5ml) were trinitrophenylated with 0.5ml of 0.1% TNBS in 0.5ml of 0.1M sodium phosphate buffer, pH 8.0 (see section 2.3.3). After incubation, a further 0.5ml of the same buffer was added to one reaction mixture. This sample and the corresponding reagent blank were diluted with an equal volume of water and the absorption spectrum of the reaction mixture (pH 8.0) was measured against the reagent blank (————). The second reaction mixture was acidified to pH 1.0 with 0.5ml of 4M HCl. Once again, the reaction mixture and the appropriate reagent blank were diluted with an equal volume of water, before the absorption spectrum of the acidified sample was read against the acidified reagent blank (· · · · · · ·).

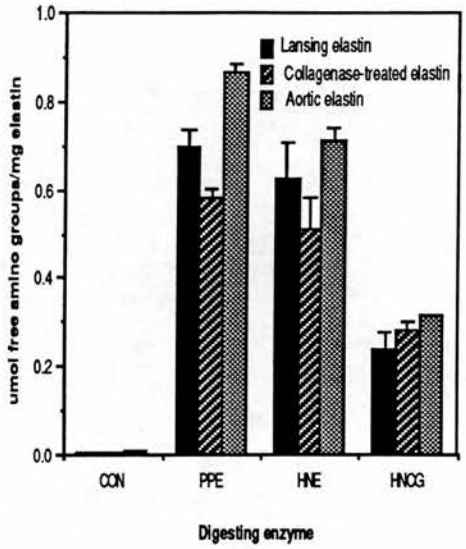
Both absorption spectra were measured in a PU8700 uv/vis spectrophotometer (Philips).

Figure 4.4 Comparison of the absorbance at 420nm of Tnp-glycine with and without acidification



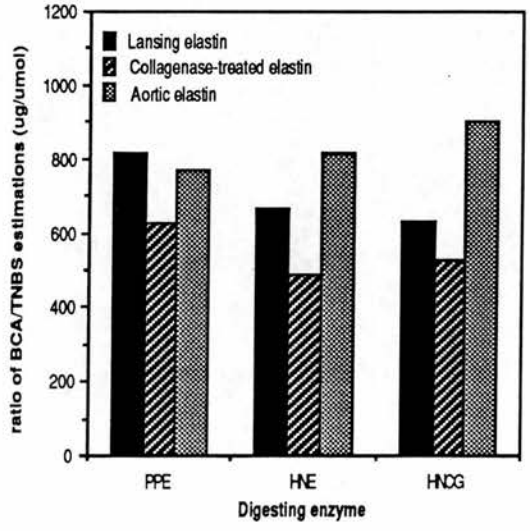
Two sets of triplicate samples of glycine (0-0.5µmol in 0.5 ml) were trinitrophenylated as in section 2.3.3. After incubation, 0.5ml of 4M HCl was added to each of one set of triplicates (giving final volumes of 2ml and approximate pH of 1), whilst the other set of triplicates remained untreated (giving final volumes of 1.5ml). The absorbance of each set of Tnp-glycine standards was measured at 420nm against the appropriate reagent blank; and the absorbance values of the neutral standards were corrected to the value expected if their volume was 2ml.

Figure 4.5 Estimation of soluble peptides as Tnp-peptides formed on reaction with TNBS



The estimation of the soluble peptides produced in section 4.1.1 by the enzymic digestion of Lansing elastin, collagenase-treated elastin and aortic elastin with no enzyme (control: CON), PPE, HNE or HNCG. The Tnp-peptides were measured as the absorbance at 420nm against a reagent blank, and estimated by reference to a standard curve prepared with glycine (n=3).

Figure 4.6 Estimation of the relative size of the soluble peptides as the ratio of the estimated mass by the BCA method and the estimated moles by the TNBS method



The estimation of the relative size of the soluble elastin-derived peptides prepared as detailed in section 4.1.1., as the ratio of the estimated mass of peptides to the estimated moles of peptides.

with TNBS at pH 8.0 and 37°C for 2 hours, which is the time necessary for the complete formation of Tnp-glycine (Satake et al, 1960). The Tnp-peptides have absorbance maxima at 350nm and 420nm, at neutral and acidic pH (Figure 4.3). The absorbance of a neutral reagent blank at 420nm is high because of the presence of picrate anions, a decomposition product of TNBS. Satake et al (1960) recommend the acidification of the samples with 4M HCl to approximately pH 1.0. The absorbance of the reagent blank is reduced by approximately 0.4 on acidification, but this is at the expense of sensitivity (Figure 4.4). The molar absorbances of Tnp-glycine, calculated from Figure 4.4, are 7120 $l.mol^{-1}.cm^{-1}$ at pH 8.0 and 4300 $l.mol.^{-1}.cm^{-1}$ at pH 1.0.

4.2.2 Application of the method to the measurement of soluble elastin-derived peptides

When the soluble peptides produced by PPE digestion of Lansing elastin and aortic elastin (see Section 4.1.1) were trinitrophenylated and acidified with HCl, the resulting solutions were visibly cloudy so that spectrophotometric measurements could not be made. So, the Tnp-peptides were measured at pH 8.0, avoiding the precipitation of the elastin-derived peptides (Figure 4.5).

4.2.3 Estimation of the relative size of the peptides

The relative size of the soluble elastin-derived peptides was estimated as the ratio of mass, equivalent to BSA, by the BCA method

(Section 4.1.2) to moles, equivalent to glycine, by the TNBS method (Section 4.2.2) (Figure 4.6). The data suggest that the peptides are between 5 and 9 residues long. Again note that the BCA method potentially underestimates the mass of the elastin-derived peptides, so the size of the peptides will also be underestimated.

4.3 Concluding remarks about the estimation of soluble elastin-derived peptides

Elastin is more susceptible to attack by PPE than by HNE (Reilly & Travis, 1980), and the digestion by HNCG is least effective. This was not unexpected. By assuming that PPE can cleave peptide bonds on the carbonyl side of glycine, valine and alanine residues, that HNE can cleave peptide bonds to the carbonyl side of valine, alanine and isoleucine, and that HNCG can cleave peptide bonds to the carbonyl side of isoleucine, leucine, methionine, tyrosine and phenylalanine; then it may be calculated that PPE, HNE and HNCG can potentially cleave 70%, 37% and 12% respectively of the residues in Lansing lung elastin . This assumes that each bond is equally susceptible to digestion, irrespective of steric effects of the neighbouring residues and of whether neighbouring bonds are cleaved. This assumption is known not to be entirely justified (Nakajima et al, 1979; and see Section 1.2 for details of the specificity of these enzymes).

Lansing elastin and aortic elastin are more susceptible to enzymic digestion than is collagenase-treated elastin. This is probably due to the contamination of collagenase-treated elastin with collagen (see Section 3.2.3), rather than to a possible increased susceptibility of Lansing elastin

and aortic elastin to attack as a consequence of very limited hydrolysis of peptide and amide bonds by sodium hydroxide or TCA during the isolation of the elastins. If hydrolysis had occurred, the peptides from Lansing elastin and aortic elastin would be smaller than the peptides from collagenase-treated elastin; this did not appear to be the case (Figure 4.6).

Since the estimation of peptides produced, in molar terms, is more meaningful than weight-based estimates related to standard proteins, the TNBS method was adopted in further investigations of elastin digestions.

CHAPTER 5

DEVELOPMENT OF A METHOD FOR THE ESTIMATION OF TOTAL PEPTIDES

CHAPTER 5

DEVELOPMENT OF A METHOD FOR THE ESTIMATION OF TOTAL PEPTIDES

Soluble peptides may be estimated spectrophotometrically as the Tnp-peptides formed by the reaction of the amino groups of peptides with TNBS (see Chapter 4). The spectrophotometric method cannot be used to measure directly the amino groups of insoluble peptides or proteins, because the absorbing Tnp-group will be sedimented on centrifugation. However, the sulphite by-product of the TNBS reaction is soluble and should be measurable. It seemed that sulphite could be estimated by allowing it to reduce a suitable chromophore if a stoichiometric reaction could be achieved.

5.1 The choice of a chromophoric oxidising reagent capable of reacting with sulphite

Ellman's reagent (5,5'-dithiobis-2-nitrobenzoate) and potassium dichromate were rejected because they both react to give products which absorb around the same wavelength as Tnp-peptides.

(a) Potassium permanganate

Potassium permanganate in acid solution was tested and rejected because the loss of absorbance at 525nm was not linearly related to the amount of sulphite added. It has also been reported by Hass (1937) that

elastin may reduce highly oxidised salts of metals and this reaction would interfere further.

(b) Iodine

Iodine was tested and rejected because a misleading decrease of absorbance at 288nm resulting presumably from the iodination of the peptides was sometimes observed. A second reason for the rejection was that the absorbance of iodine overlaps with the absorbance of the cross-linking residues and aromatic residues.

(c) NAD^+

NAD^+ was rejected because the product of the reaction between NAD^+ and sulphite absorbs at 320nm, which overlaps with the absorbance of Tnp-peptides.

(d) Basic fuchsin

Basic fuchsin was reduced by sulphite giving a linear decrease of absorbance at 540nm. It was rejected because it has a high chemical affinity for elastin (Hass, 1937), which is exploited by its inclusion in resorcin-fuchsin, a biological stain for elastin (Weigert, 1898). The binding of the dye to elastin and elastin-derived peptides would cause a misleading decrease of absorbance.

Figure 5.1 The expected mechanism of the reduction of DCPIP with sulphite

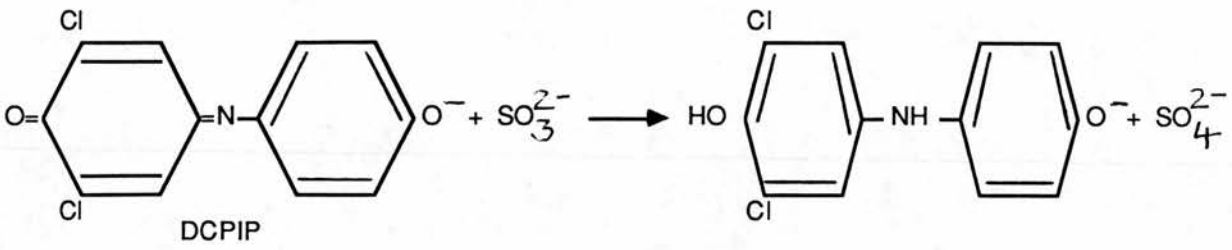
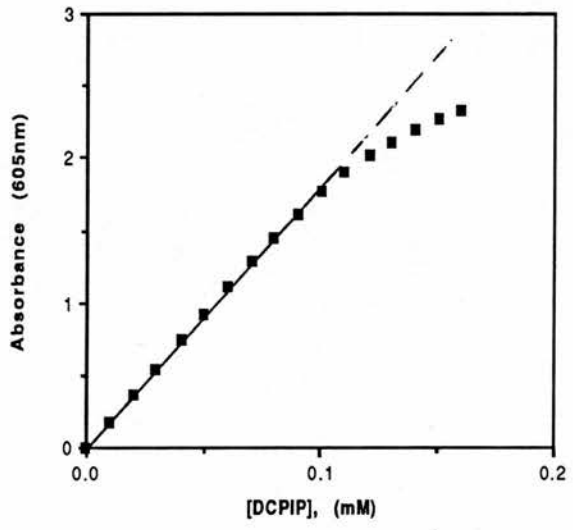


Figure 5.2 The relationship between the concentration of DCPIP and the absorbance at 605nm



The absorbance of DCPIP (0-0.16mM) in 0.05M sodium phosphate buffer, pH 8.0 was measured against the phosphate buffer.

5.2 Investigation of the properties of 2,6-dichlorophenol indophenol and its response to sulphite

The absorption spectrum of DCPIP (λ_{max} 605nm) does not overlap with the absorption spectrum of Tnp-peptides and DCPIP does not bind significantly to elastin.

5.2.1 Determination of the optimum DCPIP concentration

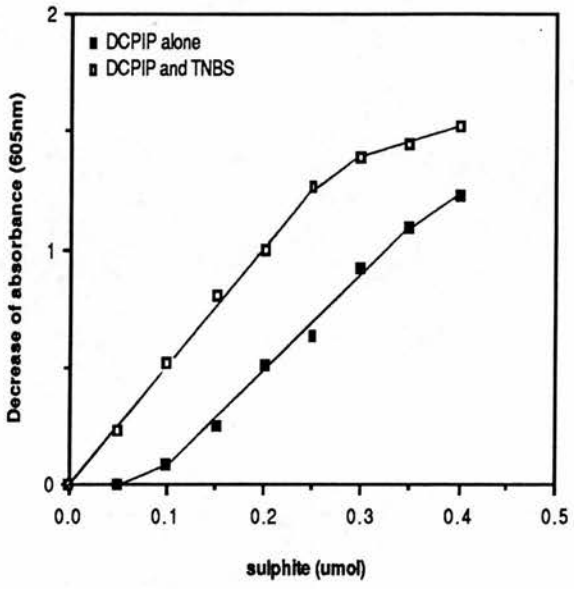
Sulphite was expected to reduce and decolourise DCPIP according to the equation shown in Figure 5.1. Estimating sulphite as a decrease of absorbance necessarily meant starting with a reagent with a high absorbance. The maximum useful initial absorbance will be limited by the capabilities of the spectrophotometer. The range of concentration of DCPIP over which the reported absorbance at 605nm and pH 8.0 is proportional to the concentration of DCPIP was investigated (Figure 5.2) using a SP6-550 UV/vis spectrophotometer (Pye Unicam). The relationship was linear up to an absorbance of approximately 1.76, corresponding to 0.1mM DCPIP. The molar absorbance of DCPIP at 605nm calculated from Figure 5.2 was 17600 $l.mol^{-1}.cm^{-1}$. The molar absorbance of the DCPIP was not affected by the presence of 0.05% TNBS. Armstrong (1964) measured the molar absorbance of DCPIP at 600nm and pH 8.0 to be 21800 $l.mol^{-1}.cm^{-1}$. The discrepancy between Armstrong's value of 21800 $l.mol^{-1}.cm^{-1}$ and 17600 $l.mol^{-1}.cm^{-1}$, the value calculated from Figure 5.2 is substantial. However, there are two possible explanations for the difference. First, the discrepancy

may indicate that the pH of the DCPIP solution was not correct. Armstrong stated that the molar absorbance of DCPIP is dependent on pH and rises from 2700 $l.mol^{-1}.cm^{-1}$ at around pH 4 to 22000 $l.mol^{-1}.\ cm^{-1}$ at approximately pH 8.5. Therefore a discrepancy of 4000 $l.mol^{-1}.cm^{-1}$ could indicate that the pH of the DCPIP solution was approximately pH 6.5, which is substantially less than expected. The pH of the DCPIP solution was checked and was correct. Alternatively, the DCPIP solution used during this investigation may have had a lower concentration than originally thought, so the actual molar absorbance would be greater than the calculated value. Armstrong standardised his solution of DCPIP against ascorbic acid solution, so this method of standardisation of the DCPIP solution was adopted. A solution of ascorbic acid (approximately 0.2mM) was standardised against exactly 0.33mM iodine solution. The 0.2mM DCPIP solution (10ml) was then standardised against the ascorbic acid solution, and the exact molarity was calculated. The absorbance at 605nm of the DCPIP solution was divided by the calculated concentration of DCPIP to give the actual molar absorbance at 605nm of the DCPIP solution, which was 18900 $l.mol^{-1}.cm^{-1}$.

5.2.2 Determination of the response of DCPIP to the addition of sulphite

A solution of sodium sulphite (approximately 10mM) was standardised. Briefly, a solution of approximately 0.1M sodium thiosulphate was standardised by titration against exactly 17mM potassium iodate

Figure 5.3 The reduction of a constant amount of DCPIP with sulphite



Sodium sulphite (0-0.45µmol) was added to 1ml of 0.2mM DCPIP in 0.05M sodium phosphate buffer, pH8.0 with (□) and without (■) 0.05% TNBS. The volume of the solutions was made up to 2ml with the buffer and they were incubated at 37°C for 2 hours.

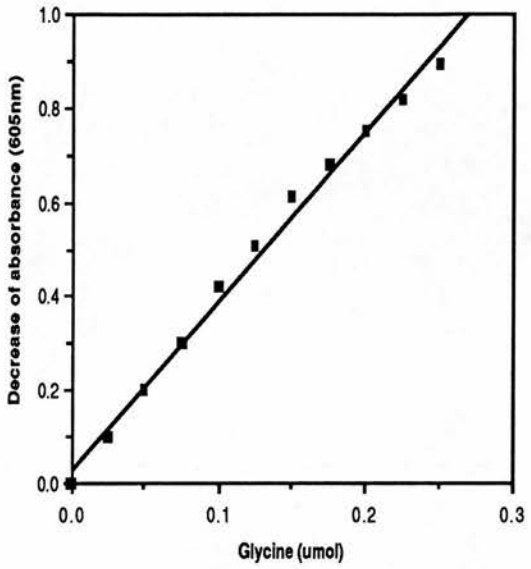
solution in the presence of potassium iodide. The standard sodium thiosulphate solution was used to standardise approximately 0.05M iodine solution. The iodine solution (25ml) was reduced with 10ml of approximately 10mM sodium sulphite and the excess iodine was measured by back-titration with the standard sodium thiosulphate. It was then possible to calculate the volume of iodine reduced by the sodium sulphite solution and the exact molarity of the sodium sulphite solution.

The reaction of a fixed concentration of DCPIP at 37°C, in the presence and absence of TNBS, with varied concentrations of sodium sulphite caused an approximately linear decrease of absorbance of DCPIP, except for a lack of response below 0.05µmol (Figure 5.3). The lack of response, which was rather variable, was initially thought to be a result of oxidation of some of the sulphite by oxygen in the reagent and buffer. However, the lack of response was not prevented by de-gassing the solutions under vacuum.

The molar absorbances at 605nm for the reduction of DCPIP in the presence and absence of TNBS, were calculated from the linear portions of Figure 5.3 to be 10110 $l.mol^{-1}.cm^{-1}$ and 8070 $l.mol^{-1}.cm^{-1}$ respectively. These values correspond approximately to half the molar absorbance of DCPIP, which surprisingly suggests that at pH 8.0 2 moles of sulphite are required to reduce a mole of DCPIP. This was rather unexpected and was investigated further. These investigations are detailed in the Appendix.

Although the actual mechanism of the reaction is not as simple as initially hoped, the reaction between sulphite and DCPIP did produce a

Figure 5.4 The reaction of DCPIP with the sulphite by-product of the reaction between TNBS and glycine



Glycine (0-0.25µmol in 0.5ml of solution) was added to 0.5ml of 0.1M sodium phosphate buffer, pH 8.0, and 1ml of 0.2mM DCPIP in 0.05M sodium phosphate buffer, pH 8.0 containing 0.05% TNBS. The solutions were incubated for 2 hours at 37°C after which the decrease of absorbance was measured.

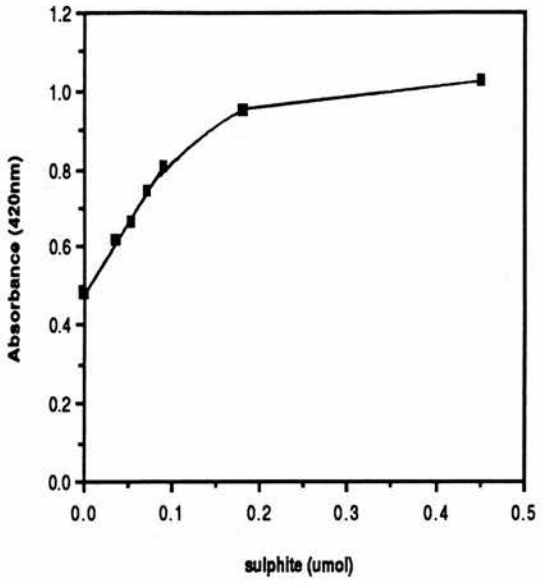
linear decline of the absorbance of DCPIP and was therefore considered useful for the measurement of sulphite liberated by TNBS during its reaction with peptides. By incubating the TNBS and DCPIP simultaneously with the peptides the DCPIP would be available for reduction as the sulphite was liberated from the TNBS. This would presumably minimise the loss of sulphite due to oxidation by oxygen in the reagents, which would be encountered if the DCPIP was not added until after the TNBS reaction had reached completion.

5.3 Measurement of amino groups with DCPIP and TNBS

The DCPIP and TNBS reagent reacted with a varied amount of glycine to give a linear decrease of absorbance at 605nm (Figure 5.4). The molar absorbance of the reduction of the DCPIP was 7230 $l.mol^{-1}.cm^{-1}$, which is slightly less than the molar absorbance for the reduction of DCPIP with sodium sulphite (8070 $l.mol^{-1}.cm^{-1}$; see Section 5.2.2). When the Tnp-glycine was measured in these standards as the increase of absorbance at 420nm, the resulting molar absorbance was calculated to be 6070 $l.mol^{-1}.cm^{-1}$. This is less than the molar absorbance of Tnp-glycine produced by the reaction of glycine with TNBS alone which was 7120 $l.mol^{-1}.cm^{-1}$ (see Section 4.2 and Figure 4.4). As expected, glycine did not produce a decline of the absorbance of DCPIP in the absence of TNBS.

An explanation for the discrepancy between the molar absorbance of TNBS with glycine in the presence and absence of DCPIP was provided by the work of Goldfarb (1966) which suggested that the absorbance at 420nm

Figure 5.5 The enhancement of the absorbance at 420nm of Tnp-glycine with sodium sulphite



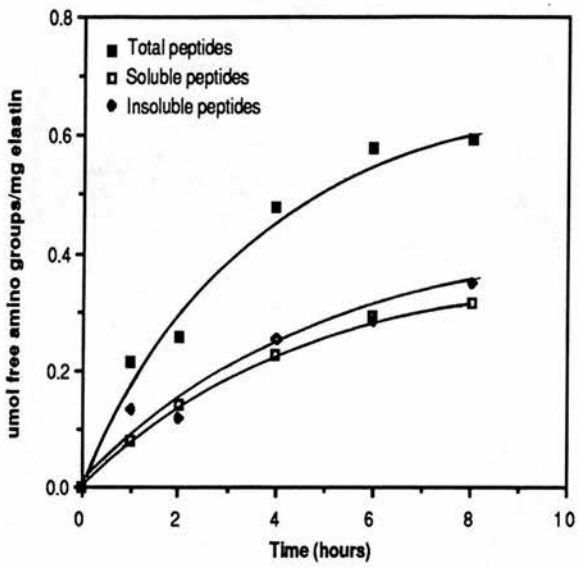
Tnp-glycine free from sulphite (0.06mM in 4% sodium bicarbonate solution) was prepared as detailed in the text. Tnp-glycine (1ml) was treated with sodium sulphite (0-0.45μmol) and the increase of absorbance at 420nm due to enhancement by the sulphite was measured. The final volume of each solution was 1.1ml.

of Tnp-glycine is enhanced by sulphite, with which it can form complexes. This was investigated by using Tnp-glycine free from sulphite prepared as detailed by Okuyama and Satake (1960). Briefly, 75mg of glycine in 200ml of 4% sodium bicarbonate solution was trinitrophenylated with 200ml of 0.1% TNBS. The solution was acidified to pH 1.0 with HCl. The solvent was removed by rotary evaporation leaving sulphite free Tnp-glycine which was re-extracted into 50% (v/v) methanol. The pure Tnp-glycine was dissolved in 4% sodium bicarbonate at a suitable concentration given that the molar absorbance of Tnp-glycine in 4% bicarbonate at 348nm is 1.54×10^4 $l.mol^{-1}.cm^{-1}$ (Okuyama and Satake, 1960).

Tnp-glycine free from sulphite had a lower absorbance at 420nm than Tnp-glycine treated with sulphite (Figure 5.5). The enhancement of the absorbance at 420nm of one mole of Tnp-glycine by equimolar sulphite was calculated from Figure 5.4 to correspond to 3050 $l.mol^{-1}.cm^{-1}$. This is more than sufficient to account for the discrepancy of 1050 $l.mol^{-1}.cm^{-1}$. The reason why the discrepancy is less than the observed molar absorbance of enhancement is probably that the TNBS solution probably contained some sulphite which resulted from the reaction of TNBS with water.

It was concluded that the soluble peptides may be measured with greater sensitivity as the increase of absorbance at 420nm after their reaction with TNBS alone. The reduction of DCPIP with the sulphite by-product of the TNBS reaction, measured as a decrease of absorbance at 605nm, was established to be a suitable method for measuring all peptides. However, it was necessary to standardise the method using a soluble amino

Figure 5.6 The measurement of peptides resulting from the digestion of Lansing lung elastin with HNE for 8 hours



Suspensions of Lansing lung elastin (3mg/ml in 0.1M sodium phosphate buffer/0.15M NaCl, pH 7.4) were digested with HNE (10mU/ml) for 8 hours at 37°C. Duplicate aliquots were removed at intervals. The soluble amino groups in the supernatant of one aliquot were measured by the TNBS method and the total amino groups in the second aliquot were measured by the DCPIP and TNBS method. The amount of total and soluble peptides were estimated by reference to the appropriate glycine standard curve, and the amount of insoluble peptides was obtained by difference.

compound e.g., glycine because a suitable insoluble model was not available.

5.4 Application of the DCPIP and TNBS method to the estimation of total elastin-derived peptides

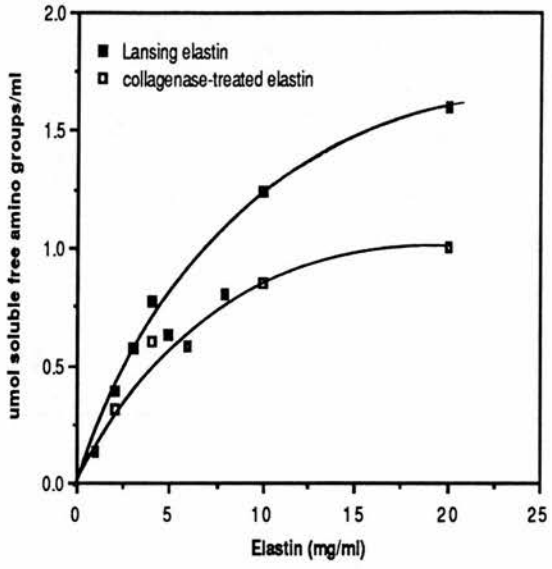
Suspensions of Lansing lung elastin were digested for 8 hours as detailed in Section 2.3.1 with HNE (10mU/ml). Aliquots were removed periodically. Half of each aliquot was used to measure the soluble peptides with TNBS, and the other half was used for total peptide estimation with DCPIP and TNBS. A typical result is shown in Figure 5.6 and suggests that during a digestion with HNE up to 8 hours, Lansing lung elastin is hydrolysed to give similar amounts of soluble and insoluble products.

Having established that the use of DCPIP and TNBS is a valid method of evaluating elastin digestion, it was applied to the study of the digestion patterns produced during the proteolysis of the various elastin preparations with HNE and HNCG.

CHAPTER 6

THE DIGESTION OF ELASTIN WITH HNE

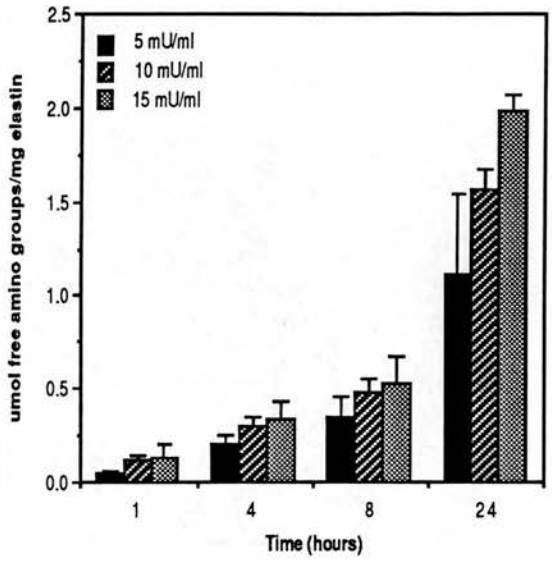
Figure 6.1 Soluble peptide production/24 hours from a varied amount of lung elastins digested with a constant amount of HNE



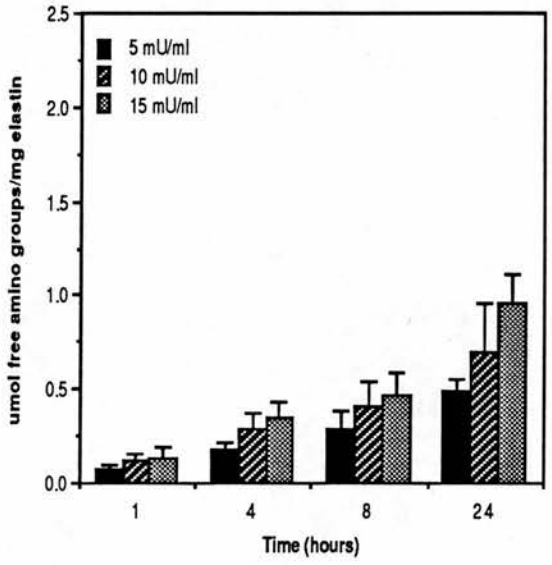
Suspensions of Lansing lung elastin (■) and collagenase-treated elastin (□) (0-20mg/ml in 0.1M sodium phosphate buffer/0.15M NaCl, pH7.4) were digested with a constant amount of HNE for 24 hours at 37°C. The soluble peptides were collected and measured by the TNBS method (section 2.3.3).

Figure 6.2 The digestion of lung elastins with HNE

a. Lansing elastin



b. Collagenase-treated elastin



Suspensions of lung elastins (3mg/ml in 0.1M sodium phosphate buffer/0.15M NaCl, pH 7.4) were digested with HNE (5, 10 or 15mU/ml) at 37°C (section 2.3.1). The amounts of soluble peptides were measured after 1, 4, 8 and 24 hours of digestion (section 2.3.3). Each data point corresponds to the average of triplicate digestions of each of the three preparations of each type of elastin.

CHAPTER 6

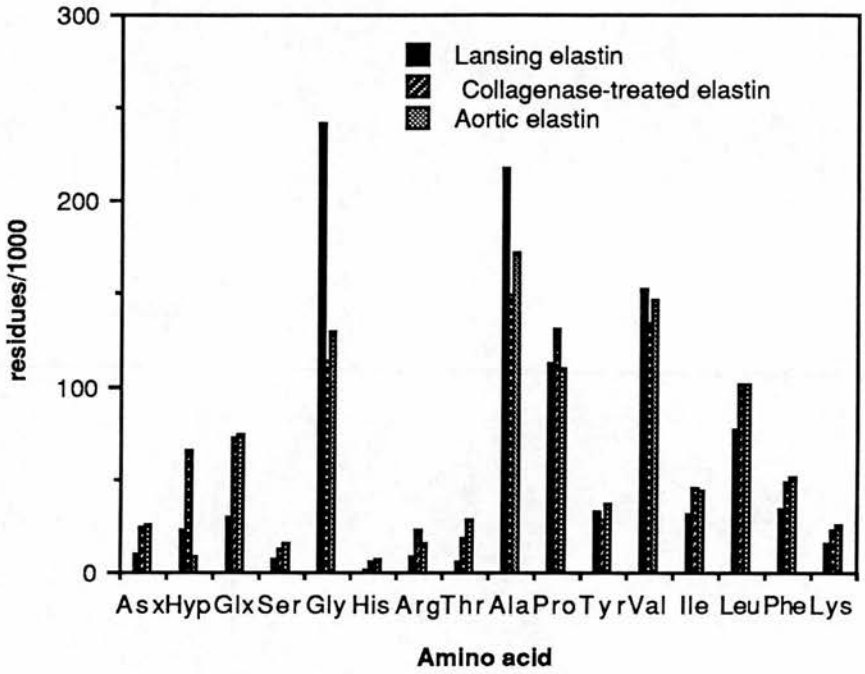
THE DIGESTION OF ELASTIN WITH HNE

The digestion patterns produced when the elastin preparations were digested with HNE were investigated.

6.1 The investigation of the difference between the susceptibilities of Lansing lung elastin and collagenase-treated elastin to digestion by HNE

In Section 4.3 it was shown that Lansing elastin was apparently more susceptible than collagenase-treated elastin to proteolysis by HNE at 37°C when the extent of proteolysis was measured at 24 hours. The difference between the preparations was most noticeable when larger amounts of elastin were digested with a constant amount of enzyme for a fixed length of time (Figure 6.1); or when a constant amount of the elastin (3mg/ml) was digested with HNE (5-15mU/ml) for more than 8 hours (Figure 6.2). The difference between the amounts of soluble peptides produced after 24 hours of digestion with HNE from Lansing elastin and collagenase-treated elastin was never more than 2.5 fold. Stone et al (1987) observed that collagen and elastin from cultured cells are capable of binding similar amounts of HNE, but collagen is less susceptible to digestion. The contamination of the collagenase-treated elastin with collagen was documented in Sections 3.2.2-3.2.3. The amino acid compositions of the soluble elastin-derived peptides released from Lansing lung elastin, collagenase-treated elastin

Figure 6.3 Amino acid composition of the soluble peptides resulting from digestion by HNE



Suspensions of Lansing elastin, collagenase-treated elastin and aortic elastin were digested with HNE (10mU/ml) for 24 hours at 37°C. The soluble peptides were collected, after which they were hydrolysed and their amino acid compositions were determined (see section 2.2.3)

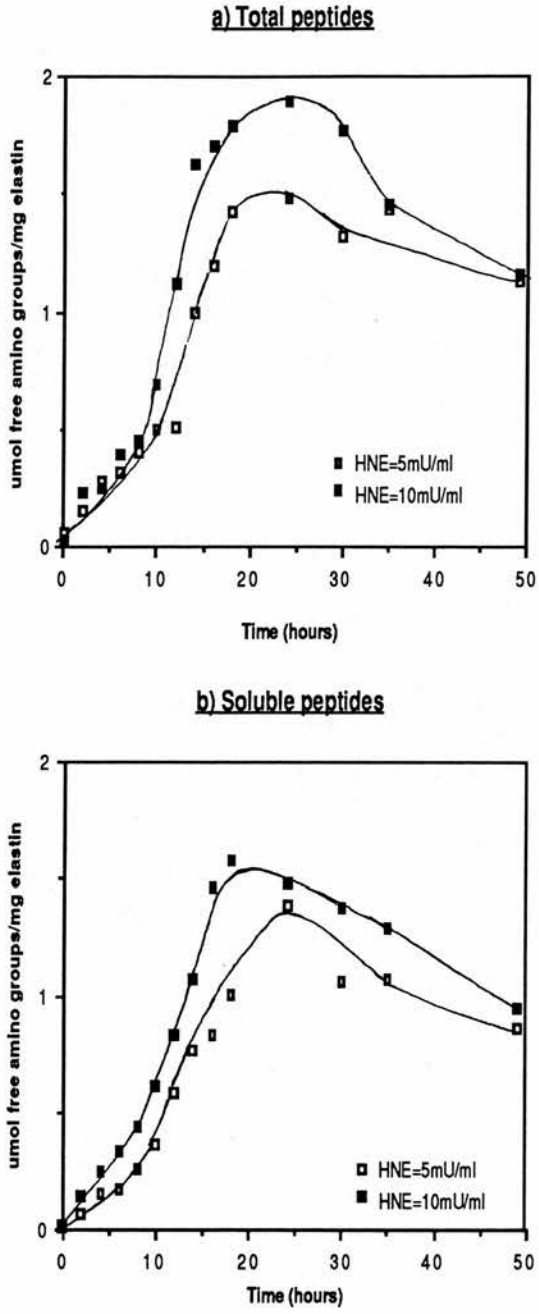
and aortic elastin after digestion with HNE (10mU/ml) for 24 hours (Section 4.1.1) were obtained as detailed in Section 2.2.3, to permit the assessment of the contribution of collagen to the peptides by means of the content of hydroxyproline (Figure 6.3). The soluble peptides produced after the digestion of collagenase-treated elastin with HNE were relatively rich in hydroxyproline, suggesting that some of these peptides were derived from collagen. Therefore, the collagen contaminating the collagenase-treated elastin probably acts as a competing substrate for HNE.

The soluble peptides released from Lansing lung elastin had essentially the same composition as the native Lansing elastin (Table 3.5). In comparison, the soluble peptides released from aortic elastin after 24 hours of digestion with HNE contained a greater amount of aspartic acid and glutamic acid and a smaller amount of glycine than the native aortic elastin. This may simply imply that the digestion of aortic elastin allows the release of any contaminating microfibril interpenetrated within the aortic elastin which was not removed during the isolation of the aortic elastin (see Section 3.2.4). The peptides derived from collagenase-treated elastin also contained a higher than expected amount of glutamic acid, this may be another consequence of the contamination of collagenase-treated elastin with collagen since collagen contains a greater amount of glutamic acid than elastin.

6.1.1 Standardisation of the digestion conditions

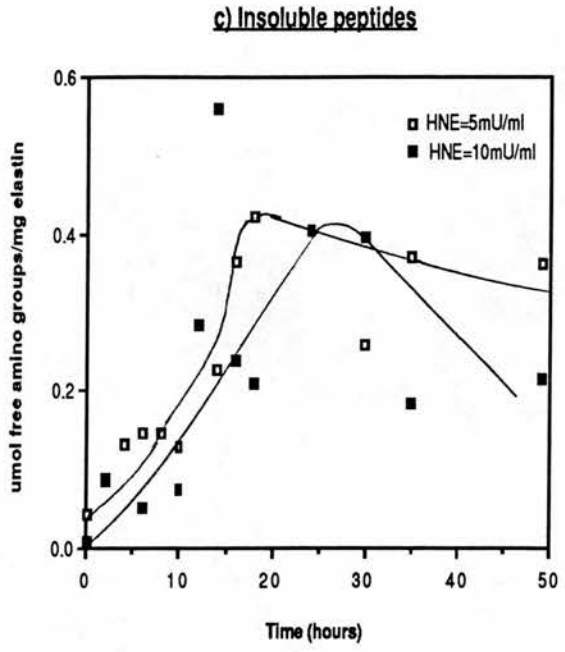
For the purposes of all future digestions, 3mg/ml suspensions of elastin (the concentration used by Boudier et al, 1981) were digested in

Figure 6.4 The digestion of Lansing lung elastin with HNE



Suspensions of Lansing lung elastin were digested with HNE (5 or 10mU/ml) for up to 48 hours, as detailed in section 2.3.1. Aliquots were removed periodically and the (a) total and (b) soluble products were measured as detailed in sections 2.3.3 and 2.3.4 .

Figure 6.4 The digestion of Lansing lung elastin with HNE (continued)



Suspensions of Lansing lung elastin were digested with HNE (5 or 10mU/ml) for up to 48 hours, as detailed in section 2.3.1. Aliquots were removed periodically and the (c) insoluble products were measured as detailed in section 2.3.5.

0.1M sodium phosphate buffer, pH 7.4, containing 0.15M NaCl, at 37°C. These conditions of temperature, pH and NaCl concentration were used by Reilly and Travis (1980) (see Section 2.3.1). The data presented in Figure 6.2 showed that some variation may be expected from digestion to digestion, so all future digestions were performed at least once on each sample of elastin, and a typical set of data is presented.

6.2 The digestion of elastin over a prolonged period of time

Boudier et al (1981) and Reilly et al (1984) were primarily interested in comparing the initial rate of soluble peptide release from elastin in response to digestion by different enzymes, so it was not necessary for them to follow the digestions to equilibrium.

In order to study the products of a digestion it is of some importance to know when the reaction has reached completion. In a case such as this where the products of the reaction will be studied, it is important to know if the peptides were intermediates or if they were end-products of a digestion which had reached completion. So preparations of elastin were digested for a prolonged period of time in an attempt to establish how long the digestion took to reach its equilibrium.

6.2.1 Digestion of Lansing lung elastin

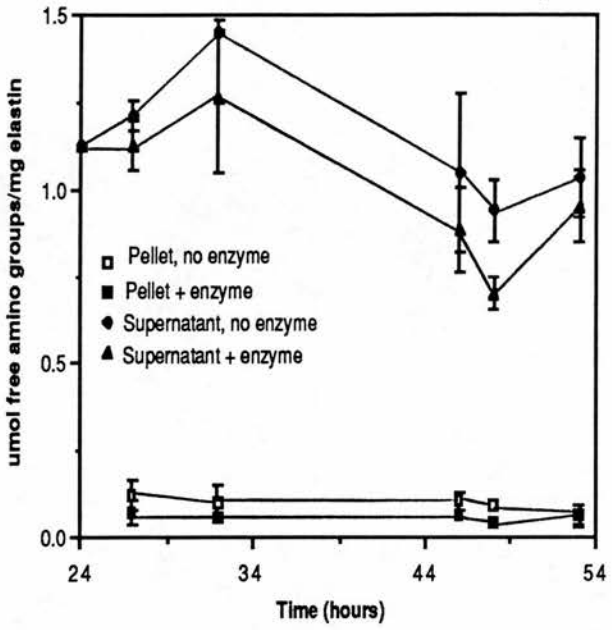
Lansing lung elastin was digested for 48 hours with two doses of HNE (5 or 10mU/ml, specific activity 0.324U/mg). The total, soluble and insoluble amino groups were measured at regular intervals (Figures 6.4 a, b and c). In

all cases the amount of peptides reached a maximum after approximately 24 hours of digestion, followed by a rather surprising decline of all the amino groups; this pattern also occurred when the elastin was digested in 0.02M sodium phosphate buffer/0.15M NaCl, pH 7.4 (phosphate buffered saline). The samples were usually frozen after collection, and the peptides measured within 48 hours.

It was assumed that freezing the samples would be sufficient to stop further digestion by the enzyme. This was investigated by measuring the amount of soluble amino groups in 3 samples before and after freezing. The amounts did not differ, so confirming that freezing was sufficient to stop the digestion. A second conclusion from this fact is that the freeze-thaw cycle inflicted on the samples was not responsible for the apparent loss of amino groups.

The fact that the amount of peptide production reached a maximum suggests that either the enzyme was no longer active or the maximum amount of digestion had occurred. Attempts were made to measure the amount of enzyme activity remaining after 24 hours of digestion. Lansing lung elastin was digested with HNE (10mU/ml) for 24 hours at pH 7.4 and 37°C. The soluble material was separated from the remaining insoluble material by centrifugation as detailed in Section 2.3.2. A sample of the supernatant (0.1ml) was added to 0.9ml of 50mM Tris-HCl/1.0M NaCl pH 8.0, and 10µl of 20mM STANA. The solution was incubated at 37°C for an hour, after which the absorbance at 410nm of the solution was measured against a control solution which was identical except that the STANA was

Figure 6.5 The effect of adding HNE to fractions of pre-digested elastin

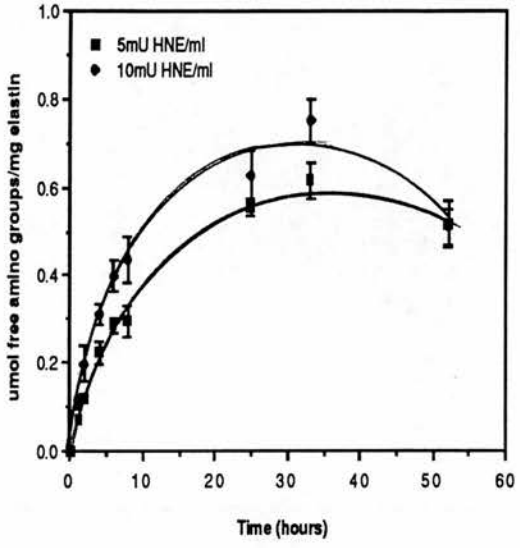


A suspension of elastin was digested for 24 hours with HNE (5mU/ml) at 37°C and pH 7.4. The soluble and insoluble material was separated by centrifugation and the insoluble pellet suspended in fresh buffer. The supernatant and the pellet suspension were divided in half. One half of each was treated with more enzyme (5mU/ml) (□ and ◇); and the other half represented an untreated control (■ and ▲). Aliquots were removed periodically up to 54 hours from the initial time of digestion, and the soluble peptides were measured by the TNBS method (see section 2.3.3).

omitted. The amount of enzyme activity, measured against STANA, which was detectible in the digestion mixture after 24 hours of digestion typically corresponded to 25% of the initial enzyme activity. This represents the minimum amount of remaining enzyme, because it does not include the enzyme which was preferentially digesting the soluble elastin-derived peptides or the enzyme which was adsorbed on the elastin and had been removed by centrifugation.

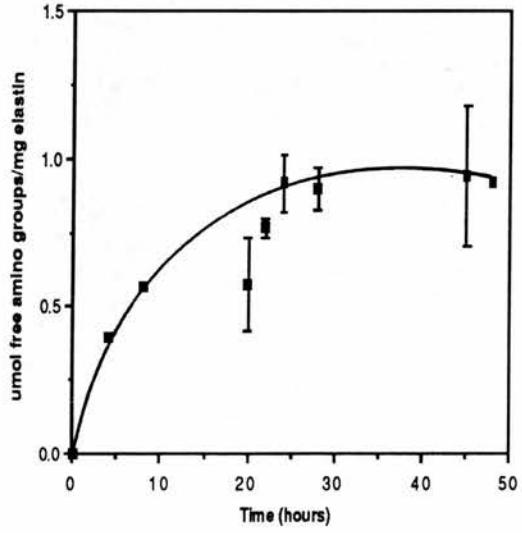
Having established that some of the enzyme activity was still present after 24 hours of digestion, it was concluded that peptide production did not cease after 24 hours because there was no active enzyme present. The possibility still remained that the maximal point of digestion after 24 hours represented the fact that no further bonds in the elastin could be hydrolysed; this was investigated. Suspensions of elastin were pre-digested with HNE for 24 hours and 37°C, after which the insoluble material was removed from the soluble peptides and suspended in fresh buffer. The pellet suspension and supernatant were divided approximately in half, and one half of each was treated with a second dose of enzyme. Aliquots were removed periodically and the soluble peptides were measured (Figure 6.5). Very little further digestion of the pellets occurred, even when a second dose of enzyme had been added, suggesting that no further enzyme could be productively adsorbed onto the elastin or that the hydrolysis of the elastase-susceptible bonds was complete. The addition of further enzyme did not produce any increase in the amount of soluble peptides or prevent the decline in free amino groups. So the apparent decline in the amino groups was not due to a lack of active enzyme.

Figure 6.6 The measurement of the soluble peptide produced by the digestion of collagenase-treated elastin with HNE



Suspensions of collagenase-treated elastin were digested with HNE (5 or10mU/ml) at 37°C and pH 7.4 for up to 54 hours (section 2.3.1). Aliquots were removed periodically and the amount of soluble peptides were measured by the TNBS method (section 2.3.3). Each data point was measured in triplicate.

Figure 6.7 The measurement of the soluble peptides produced by the digestion of aortic elastin with HNE



Suspensions of aortic elastin were digested with HNE (10mU/ml) at 37°C and pH 7.4 for 48 hours (section 2.3.1). Aliquots were removed periodically and the amount of soluble peptides were measured by the TNBS method (section 2.3.3). Each data point was determined at least in duplicate.

6.2.2 Digestion of collagenase-treated elastin

As expected, when collagenase-treated elastin was digested with HNE (5-10mU/ml) the maximum amount of detectible amino groups was less than the maximum amount of soluble peptides from Lansing elastin; probably as a result of the contamination with collagen (see Section 6.1). Once again, a decline in the amount of soluble amino groups was observed (Figure 6.6) although this was later than the observed declines in Lansing elastin-derived peptides.

6.2.3 Digestion of aortic elastin

When aortic elastin was digested with HNE (10mU/ml) the initial amount of soluble peptides (Figure 6.7) increased in a hyperbolic way. Maximal digestion was reached after 24 hours, and there was no significant decline of the peptides between 24 and 48 hours of digestion.

6.2.4. Comments and comparisons with the work of other authors

The hyperbolic relationship between the amount of lung elastin and the amount of soluble peptide production per 24 hours of digestion (Figure 6.1) was not surprising since Baici (1990) stated that there is a hyperbolic relationship between the initial velocity of digestion with HNE and the amount of bovine neck ligament elastin which was digested. Although it should be noted that a direct comparison between initial velocity and an apparent velocity is not totally justified because the calculation of the

apparent velocity assumes that the velocity over 24 hours was constant, when in actual fact it is not (Figure 6.4b).

Baici (1990) also presented kinetic data which supported the mechanism of digestion proposed by Robert et al (1974). They suggested that elastase is initially adsorbed onto the surface of the elastin, predominantly by hydrophobic interaction, after which there is a limited proteolysis to expose the interior of the elastin, providing further sites for enzymic attack. As the amount of sites increases, so the amount of products increases almost exponentially until a steady state is approached. The digestion of Lansing lung elastin with HNE (Figure 6.4) was initially slow followed by a rapid increase in the amount of products up to 24 hours of digestion, so it appeared to comply with the proposed mechanism of elastin digestion.

The digestion of elastin produces a conformational change, disrupting the stability and hydrophobic interactions within the elastin (Robert et al, 1974). This is accompanied by a decrease in the affinity between the enzyme and elastin. Therefore, it is reasonable to suggest that insoluble pre-digested elastin (Figure 6.5) was not digested any further by the addition of fresh enzyme because few susceptible bonds remained.

The digestion of collagenase-treated elastin appeared to follow a normal reaction course without an initial accelerating stage. This is probably because the early products of enzyme attack are derived from collagen (see Section 6.1.2) which obscures the pattern of elastin digestion.

Similarly, the initial digestion of aortic elastin appeared to follow a normal reaction course unlike the initial accelerating progression of the

Lansing lung elastin digestion. This may be a consequence of the different methods used for the isolation of the two types of elastin, which provided an aortic elastin preparation which may be contaminated with microfibril (see Section 3.2.4). Alternatively, it could be related to the morphological difference between the elastins. Lung elastin, like neck ligament elastin, exists as fibres, which may be unidirectional, whereas aortic elastin is composed of fenestrated lamellae (Cleary and Cliff, 1978). Evidence to suggest that the morphology may be significant was presented by Baici (1990) who observed that porcine aortic elastin was digested with HNE at a greater initial velocity than bovine neck ligament elastin. In comparing the apparent velocity of the digestion of Lansing lung elastin and aortic elastin as the amount of soluble peptides released over the first 4 hours of digestion (Figures 6.4b and 6.7) it may be tentatively suggested that the aortic elastin is initially digested with a greater velocity than Lansing lung elastin. However, this could only be confirmed if the preparation of aortic elastin used in the digestion was confidently shown to be devoid of any possible signs of glycoprotein contamination.

More direct evidence to suggest that the peptides from aortic elastin may differ from peptides derived from Lansing elastin was presented in Figure 4.1. The quantity of peptides from aortic elastin detected by the BCA reagent was greater than that with biuret reagent, but with Lansing lung elastin peptides the reverse was true. This would suggest that the large peptides from aortic elastin and Lansing elastin are different in composition (detailed in Section 4.1.2). Although it is possible that this difference is simply a reflection of the fact that some of the digestion products of the aortic

elastin may be derived from the microfibril component which is interpenetrated in the elastin.

6.3 The investigation of the apparent disappearance of the amino groups

The apparent decline of the response of the peptidyl amino groups to TNBS between 24 and 48 hours of digestion of Lansing lung elastin with HNE suggests that there is a disappearance of the amino groups which was unexpected and deserved further attention.

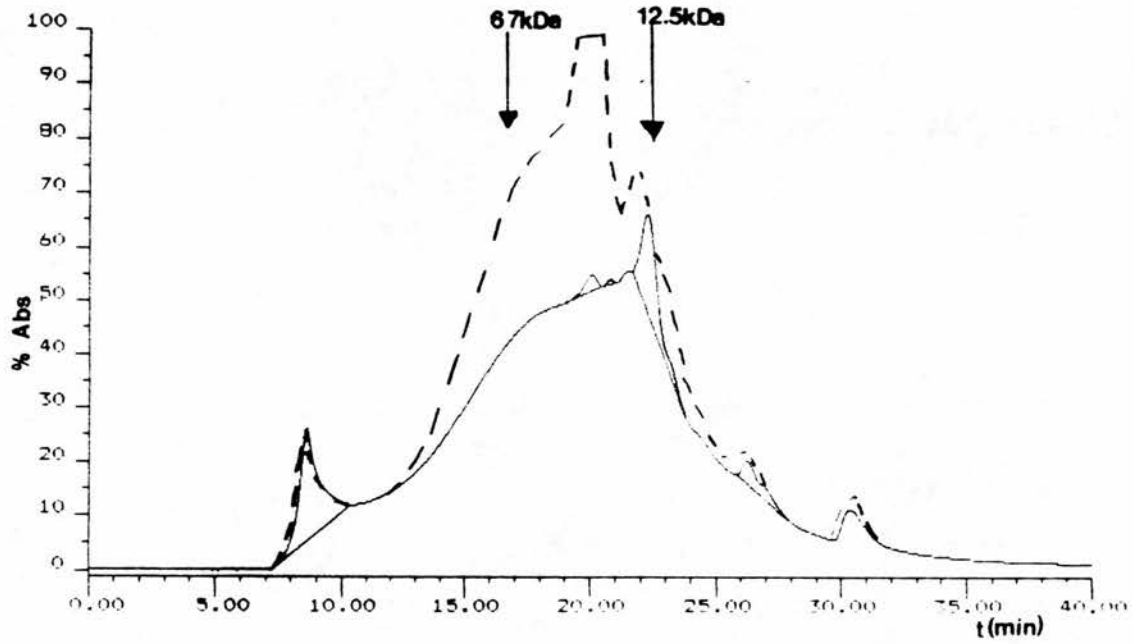
A simple explanation would be that the amino groups are slowly modified by a component in the digestion buffer, but a phosphate buffer contains no components likely to modify amino groups so this explanation was rejected. A second explanation would be that the amino groups become physically buried within an aggregate of peptides. This possibility would appear unlikely, but for the fact that elastin-derived peptides are known to associate together hydrophobically to form coacervates (Partridge et al, 1955a). Another explanation would be that the amino groups undergo a chemical reaction with some component of the elastin. This is possible because aldehyde cross-link intermediates may be present in some of the peptides (see Section 1.3), and are capable of forming Schiff's bases with the amino groups of other peptides. The cross-link intermediates of undigested elastin are locked in a situation which is inaccessible to amino groups. So, the apparent loss of amino groups may be a consequence of coacervation and/or new bond formation via the amino groups. The problem was investigated further.

6.3.1 Coacervation of elastin

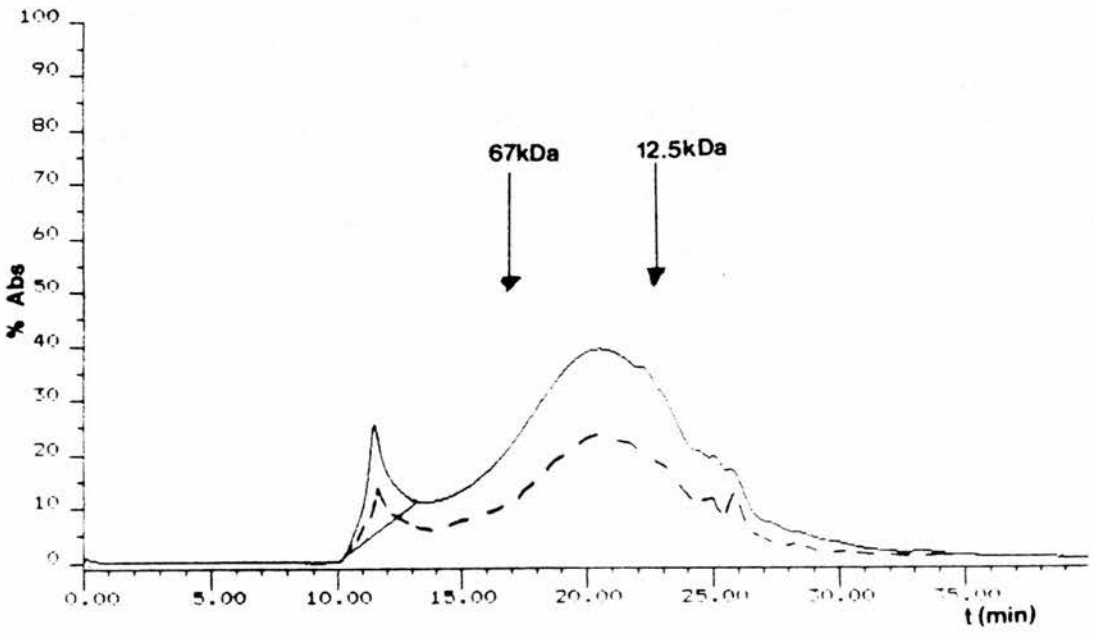
Partridge et al (1955a) chemically digested bovine ligamentum nuchae elastin with 0.25M oxalic acid at 100°C for several hours. The resulting soluble peptides were termed α-elastin and β-elastin. β-Elastin had a mean molecular weight of 5500 and consisted of 2 chains each containing 27 amino acids (Partridge et al, 1955b). α-Elastin was much larger, having a mean molecular mass of 60,000-84,000 and consisted of 17 chains each containing 35 residues. Partridge et al (1955a) observed that under certain conditions of pH, temperature and ionic strength, molecules of α-elastin were capable of associating together hydrophobically to form coacervates. Speculative evidence to suggest that the peptides produced by the digestion of Lansing lung elastin with HNE for 24 hours at 37°C form coacervates was presented by Kucich et al (1985). They used polyclonal antibodies to measure elastin-derived peptides and observed that the recovery of peptides incubated in plasma, which contained inhibitors of the major classes of proteolytic enzymes, for 24 hours at 37°C was less than the recovery at 16°C. The loss was presumed to be due to coacervation. This assumes that some of the peptides were buried within coacervates making them inaccessible to the antibodies.

Figure 6.8 Molecular exclusion chromatography of soluble elastin-derived peptides resulting from the digestion of Lansing lung elastin with HNE

a) Detection of the absorbance at 220nm of the eluate



b) Detection of the absorbance at 270nm of the eluate



Suspensions of Lansing lung elastin were digested with HNE (10mU/ml) for 48 hours. The soluble peptides (0.2ml) were isolated after 24 (———) and 48 (– – – –) hours of digestion. The peptides were examined by molecular exclusion chromatography as detailed in the text. The absorbance of the eluate was measured at (a) 220nm and (b) 270nm. 100% corresponds to an absorbance at 220nm of 0.4 or an absorbance at 270nm of 0.1. The path length of the flow cell was 1cm.

6.3.2 The investigation of an aggregation of the soluble peptides

Wood (1958) stated that a production of a coacervate of elastin may be observed as the formation of a cloudy suspension which can be dissolved immediately after formation by heating to 40°C in 0.075M sodium acetate buffer, pH 5.0. The solution of soluble elastin-derived peptides produced after the digestion of Lansing lung elastin with HNE for 24 hours appeared to be cloudy, suggesting that some of the peptides had formed coacervates. It was presumed that the coacervates could be isolated by high speed centrifugation, dissolved in buffer and then the amount of peptides in the coacervate measured by the methods detailed in Sections 2.3.2-2.3.3

Lansing lung elastin was digested with HNE (10mU/ml) as detailed in Section 2.3.1 for 48 hours. After 24 hours of digestion the suspension of peptides was visible cloudy, even after they had been filtered through a 0.45μm filter. Samples of the soluble peptides were removed after 24 and 48 hours of digestion, and examined by molecular exclusion chromatography using an Anagel-TSK G3000 SWXL column which was developed with 50mM sodium phosphate buffer/0.15M NaCl, pH 7.4 as detailed in Section 2.4.1 (Figure 6.8). The resulting elution profiles suggested that the proportion of large peptides increased between 24 and 48 hours of digestion. It also suggested that the peptides are significantly larger than predicted in Chapter 4 (see Section 4.3), confirming that the protein assay underestimates the amount of peptides. Coacervates are

large molecular weight aggregates. So, it seemed possible that the increase in the proportion of larger peptides may result from an increase of coacervation of the soluble peptides between 24 and 48 hours of digestion.

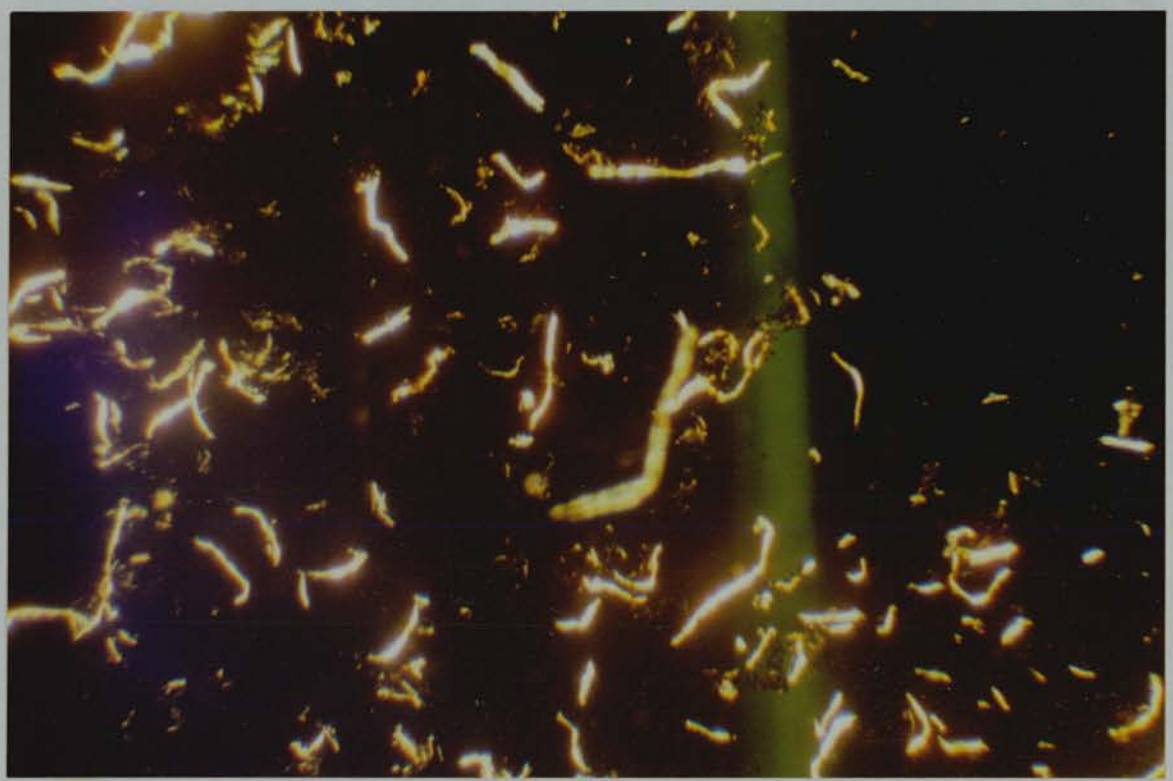
Further samples were removed after 24 and 48 hours of digestion, and the cloudy supernatant was decanted from the remaining insoluble elastin. The supernatants (0.5ml) were centrifuged at 100,000 rpm for 15 minutes at 4°C in a TL-100 Ultracentrifuge (Beckman). The precipitate was dissolved in 0.1M sodium phosphate buffer/0.15M NaCl pH 7.4 (0.1ml) and the amounts of peptides in both the supernatant and dissolved precipitate were measured. The percentage of peptides which precipitated was greater in the case of the products of 48 hours of digestion, when measured in molar terms (by the TNBS method) and in terms of protein (by the BCA method). Therefore the amount of peptides capable of coacervating appeared to increase between 24 and 48 hours of digestion.

6.3.3 The investigation of the aggregation of the insoluble material remaining after digestion

The apparent loss of the total and insoluble amino groups (Figures 6.4b and c) suggests that the amino termini of insoluble pellet were also capable of some form of aggregation or coacervation. Kham et al (1982) suggested that elastic coacervates are birefringent, if so the coacervates or aggregates of the insoluble material could be examined microscopically under polarised light. The insoluble material from elastin which had been digested with HNE for 6, 24 and 48 hours was examined by light microscopy

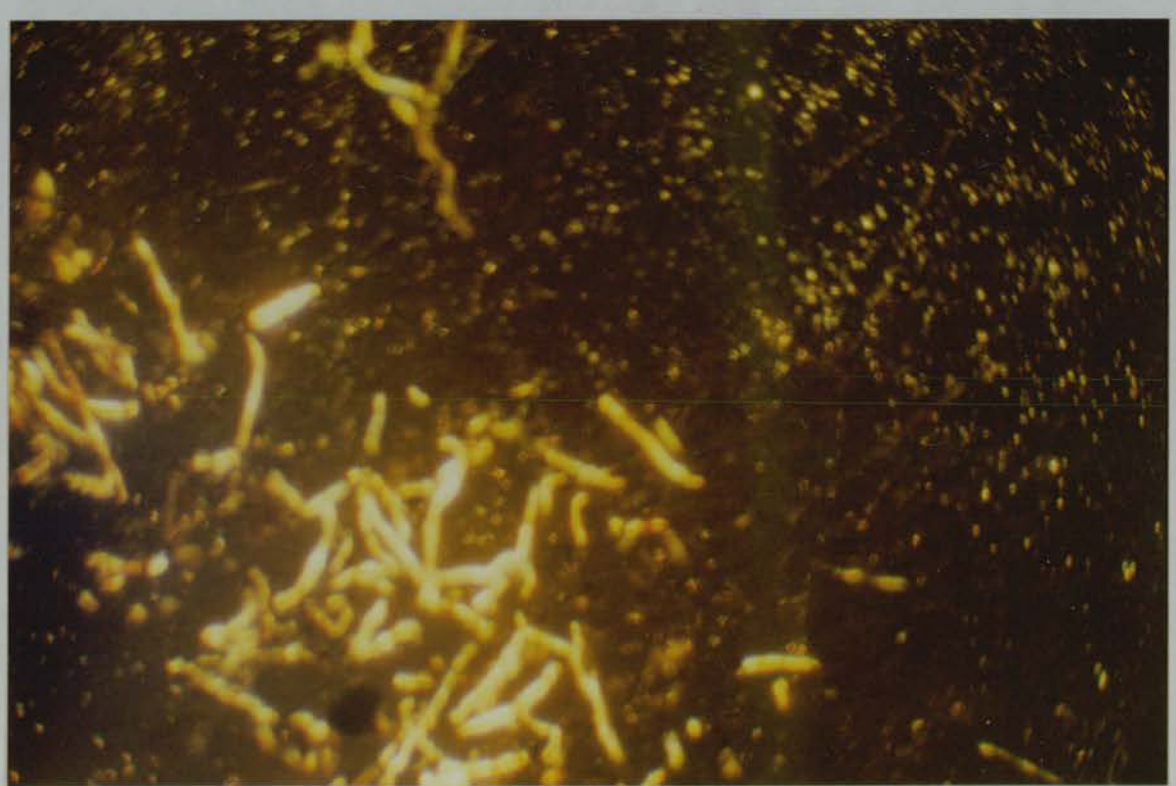
Figure 6.9 Light microscopic examination of the insoluble material remaining after the digestion of elastin with HNE

a) 6 hours of digestion



13.4 x magnification

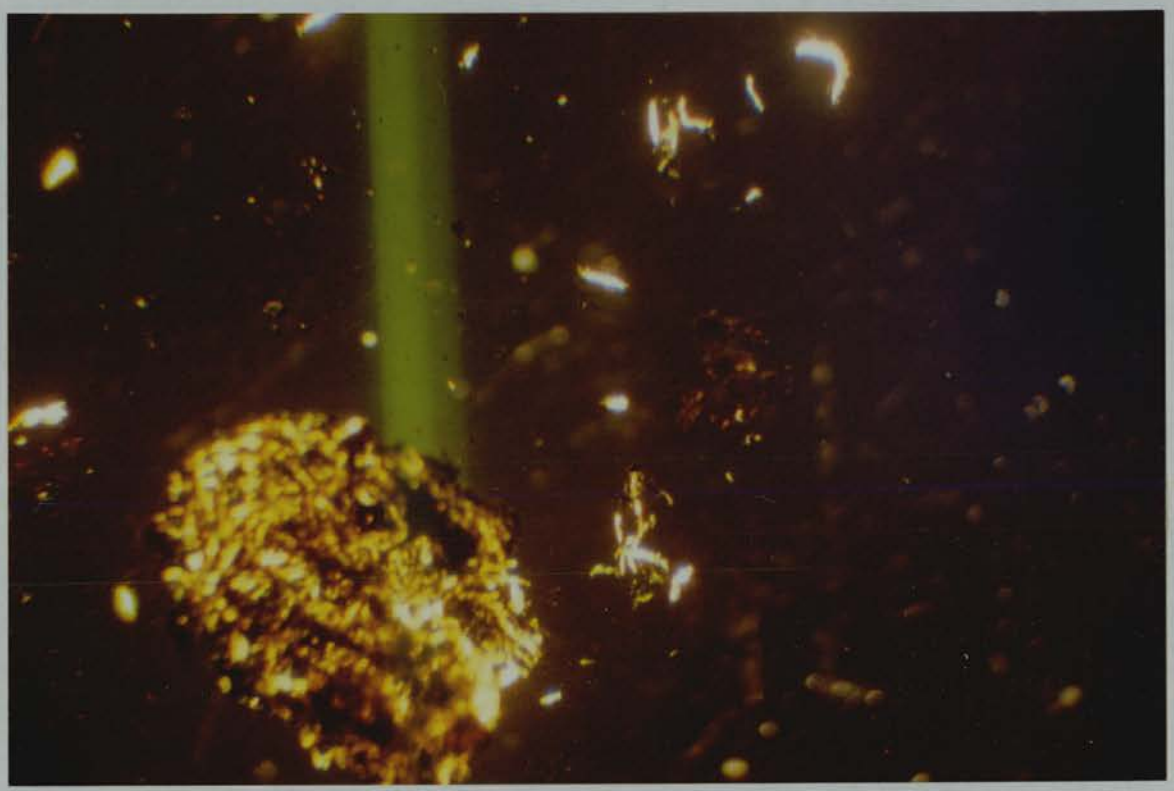
b) 24 hours of digestion



13.4 x magnification

Figure 6.9 **Light microscopic examination of the insoluble material remaining after the digestion of elastin with HNE (continued)**

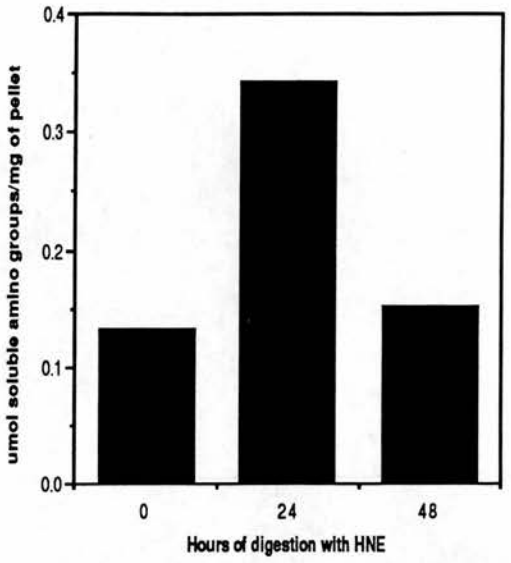
c) **48 hours of digestion**



13.4 x magnification

The insoluble elastin was isolated after (a) 6, (b) 24 and (c) 48 hours of digestion with HNE as detailed in the text. The material was suspended in 1% (w/v) resorcin-fuchsin/4M HCl (Weigert's stain; BDH) and viewed under polarised light using a Nikon SMX/10 microscope. The image was photographed using an attached camera (Nikon) on 35mm, 100ASA film (Kodak). The same pattern was observed when the resorcin-fuchsin was replaced by 0.1M sodium phosphate buffer, pH 8.0.

Figure 6.10 Measurement of the soluble peptides solubilised from digested elastin by 0.1M NaOH



Undigested Lansing lung elastin, and the insoluble material remaining after 24 or 48 hours of digestion with HNE (approximately 2mg of each sample) were subjected to alkaline hydrolysis with 0.1M NaOH as detailed in the text.

under polarised light (Figure 6.9). It was evident that the birefringent elastin particles seemed to associate together in a disordered fashion after approximately 24 hours of digestion. A visible change in the consistency of the insoluble material was also noticed. The insoluble material was seen to associate together like rubber and was very difficult to disrupt. The implication was that partially digested insoluble elastin is capable of re-associating in a disordered but stable fashion.

According to Gotte et al (1963) partially digested insoluble elastin becomes susceptible to alkaline hydrolysis because the previous stabilisation by hydrophobic interaction and covalent cross-links is no longer as strong. Therefore, after 24 hours of digestion of Lansing lung elastin with HNE the insoluble remaining elastin is probably sensitive to alkaline hydrolysis. If coacervation or aggregation of the remaining fragmented material then followed and there was some form of stabilisation of the aggregate it could be predicted that resistance to alkaline hydrolysis may be re-established by 48 hours of digestion. This was investigated.

Elastin was digested with HNE (10mU/ml) for 24 and 48 hours, and the remaining insoluble material was washed several times with distilled water before freeze-drying. Attempts were made to hydrolyse the pellets (1-2mg) by suspending them in 0.75ml of 0.1M sodium hydroxide solution and boiling for 45 minutes. The amount of soluble peptides resulting from alkaline hydrolysis of the insoluble material was measured by the TNBS method (see Section 2.3.3) in 0.2ml of supernatant after it had been neutralised by the addition of 5µl of 4M HCl (Figure 6.10). The results suggested that by 24 hours further enzymic digestion decreased the quantity

of insoluble products so much that the amount that could be solublised after 48 hours of digestion was less than that at 24 hours. Alternatively, between 24 and 48 hours of digestion the insoluble material may be stabilised in such a way as to re-establish alkaline resistance. Such a stabilisation is unlikely to be produced by coacervation alone suggesting that some kind of new bond formation must occur in order to make this a valid explanation.

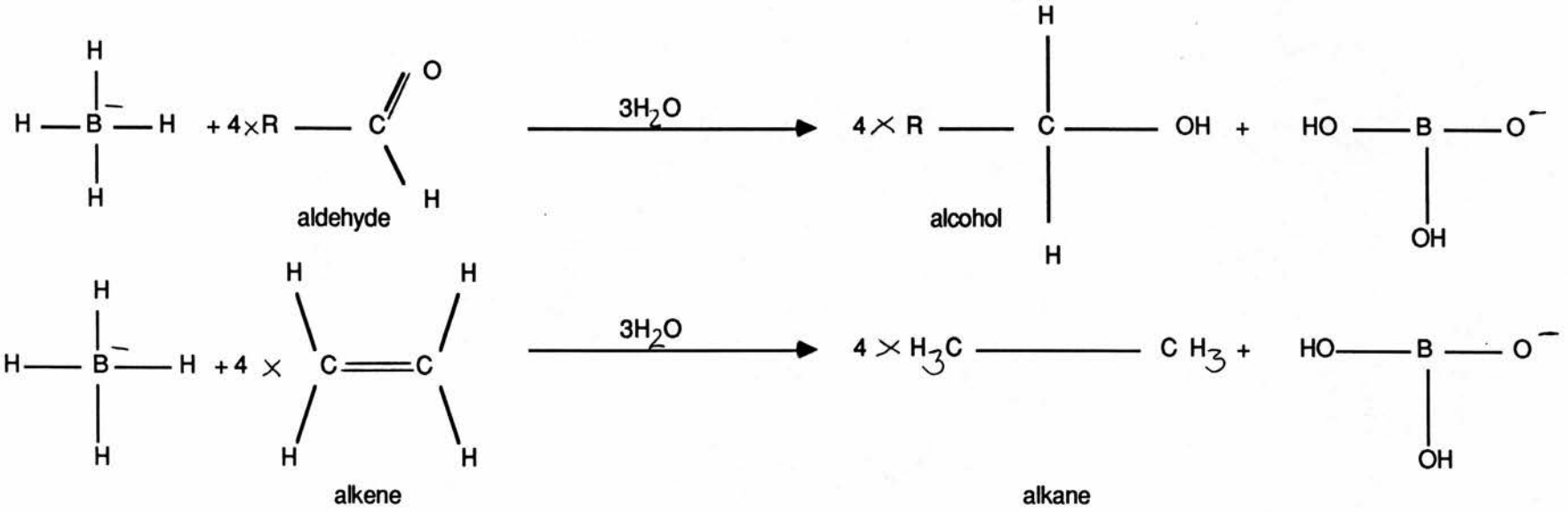
A temporary loss of resistance to alkaline hydrolysis was also observed by Stone et al (1988) who studied stabilised layers of rat aortic smooth muscle cells in continuous culture. The cell layers were treated with PPE (50μg or 75μg). At various times after the addition of the enzyme, a cell layer was homogenised and incubated with 0.1M sodium hydroxide at 98°C for 45 minutes. The amount of insoluble elastin which remained after this treatment was measured by amino acid analysis and expressed as a percentage of the amount of elastin found in control cell layers which had not been subjected to proteolysis. They found that more than 90% of the remaining elastin was alkaline sensitive immediately after proteolysis. However, 2 weeks after treatment with 50μg of PPE or 4 weeks after treatment with 75μg of PPE the cell layers had re-established their resistance to alkali.

6.3.4 Investigation of the stabilisation of the aggregates or coacervates

The re-establishment of alkali resistance suggests that a stabilisation of aggregates of insoluble partially digested elastin may have occurred. It

has been stated that coacervation is reversible (Partridge et al, 1955a). However, Wood (1958) stated that coacervates can be difficult to dissolve in 0.075M sodium acetate buffer pH 5.0 if some time has elapsed since their formation. This suggested that a slow stabilisation occurred, which Wood (1958) concluded to be due to an increase in the extent of aggregation. Stone et al (1988) suggested that the restoration of alkaline resistance was only in part due to the formation of hydrophobic interactions. They speculated that the elastin may be repaired and stabilised by the formation of Schiff's bases between aldehyde intermediates of cross-link formation within the damaged elastin or newly synthesised tropoelastin, and amino termini of the elastin-derived peptides generated during proteolysis (see Section 1.3). Cross-link intermediates, such as residues of allysine (α-aminoadipic-δ-semialdehyde) and aldol condensation products, have been detected in the amino acid hydrolysate of human and bovine elastin and measured by several authors including Lent et al (1969) and Paz et al (1976). The existence of these intermediates has been used to explain the discrepancy between the lysine content of tropoelastin and that of mature elastin, including the lysine accommodated within cross-links (see Section 3.2.2 and Francis et al 1973). The possibility arises that the restoration of resistance to alkali of the aggregate may be due to the formation of Schiff's bases such as lysinonorleucine (Franzblau et al, 1969) between aldehyde intermediates of some peptides and the amino termini of other peptides. This would account for the apparent loss of amino groups, and is a more satisfactory explanation than coacervation alone. Coacervation would

Figure 6.11 The reduction of aldehydes and alkenes with borohydride



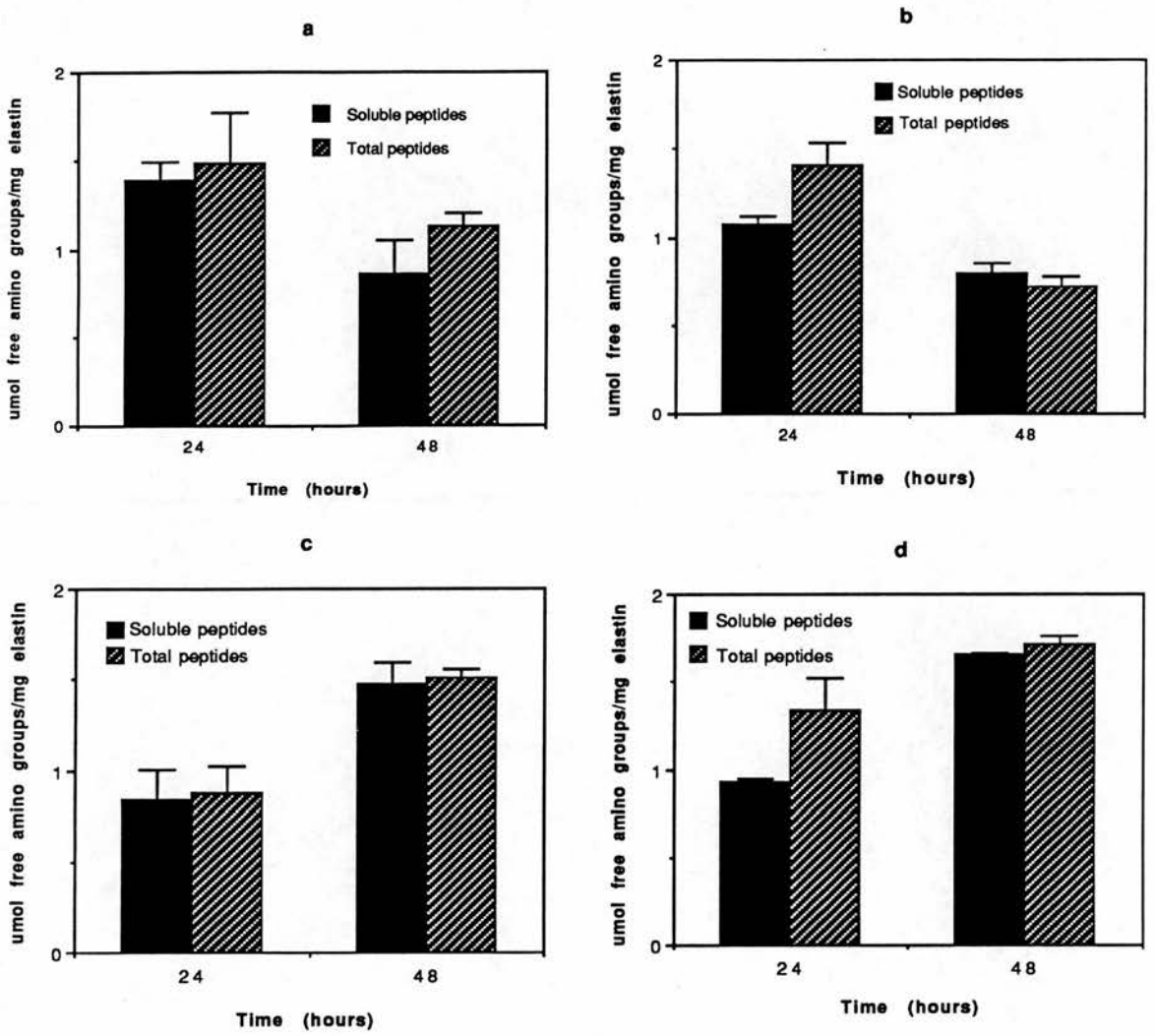
cause an aggregation of the peptides but it is unlikely that the aggregate would be capable of excluding TNBS, which is a small molecule, from reacting with amino groups buried within the coacervate.

6.3.5 The reduction of elastin-derived peptides with sodium borohydride

The apparent loss of amino groups (Sections 6.2.1-6.2.2) could be a consequence of the formation of Schiff's bases between new amino termini and the aldehyde groups or reactive double-bonds in some cross-link intermediates, rather than coacervation alone. If this was the case the apparent loss would be prevented by reducing the aldehydes to alcohols or -ene to -ane with sodium borohydride (Figure 6.11) which is generally used to stabilise cross-link intermediates (reviewed by Robins, 1982).

In order to investigate this possibility it was necessary to establish whether HNE was inactivated by the reducing conditions used. This was of importance because HNE contain 8 cysteine residues (Farley et al, 1989). Residues of cysteine can potentially form disulphide bridges which stabilise the conformation of the enzyme, and disulphide bridges can be reduced with sodium borohydride. A reduction may potentially lead to a change of the enzyme conformation which causes an inactivation of the enzyme. A solution of HNE (5mU in 1ml of 50mM Tris-HCl/1.0M NaCl, pH 8.0) was added to 0.75mg of sodium borohydride. The solution was treated with 10µl of STANA. The amount of STANA hydrolysis was measured as detailed in Section 2.1.1. The amount of STANA hydrolysis was the same as a control solution of enzyme which had not been treated with sodium borohydride.

Figure 6.12 The effect of sodium borohydride reduction on elastin-derived peptides



Suspensions of Lansing lung elastin were digested with HNE (5mU/ml; see section 2.3.1). One suspension (a) was not treated further. Sodium borohydride was added to the other suspensions (b) prior to digestion; (c) after 18 hours of digestion; and (d) after 24 hours of digestion. The soluble and total products were measured after 24 and 48 hours of digestion (sections 2.3.3 and 2.3.4).

Sodium borohydride was added to suspensions of Lansing lung elastin before digestion, or after 18 or 24 hours of digestion with HNE (5mU/ml) (Figure 6.12), at an elastin to sodium borohydride ratio of 4:1 (w/w). Sodium borohydride is unstable at pH 7.4, so any borohydride not involved in the reduction of the cross-link intermediates would decompose within minutes.

The apparent decline in the amount of amino groups was prevented if the reduction was performed after 18 or 24 hours of digestion, but not when the elastin was treated with borohydride before digestion. This suggested that proteolysis causing the exposure of internal chemical groups was necessary for the apparent decline of amino groups and for permitting borohydride to prevent the loss of amino groups. These chemical groups in the undigested elastin were not accessible to the borohydride, so explaining why borohydride reduction of the elastin before digestion was ineffective in preventing the decline of amino groups.

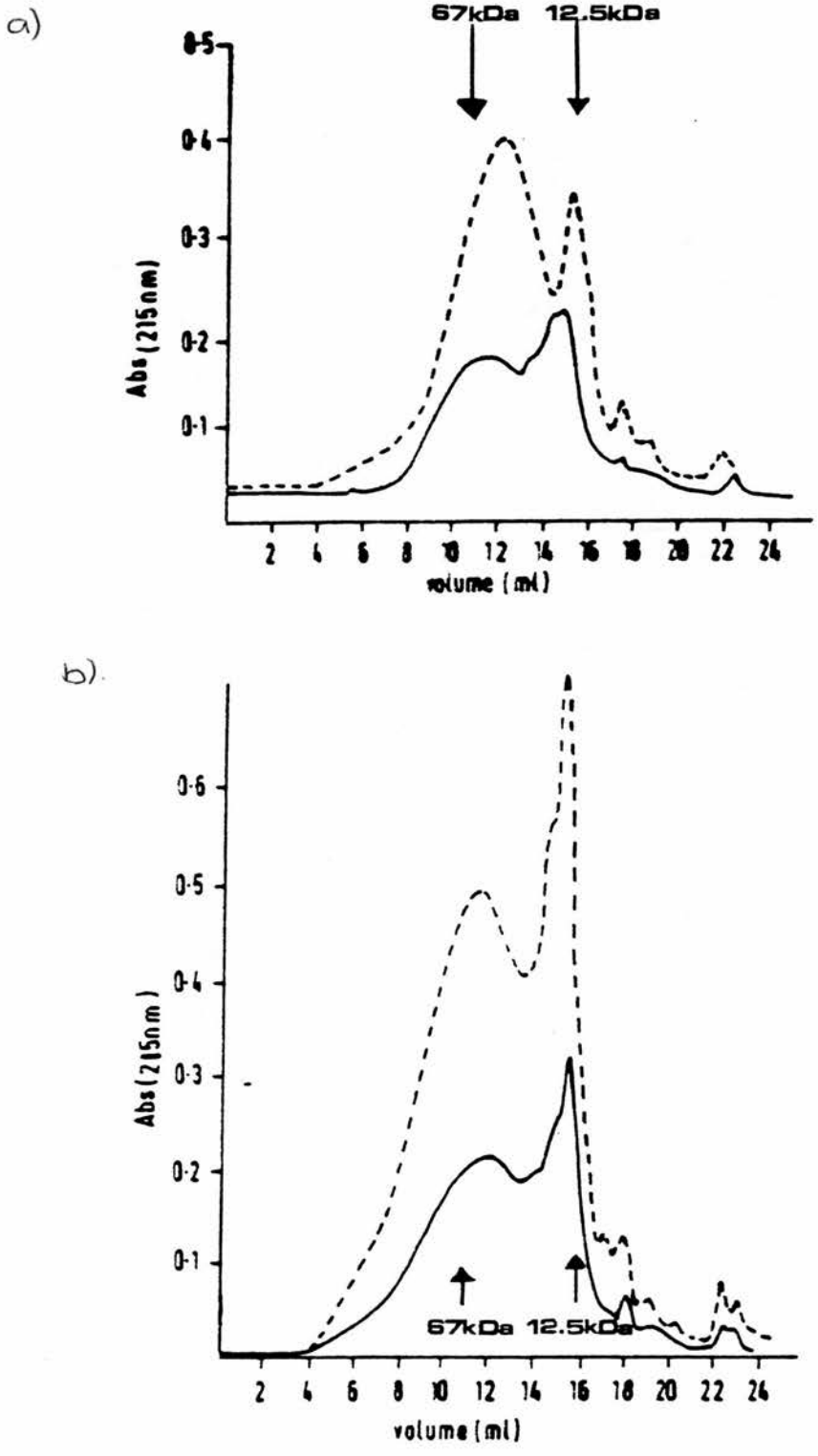
Sodium borohydride reduction after 24 hours of digestion also prevented the apparent decline of amino groups observed when a larger dose of HNE (10mU/ml) was used.

6.3.6 Examination of the products of digestion

Having established that borohydride was capable of preventing the loss of amino groups, it could be predicted that the treatment may also be capable of preventing the shift of molecular weight (Section 6.3.2 and Figure 6.8) which appeared to be associated with the loss of amino groups.

The soluble peptides from the reduction experiments (100μg, as measured by the Bradford method, in 0.1ml of 50mM sodium phosphate buffer, pH 7.4) which had

Figure 6.13 Molecular exclusion chromatography of peptides with and without reduction



Suspensions of Lansing lung elastin were digested with HNE (5mU//ml) for 48 hours (see section 2.3.1). The suspensions were either (a) untreated controls or (b) reduced by the addition of sodium borohydride after 18 hours of digestion. The soluble peptides (100µg by Bradford assay) were isolated after 24 (———) and 48 (– – – –) hours of digestion and examined by molecular exclusion chromatography as detailed in the text.

Figure 6.14 The effects of sodium borohydride reduction on elastin which had been partially digested with HNE

27 x magnification

A suspension of Lansing lung elastin was digested with HNE (see section 8.3.1) and treated with sodium borohydride after 24 hours of digestion as detailed in the text. The insoluble material was isolated after 48 hours of digestion, suspended in 1% (w/v) resorcin-fuchsin/4M HCl (Weigert's stain; BDH) and examined by polarised light microscopy using a NikonSMX/10 microscope. The image was recorded on 35mm, 100ASA film (Kodak) using a Nikon camera.

been digested with HNE (5mU/ml) for 24 or 48 hours were examined by molecular exclusion chromatography using a Superose-12 column (Pharmacia). The column was developed with 50mM sodium phosphate buffer, pH 7.4 as detailed in Section 2.4.1 (Figure 6.13). As expected, the shift of molecular weight associated with the decline of free amino groups between 24 and 48 hours of digestion, was prevented by reducing the peptides after 18 hours of digestion. On examining the elution profiles it would appear that a greater amount of peptides resulting from 48 hours of digestion was loaded on the column as compared with the amount of peptides resulting from 24 hours of digestion, although they had both been equated to 100µg by the Bradford method. This suggests that a greater proportion of the peptides resulting from 48 hours of digestion cannot be detected by the Bradford method presumably because they do not contain the residues necessary for detection (see Section 4.1.2). These peptides may result from a small amount of digestion which occurs between 24 and 48 hours, although the results presented in Figure 6.5 suggests any additional digestion between 24 and 48 hours is minimal in terms of the amount of new amino termini released.

The effect of borohydride on the formation of birefringent insoluble complexes (Section 6.3.3 and Figure 6.9) was also examined. A suspension of elastin was digested with HNE and reduced with sodium borohydride after 24 hours of digestion. The insoluble material was collected after 48 hours of digestion and examined by microscopy under polarised light (Figure 6.14). Although some association between the particles was evident, it was much less pronounced than the association

between the unreduced particles (Figure 6.9). The limited association observed in the reduced case (Figure 6.14) may represent association due to coacervation alone, but the stabilisation of the aggregate (Figure 6.9) probably requires the formation of new cross-links.

6.4 The implications of the ability of elastin-derived peptides to produce aggregates

The peptides produced by the digestion of Lansing lung elastin with HNE would appear to be capable of associating together to form a disorganised complex. The stabilisation of the complex could be prevented by reducing the components with sodium borohydride. Soluble peptides (0.1ml) resulting from 48 hours of digestion were suspended in 50% (v/v) organic solvent (methanol, ethanol or acetonitrile). The amount of peptides was then measured as detailed in Section 2.3.3 and related to a standard curve of glycine which was prepared in 50% (v/v) of the appropriate solvent. In all three cases the amount of detected peptides did not differ from the amount in a control sample of peptides which had been prepared in 0.1M sodium phosphate buffer pH 8.0. So it was concluded that the stable peptide complex was not dissociated with simple organic solvent (50% (v/v) methanol, ethanol or acetonitrile). It was further concluded that the apparent loss of amino groups detected *in vitro* may be a consequence of the ability of elastin-derived peptides to associate together by hydrophobic interaction to form a coacervate, which is stabilised by the formation of Schiff's bases.

This phenomenum may also occur *in vivo*. Stone et al (1988) speculated that damaged elastic fibres are partially repaired by the above

mechanism, rather than by complete synthesis of a new fibre. Since desmosine is not re-used by the body (Harel et al, 1980) and peptides from the elastic, non-cross-linked, region of the elastin molecule have been implicated in coacervation (reviewed by Urry, 1982) it appears likely that damaged elastin is partially repaired by coacervation followed by Schiff's base formation between the amino termini of peptides from the elastic region of the elastin and aldehyde cross-link intermediates of the frayed elastin which had been exposed during proteolysis. If so, the resulting repaired elastin would have altered elastic properties and cross-linking profile. Evidence to support this hypothesis was provided by Karlinsky and Snider (1978) who stated that proteolytic damage to hamster lungs was repaired within 2 months but the new tissue had an altered architecture. Therefore, cycles of proteolytic damage followed by inadequate repair over several years may eventually cause the complete breakdown of localised areas of the extracellular matrix; which could be the underlying mechanism of degenerative diseases such as emphysema.

Peptides derived from aortic elastin did not appear to aggregate in the same conditions as the peptides from Lansing lung elastin (Section 6.2). However, it has been documented that peptides from aortic elastin are capable of associating together in the presence of calcium and lipids; and become incorporated into arteriosclerotic plaques which form over the site of aortic damage (Lansing et al, 1950).

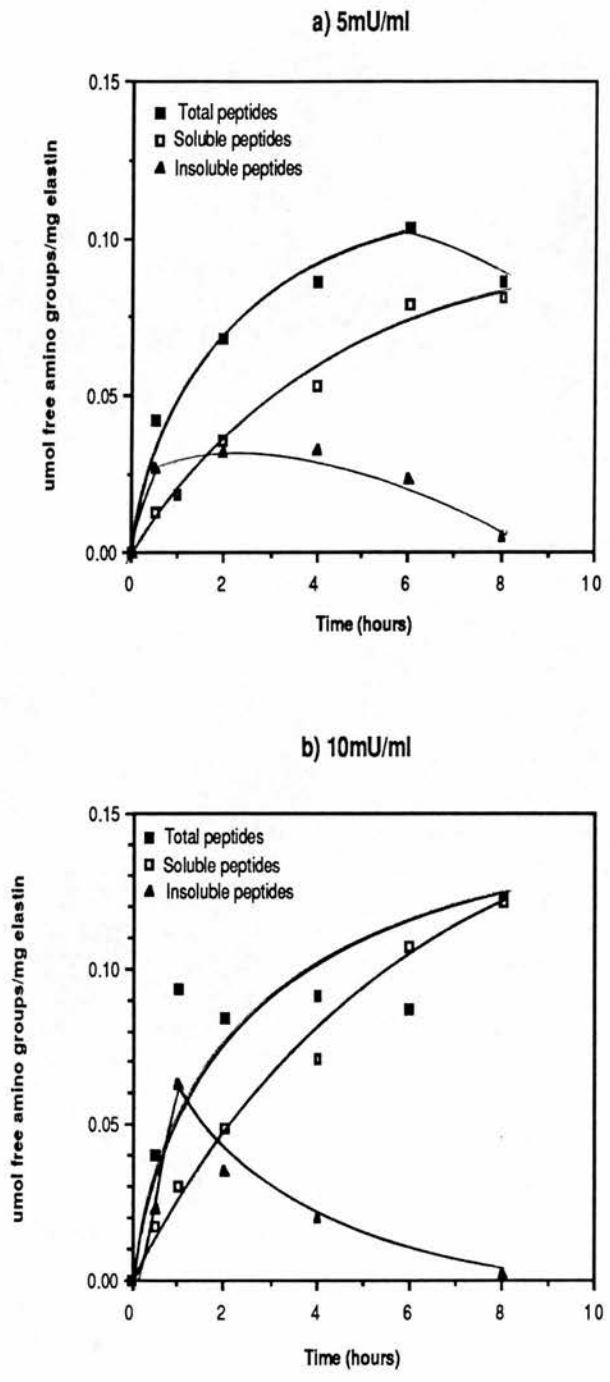
In conclusion, it would appear that elastin-derived peptides may be capable of interacting to form stable aggregates either with or without other compounds *in vitro*. The formation of such aggregates *in vivo* may have far

reaching consequences in arteriosclerosis and in the repair of damaged elastin in lung tissue.

CHAPTER 7

THE DIGESTION OF ELASTIN WITH HNCG

Figure 7.1 The digestion of Lansing lung elastin with HNCG for 8 hours



Suspensions of Lansing lung elastin were digested with HNCG (5 or 10mU/ml) at 37°C and pH 7.4 as described in section 2.3.1. The total, soluble and insoluble products were measured as detailed in sections 2.3.3-2.3.5.

CHAPTER 7

THE DIGESTION OF ELASTIN WITH HNCG

Having established the pattern of events when elastin was digested with HNE, attention now turned to the investigation of elastin digestion with HNCG.

7.1 The susceptibility of elastin to digestion by HNCG

In Chapter 4 (Section 4.2.4 and Figure 4.5) it seemed that Lansing lung elastin, collagenase-treated lung elastin and aortic lung elastin were less susceptible to digestion by HNCG than by HNE; and there was no appreciable difference between the susceptibility of the different preparations to digestion with HNCG.

7.1.1 The digestion of Lansing lung elastin with HNCG for 8 hours

Suspensions of Lansing lung elastin were digested with HNCG (5-10mU/ml) as detailed in Section 2.3.1. Aliquots were removed periodically and the amounts of total, soluble and insoluble amino groups were measured as detailed in Section 2.3.3-2.3.5 (Figure 7.1). A mixture of both soluble and insoluble products was produced throughout the 8 hours of digestion, similar to the trend resulting from the digestion of the elastin with HNE (Figure 5.8), and could be considered typical of endopeptidase action.

The amount of new insoluble products was initially greater than the

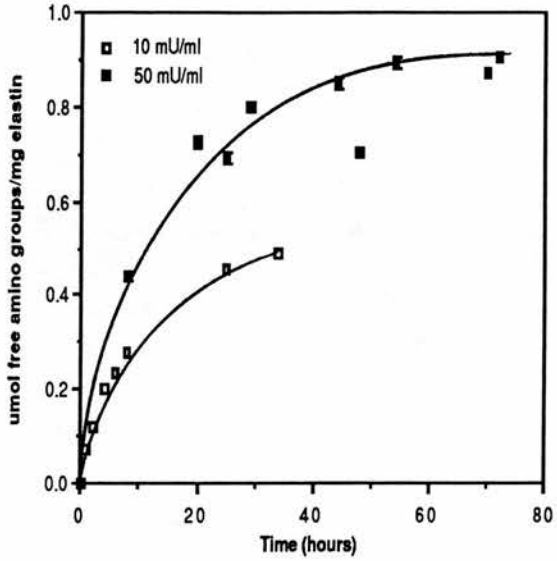
amount of soluble products indicating that the initial stage of hydrolysis involved the "opening" of the elastin network to expose the interior of the macromolecule. As the digestion continued the amount of soluble amino groups exceeded the amount of insoluble amino groups, and the insoluble amino groups appeared to decline. This observation is tentative, particularly when the elastin was digested with 5mU of HNCG/ml of digestion suspension because the decline is weighted upon a single point. Since there was no visible decrease in the amount of insoluble material it was assumed that this loss of amino groups may indicate that some coacervation and Schiff's base formation may have occurred in the early stage of digestion. However, the actual amount of insoluble amino groups lost during this decline is a great deal less than the decline observed between 24 and 48 hours of digestion of Lansing lung elastin with HNE (Section 6.2.1 and Figure 6.4), and therefore less significant.

7.1.2 Prolonged digestion of Lansing lung elastin with HNCG

Having established that the pattern of digestion of Lansing lung elastin by HNCG over 8 hours bears some similarities to the pattern of digestion with HNE over 8 hours, the next consideration was to investigate if the soluble products of digestion showed any apparent decline during the later stages of digestion.

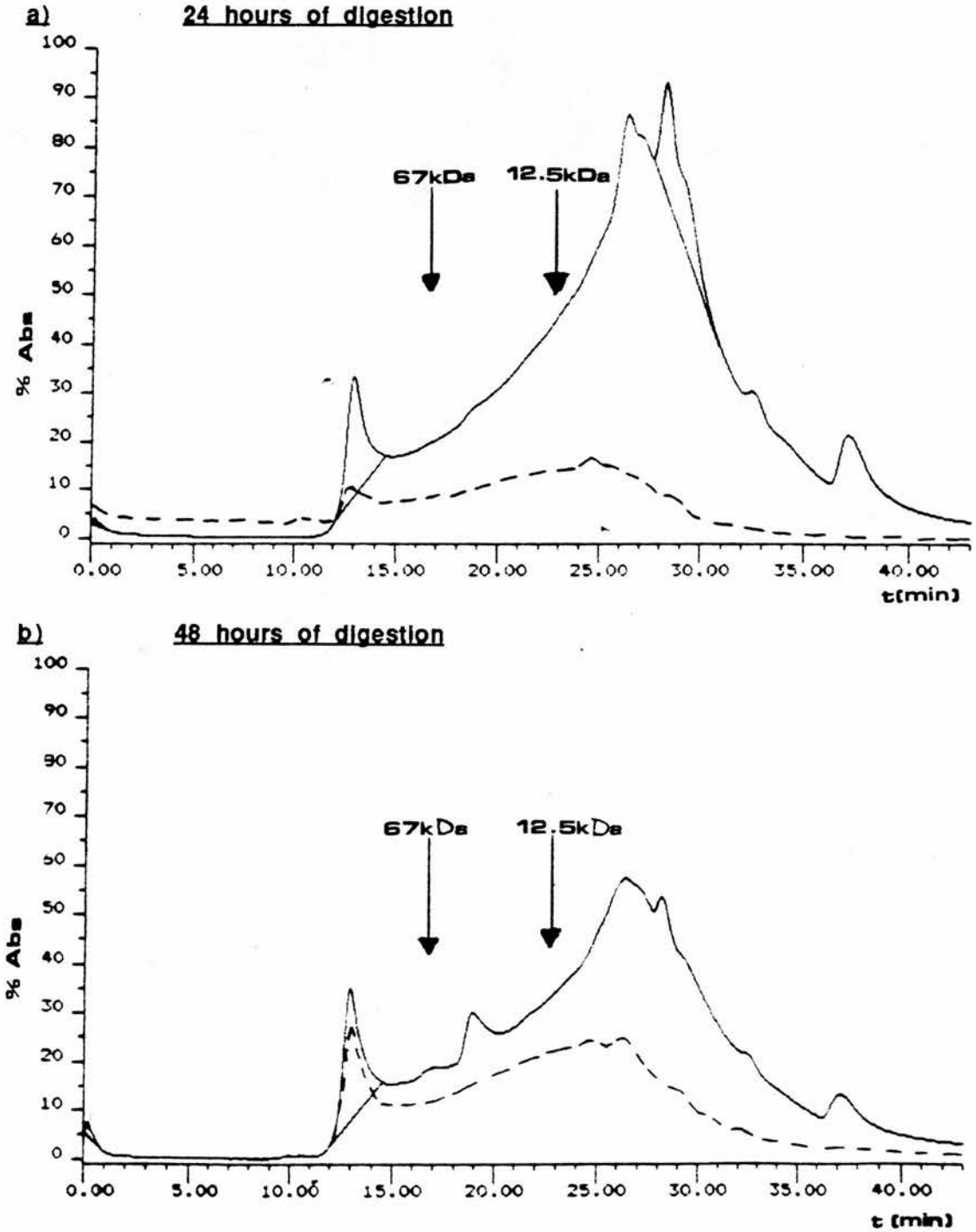
Suspensions of Lansing lung elastin were digested with HNCG (10 or 50mU/ml) at pH 7.4 and 37°C for up to 72 hours as detailed in Section 2.3.2;

Figure 7.2 The digestion of Lansing lung elastin with HNCG for a prolonged period of time



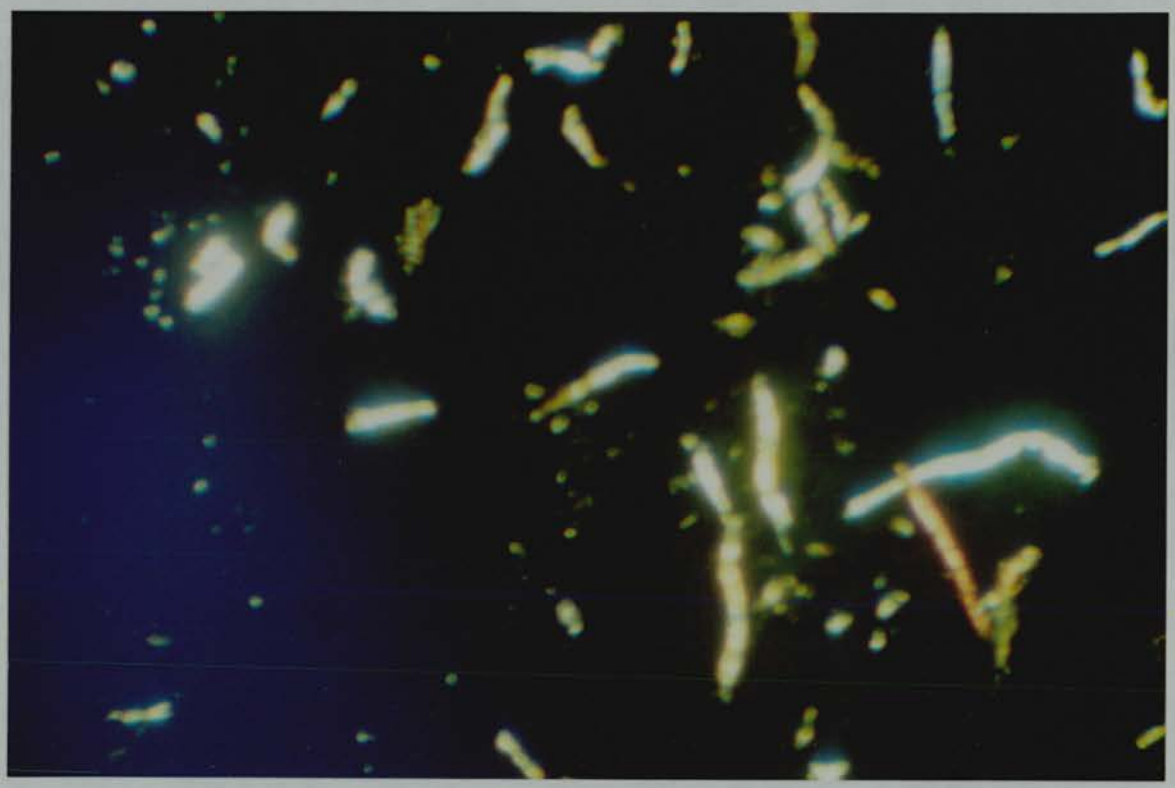
Suspensions of Lansing lung elastin were digested with HNCG (10 or 50mU/ml) as detailed in section 2.3.1. The soluble peptide release was measured as described in section 2.3.3.

Figure 7.3 Molecular exclusion chromatography of soluble elastin-derived peptides resulting from the digestion of Lansing lung elastin with HNCG



Suspensions of Lansing lung elastin were digested with HNCG (10mU/ml) for 48 hours. The soluble peptides (0.2ml) were isolated after (a) 24 and (b) 48 hours of digestion. The peptides were examined by molecular exclusion chromatography as detailed in the text. The absorbance of the eluate was measured at 220nm (————) and 270nm (– — — —). 100% corresponds to an absorbance at 220nm of 0.2 or an absorbance at 270nm of 0.1. The path length of the flow cell was 1cm.

Figure 7.4 Examination of the insoluble material remaining after elastin was digested with HNCG for 48 hours



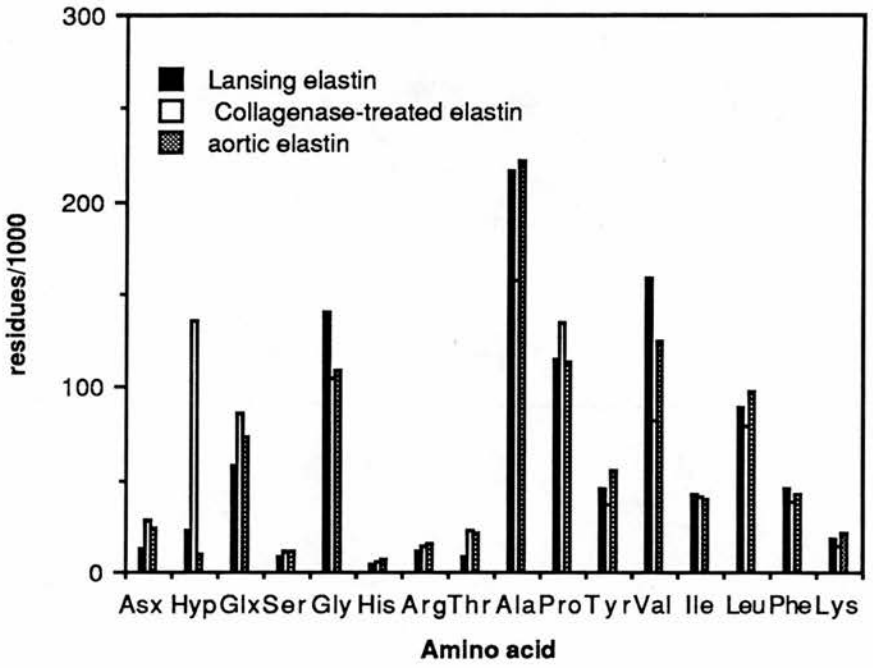
27 x magnification

Lansing lung elastin was digested for 48 hours with HNCG (10mU/ml) as detailed in section 4.3.1. The remaining insoluble material was removed, suspended in 1% (w/v) resorcin-fuchsin/4M HCl (Weigert's stain; BDH) and examined under polarised light using a Nikon SMX/10 microscope. The image was recorded on 35mm, 100ASA film (Kodak) using a Nikon camera.

and the soluble products were measured as detailed in Section 2.3.3 (Figure 7.2). The maximal soluble peptide production was achieved by approximately 40 hours of digestion. However, the maximum amount of peptides, even when digested with 50mU/ml, was less than the maximal peptide production with HNE (10mU/ml), and there was no significant decline in the free amino groups. The soluble peptide products of 24 and 48 hours of digestion with HNCG (10mU/ml) were examined by molecular exclusion chromatography using an Anagel TSK G3000 SWXL column developed with 50mM sodium phosphate buffer/0.15M NaCl, pH 7.4 as detailed in Section 2.4.1 (Figure 7.3). The peptides appeared to be smaller than the peptides produced by the digestion of Lansing lung elastin with HNE (Figure 6.8). The proportion of the peptides which absorbed at 270nm was greater after 48 hours of digestion than after 24 hours of digestion. Between 24 and 48 hours of digestion with HNCG there appeared to be a slight shift to a higher molecular weight.

The insoluble material which was present after 48 hours of digestion with HNCG (10mU/ml) was examined microscopically under polarised light (Figure 7.4), and little sign of aggregation was observed. So, if the decline in the insoluble amino groups observed in Figure 7.1 and the shift of molecular weight (Figure 7.3) are due to coacervation followed by new bond formation, then the actual extent of this aggregation is insufficient to be viewed by light microscopy.

Figure 7.5 Amino acid analysis of elastin-derived peptides produced after digestion with HNCG for 48 hours



Suspensions of elastin were digested with HNCG (10mU/ml) at 37°C and pH 8.0 for 48 hours. The soluble peptides were collected, hydrolysed and analysed as detailed in the text.

7.1.3 Comparison of the products of Lansing lung elastin digestion with HNCG with those resulting from the digestion of collagenase-treated elastin and aortic elastin with HNCG

The soluble peptides resulting from the digestion of Lansing lung elastin, collagenase-treated elastin and aortic elastin with HNCG (10mU/ml) for 48 hours at pH 7.4 and 37°C were hydrolysed and examined by amino acid analysis as detailed in Section 2.2.3. (Figure 7.5)

Once again the examination of the amount of hydroxyproline suggested that many of the peptides released from collagenase-treated elastin were collagenous. There was little difference between the amounts of glutamic acid in the hydrolysates of peptides from Lansing lung elastin and from aortic elastin so there is no evidence to suggest that the peptides from aortic elastin are derived from the glycoproteins of the microfibril component of the elastic fibres. This would not be surprising if the glycoprotein was interpenetrated within the elastin as extensive digestion would be required to release the glycoprotein. Since HNCG is weakly elastinolytic in comparison with HNE it may not be able to digest elastin to the extent needed for the release of any glycoprotein contaminants.

7.2 Concluding remarks about HNCG

HNCG has been implicated in platelet aggregation (Selak and Smith, 1990; Ferrer-Lopez et al, 1990) and in antimicrobial mechanisms (Odeberg and Olsson, 1975), which is of particular importance in the study of chronic granulomatous disease where the oxygen-dependent antimicrobial

mechanisms are defective. The antimicrobial action of HNCG was still evident after the enzymic activity had been inhibited by diisopropylphosphorofluoridate (see Section 1.2) so the antimicrobial activity is distinct from the enzymic activity (Odeberg and Olsson, 1975). The enzyme was digested with clostripain and two antibacterial sequences were identified (Bangalore et al, 1990). One sequence is found at the N-terminus of HNCG (Ile-Ile-Gly-Gly-Arg) and the other starts at amino acid 77 (His-Pro-Gln-Tyr-Asn-Gln-Arg). Both of the peptides were capable of killing *Neisseria gonorhoae* and *Staphylococcus aureus* more effectively than tetracyclin and streptomycin. Bangalore et al (1990) speculate that the internal sequence may be found on the exterior surface of the enzyme and would therefore be accessible for binding to the target bacteria. Interestingly this internal antimicrobial sequence of HNCG is similar to an antibacterial sequence within granzyme B, a serine proteinase found in cytotoxic T lymphocytes (Shafer et al, 1991). In contrast, HNE is not antibacterial and does not contain a sequence similar to the antimicrobial sequence of HNCG (Bangalore et al, 1990). However, HNE is capable of digesting some bacterial proteins (Blondin et al, 1978; see Section 1.1).

Although HNCG is capable of hydrolysing extracellular matrix molecules such as elastin (Reilly and Travis, 1980) and laminin (Heck et al, 1990), it is much less effective than HNE in this role. The data presented in this chapter confirms that HNCG is relatively ineffective in hydrolysing elastin, and the resulting products do not appear to aggregate together, probably because they are not produced at a high enough concentration under the conditions used here.

CHAPTER 8

THE DIGESTION OF ELASTIN WITH BOTH HNE AND HNCG

CHAPTER 8

THE DIGESTION OF ELASTIN WITH BOTH HNE AND HNCG

Boudier et al (1981) suggested that the activity of HNE on Lansing lung elastin in 50mM Tris-HCl, pH 8.0 at 37°C could be increased 7-fold by the presence of HNCG at a 1:2 (mol/mol) ratio of HNE to HNCG when measuring activity as the increase of absorbance at 280nm of the soluble products of digestion. This was disputed by Reilly et al (1984) who used three different methods of analysis to reach the conclusion that the stimulation of the digestion of Lansing lung elastin with HNE by HNCG (1:2 mol/mol ratio of HNE:HNCG) was 1.9-2.9 fold in 50mM Tris-HCl, pH 8.0 at 25°C or 1.1 fold at physiological conditions of temperature, pH and ionic strength. Both Boudier et al (1981) and Reilly and Travis (1984) measured the stimulation of HNE by HNCG as follows:-

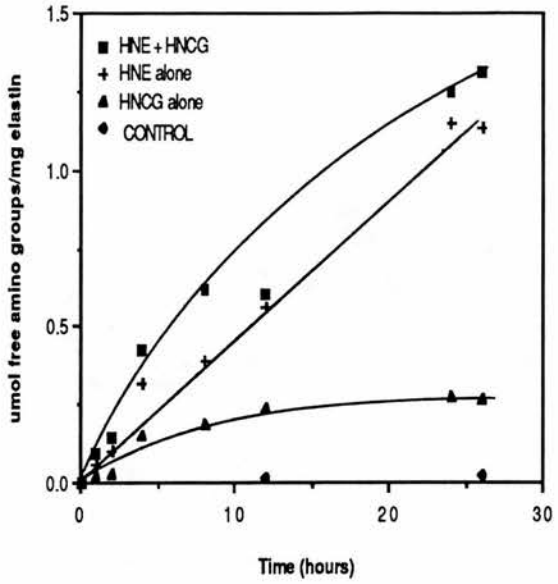
$$\text{Stimulation factor} = \frac{V_{E.C} - V_C}{V_E}$$

where $V_{E.C}$, V_C and V_E are the amounts of products formed over a given period of time in response to digestion by HNE and HNCG, HNCG alone and HNE alone, respectively.

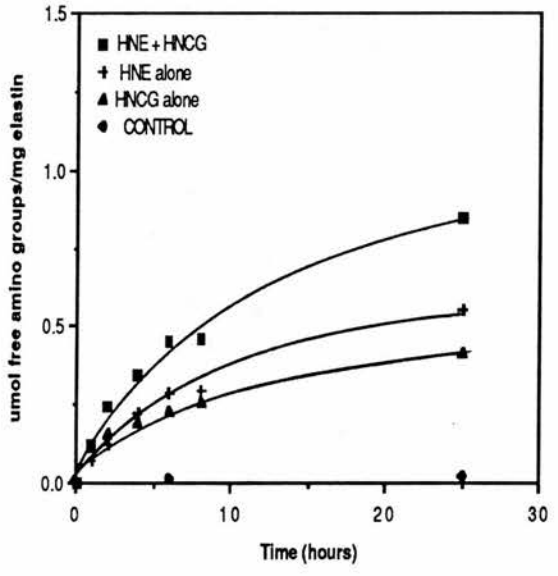
The ability of HNCG to stimulate the action of HNE is potentially very important in the understanding of the physiological mechanisms of elastin destruction in response to excessive amounts of these enzymes, but the

Figure 8.1 The effect of HNCG on HNE digestion

a) Lansing elastin



b) Collagenase-treated elastin



Lansing lung elastin and collagenase-treated elastin were digested with HNE (5mU/ml) and/or HNCG (5mU/ml) at 37°C and pH 7.4 as detailed in section 2.3.1. The soluble peptides were measured as detailed in section 2.3.3.

discrepancy between the two publications was a cause of some concern. Therefore the effect of HNCG on HNE during the digestion of elastin was investigated.

8.1 The digestion of elastin with both HNE and HNCG added simultaneously

Suspensions of Lansing elastin and collagenase-treated elastin were digested with HNE and/or HNCG (5mU/ml) for 24 hours at pH 7.4 and 37°C (Figure 8.1). The amounts of soluble peptides resulting from digestion of the elastins for 4 and 24 hours with HNE and HNCG, HNE alone and HNCG alone were applied to the above equation for the calculation of the stimulation factor. The stimulation factors for the digestion with HNE by HNCG of Lansing elastin and collagenase-treated elastin after 4 hours of digestion were 0.89 and 0.67 respectively. After 24 hours of digestion the stimulation factors for the digestion of Lansing elastin and collagenase-treated elastin with HNE by HNCG were 0.87 and 0.78 respectively. This suggests that at a 3:2 (mol:mol) ratio of HNE:HNCG there is no evidence to suggest that HNCG can stimulate the action of HNE on elastin, which is comparable with the results presented by Reilly et al (1984).

8.2 The digestion of elastin with both enzymes added at intervals

The possibility remained that pre-digestion with HNCG may enhance the activity of HNE; although Boudier et al (1981) stated that their observed enhancement of the activity of HNE by HNCG of 7-fold when the enzymes

were added simultaneously was reduced to 27% of this enhancement if the HNCG was added 40 minutes before the HNE. However, the effect of pre-digesting the elastin with either HNE or HNCG before the attention of the second enzyme was considered to be worthy of further investigation.

Suspensions of each preparation of elastin were pre-digested with HNE or HNCG (10mU/ml) for 2 hours at pH 7.4 and 37°C (see Section 2.3.1), before the addition of the alternative enzyme (10mU/ml). The suspensions were digested for a further 2 hours after which the amounts of soluble peptides were measured as detailed in Section 2.3.3.

In order to measure any stimulation of the first enzyme on the activity of the second enzyme one of two assumptions must be made. First, it may be assumed that the rate of the digestion of elastin by the first enzyme remained constant over 4 hours so that the amount of products due to digestion by this enzyme during the 2 hours of pre-digestion was equal to the amount of products released during the following 2 hours when the second enzyme was present. In this case:-

$$\text{Stimulation Factor (1)} = \frac{\text{Peptides}_{(2\text{-}4\ \text{hrs})} - \text{Peptides formed by enz.}_{1\,(0\text{-}2\ \text{hrs})}}{\text{Expected digestion by enz.}_{\,2\ (2\text{-}4\ \text{hrs})}}$$

The expected amount of digestion by the second enzyme between 2-4 hours of digestion was equated to the amount of peptides which would have been produced if undigested elastin was treated with the second enzyme only for 2 hours.

Alternatively, it could be assumed that after two hours of digestion all

Table 8.1 The stimulation of HNE or HNCG digestion of elastin by the pre-digestion of the elastin with HNCG or HNE respectively

a) The stimulation of HNE digestion by pre-digestion with HNCG

Elastin preparation	Stimulation factor	
	by Equ.1	by Equ.2
Lansing lung elastin	0.150	0.750
Collagenase-treated elastin	0.510	1.08
Aortic elastin	1.23	1.70

b) The stimulation of HNCG digestion by pre-digestion with HNE

Elastin preparation	Stimulation factor	
	by Equ. 1	by Equ. 2
Lansing lung elastin	0.940	2.61
Collagenase-treated elastin	1.01	2.71
Aortic elastin	1.49	3.65

Suspensions of elastin were pre-digested with HNE (10mU/ml) or HNCG (10mU/ml) for 2 hours at 37°C and pH7.4 (see section 2.3.1) before the addition of the other enzyme. The stimulation factor was determined after a further 2 hours of digestion using the equations detailed in the text.

peptide bonds which were susceptible attack by the first enzyme had been cleaved. So, during the following two hours the first enzyme would provide a negligible amount of further soluble products in the presence of the second enzyme, therefore:-

$$\text{Stimulation factor (2)} = \frac{\text{Peptides}_{(2\text{-}4\ \text{hrs})}}{\text{Expected digestion by enz.}_{2\ (2\text{-}4\ \text{hrs})}}$$

It should be stated that these methods would be satisfactory if a simple digestion system with known kinetics was being considered, but the kinetics of the digestion of elastin with these enzymes is far from simple and so both methods over-simplify the situation. The "stimulation" of HNE by HNCG and vice versa using both these equations was calculated (Table 8.1).

It can be seen from Figures 5.6 and 6.4b that the amount of soluble peptide production by HNE over a 4 hour period appears to occur at a reasonably constant rate. Assuming that the addition of HNCG after 2 hours of pre-digestion with HNE does not induce an early acceleration stage (in Figure 6.4b the acceleration stage is seen to occur between 8-20 hours of digestion), then the effect of pre-digestion by HNE on digestion with HNCG is most likely to conform to the situation expressed in stimulation factor (1). When investigating the effect of pre-digestion with HNCG the consideration may be more complex. In Figures 7.1 and 7.2 it can be seen that the amount of digestion over the first 4 hours by HNCG remains at an approximately constant rate, so stimulation factor (1) could once again be considered a

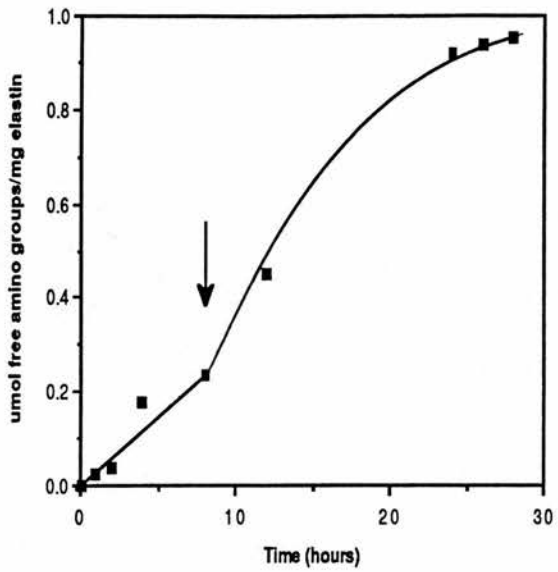
justified simple model of this digestion. However, HNCG digests elastin to produce less than half the amount of products released by HNE over the same period of time. So although the contribution to peptide production by HNCG from 2-4 hours is not negligible it is not as significant as the production of peptides by HNE. Therefore the pattern of HNCG pre-digestion followed by HNE digestion probably lies somewhere between the two situations described by stimulation factor (1) and stimulation factor (2).

Even when allowing for the fact that the methods of measuring the "stimulation" are overly simple, it would seem that HNCG did not immediately stimulate the action of HNE and may even have acted as a poor, alternative substrate for HNE. This would explain why the stimulation of elastin digestion with HNE by HNCG was less than 1 (Table 8.1). The data presented in Table 8.1 imply that it would be more reasonable to suggest that HNE enhanced the activity of HNCG.

So, the presence of HNCG appeared to be of little benefit to the digestion of elastin with HNE when the enzymes were added simultaneously or when there was a 2 hour delay before the addition of the second enzyme. Robert et al (1974) suggested (Section 6.2.4) that HNE initially binds to elastin, hydrolyses the protein to a limited extent to "open" the insoluble network, exposing potential internal sites of proteolysis. This theory was used to explain the apparent slow response of HNE when digesting Lansing lung elastin (see Section 6.2.1 and Figure 6.4). It is possible that pre-digestion of elastin with HNCG may be sufficient to open the elastin so avoiding this initial lack of response to the action of HNE.

Lansing lung elastin was pre-digested with HNCG for 8 hours

Figure 8.2 Digestion of elastin with HNE after pre-digestion with HNCG



Lansing lung elastin was digested with HNCG (10mU/ml) for 8 hours at 37°C and pH7.4, then HNE (10mU/ml) was added to the digestion mixture (↓). Aliquots were removed periodically and the amount of soluble peptides was measured as detailed in section 2.3.3.

(10mU/ml) before the addition of HNE (10mU/ml) (Figure 8.2), as detailed previously in this Section, and in Section 2.3.1. The amount of soluble peptides present after 20 hours of digestion with HNE and pre-digestion with HNCG, was not greater than would be expected if the elastin was digested with HNE alone. However, the initial lack of response to the action of HNE on Lansing lung elastin (Figure 6.4) was not present. This would suggest that HNCG may indeed perform some limited hydrolysis which opened the elastin network, so that the internal sites susceptible to HNE hydrolysis were exposed when the HNE was added to the digestion suspension.

8.3 Interpretation of the results and comparison with the literature

It may be concluded that HNCG does not greatly enhance elastolysis by HNE, which is in contradiction of the finding of Boudier et al (1981). Boudier et al (1981) measured the elastin-derived peptides as the increase of absorbance at 280nm, produced when desmosine-containing peptides were solublised. Therefore, any small peptides which do not contain desmosine would not be detected. This may explain why they did not reach the same conclusion as Reilly et al (1984). Reilly et al (1984) measured the release of peptides in three ways. First, as an increase of absorbance at 280nm, secondly as the release of tritium from tritiated elastin, and thirdly as the liberation of amino termini with ninhydrin, which is analogous to the TNBS method which also measures amino groups. They calculated that the stimulation factor was no more than 2.9, by any of these methods but the measurement of the increase of absorbance at 280nm did give the greatest

value for the stimulation factor. They suggested that any slight enhancement of the activity of HNE by the action of HNCG may indicate that HNCG can act to enhance HNE activity slightly by binding to non-productive anionic sites on the elastin so that only hydrophobic productive binding sites are available to HNE (Reilly et al, 1984). Relevant to this argument, are the observations of Lonky and Wohl (1983) who state that PPE adsorbs electrostatically to elastin to produce productive cleavage. This binding is inhibited by high sodium chloride concentrations. The adsorption of PPE onto elastin is increased if the elastin is coated with SDS (Kagan et al, 1972). Conversely, electrostatic sites are non-productive for HNE which interacts hydrophobically with elastin. The activity of HNE is enhanced in high sodium chloride concentrations and by the occupation of the anionic sites of elastin with basic compounds such as poly-lysine. Since HNCG is more basic than HNE, it will presumably be more susceptible to the electrostatic binding with elastin. In a typical digestion mixture 3mg of elastin in a ml of suspension was treated with 10mU of HNCG which corresponds to a 1:0.018 mol/mol ratio of elastin:enzyme. Since tropoelastin contains 2 residues/mol of aspartic acid and 5 residues/mol of glutamic acid (Indik et al, 1987) there is not sufficient HNCG to saturate the negatively charged residues of elastin. Therefore the suggestion that HNCG aids HNE activity by binding to the non-productive ionic sites of elastin seems unlikely. However, if the ability of HNCG to enhance the digestion of elastin by HNE in certain circumstances was simply due to electrostatic interaction then the enhancement should also be noted if inactive HNCG was added to the elastin. Boudier et al (1991) found that HNCG which had been inactivated

with peptide chloromethyl ketone impaired the activity of HNE on dermal elastin, so it would seem that the HNCG must be active in order to produce any positive effect on the digestion of elastin with HNE. Boudier et al, (1991) measured the digestion of the elastin in sections of skin as a change in the area of specifically stained elastic fibres using a process of computerised morphometry. It is possible that the inactive HNCG was digested by the HNE in preference to the elastin. This digestion would not be measured be the morphometric assay and would give the impression that the inactivated HNCG had an inhibitory effect on the HNE.

In conclusion, the most likely explanation for the effect of HNCG on digestion of elastin by HNE is that the HNCG binds and digests the elastin to a limited extent to open the network. When HNE is added to elastin which has been pre-digested it will bind to the elastin, and since the network has already been cleaved to a limited extent, the initial lack of response to digestion by HNE would not longer be evident.

CHAPTER 9

THE SEPARATION AND CHARACTERISATION OF SOLUBLE ELASTIN-DERIVED PEPTIDES

CHAPTER 9

THE SEPARATION AND CHARACTERISATION OF SOLUBLE ELASTIN-DERIVED PEPTIDES

The main aim of this investigation was the separation and characterisation of elastin-derived peptides specific for digestion with a particular enzyme (see Section 1.4).

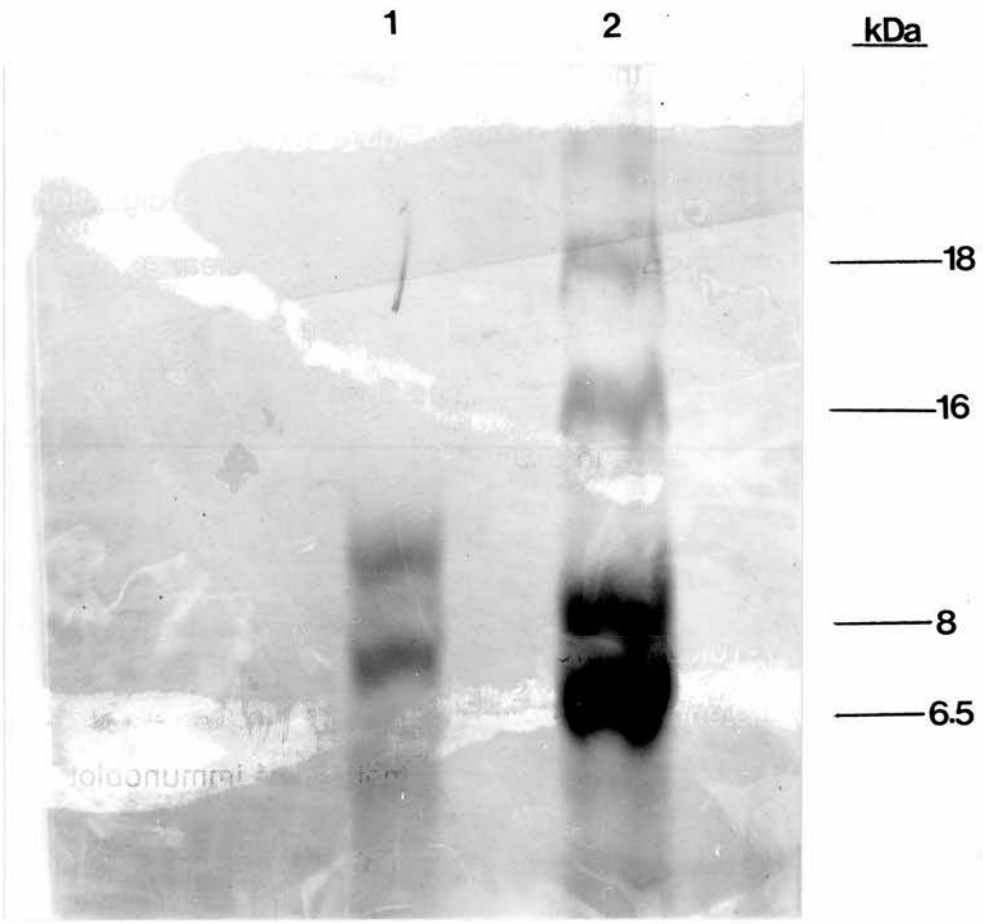
9.1 Unsuccessful methods of separation

9.1.1 Separation using polyacrylamide gels

Attempts were made to examine the soluble elastin-derived peptides resulting from 24 hours of digestion with HNE and/or HNCG (10mU/ml) at pH 7.4 and 37°C (see Section 2.3.1) using polyacrylamide gel electrophoresis.

Unsatisfactory results were achieved with 6% and 15% SDS polyacrylamide gels in the presence of SDS and mercaptoethanol (pH 8.8, according to Laemmli, 1970); 5%-20% non-reducing, gradient polyacrylamide gel at pH 8.8; and 5%-20% non-reducing, gradient polyacrylamide gel at pH 4.5 (according to Reisfeld et al, 1962). In all cases the peptide material was detected by Coomassie blue stain as a smear of material. This was also observed by Smyrlaki et al (1987) who concluded it indicated the presence of aggregates of peptides which ranged between 150kDa and 20kDa. This range is similar to the range observed in the

Figure 9.1 SDS Urea-PAGE of elastin-derived peptides



Elastin-derived peptides (50µg by Bradford assay) resulting from the digestion of Lansing lung elastin with HNE and HNCG (10mU/ml each) for 24 hours (see section 2.3.1) were separated on a 12.5% SDS-urea polyacrylamide gel (0.15cm x16cm x 20cm), at 100V and 42mA for 15 hours. The peptides were visualised by silver staining (Wray et al, 1971).

Track 1: Peptides from elastin

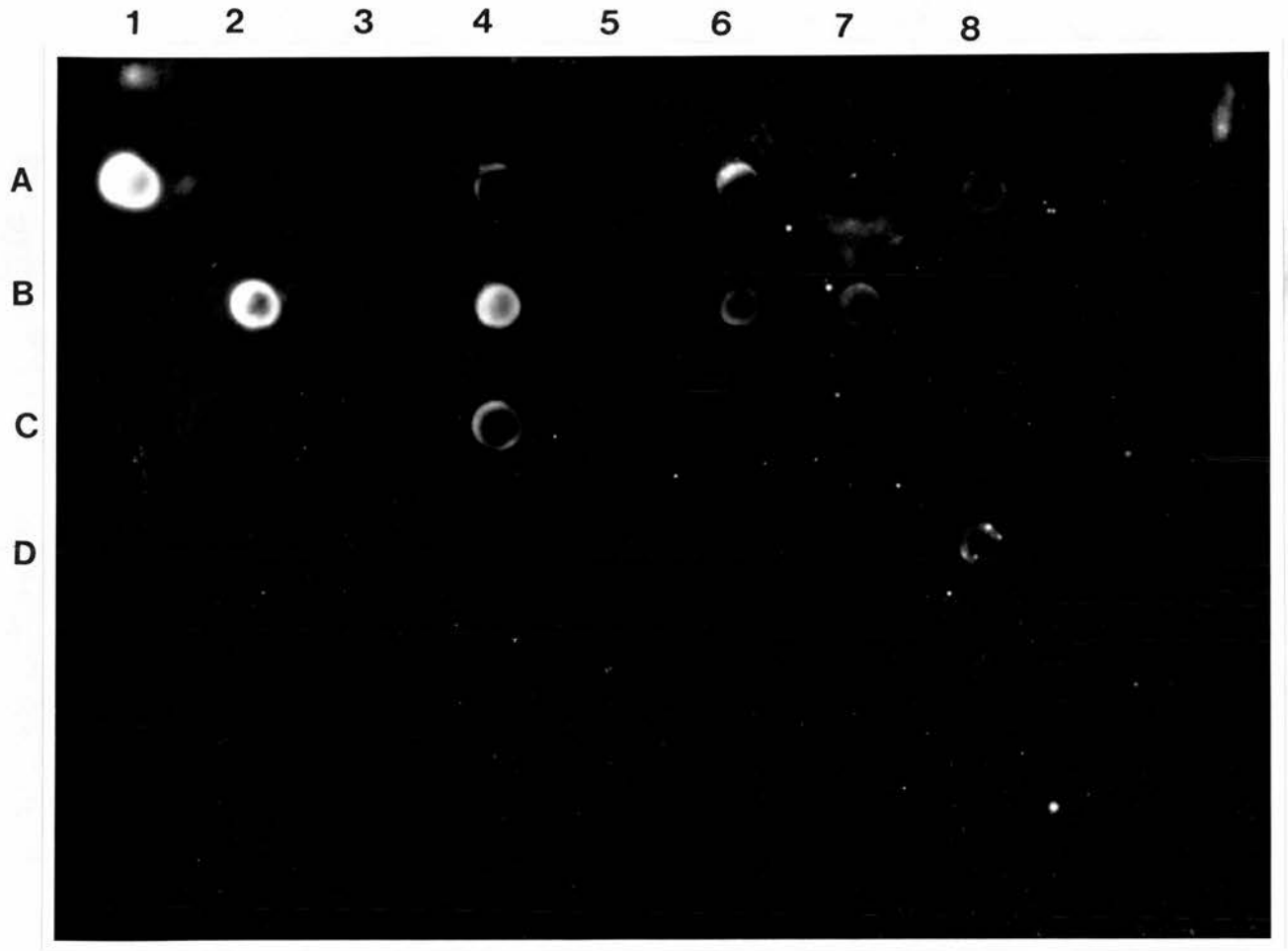
Track 2: Molecular mass markers

molecular exclusion experiments (see Section 6.3.2, Figure 6.8, Section 7.1.2 and Figure 7.3). These results were considered unsatisfactory especially in the light of the fact that small peptides generally do not bind Coomassie blue (see Section 4.1.2 and Figure 4.1) or are washed out of the gel during fixing, or both. The peptides resulting from the digestion of elastin by HNE would be expected to be small since HNE cleaves at the carbonyl side of alanyl-, isoleucyl- and valyl-residues (see Section 1.2) which account for 37% of the composition of elastin (see Section 3.2.2)

Attempts were made to examine the small elastin-derived peptides by using 12.5% SDS urea-polyacrylamide gels (Swank and Munkres, 1971) which were stained with silver (Wray et al, 1981) (Figure 9.1). Peptides of approximately 7-10kDa were often observed but their presence was somewhat inconsistent.

Wrenn et al (1986) developed a method of immunoblot analysis to detect tropoelastin using a mouse monoclonal antibody to the Val-Gly-Val-Ala-Pro-Gly hexapeptide found in elastin. The monoclonal antibody is commercially available (Sigma) so attempts were made to detect elastin-derived peptides containing this particular epitope by Western blotting as detailed by Wrenn et al (1986). This technique proved unsuccessful when attempting to detect elastin-derived peptides which had been separated in 15% SDS-polyacrylamide gel (Laemmli, 1970). This suggested that either the soluble elastin-derived peptides did not contain the epitope recognised by the antibody, or the peptides containing the epitope were small and were eluted from the gel during electrophoresis or lost during the transfer to nitrocellulose.

Figure 9.2 Detection of the elastin-derived peptides recognised by a monoclonal antibody



Soluble elastin-derived peptides (0.1ml) were transferred onto nitrocellulose (13cm x 9cm, Amersham) and probed with a monoclonal antibody directed against Val-Gly-Val-Ala-Pro-Gly then sheep anti-mouse antibody conjugated to horseradish peroxidase as detailed in the text. The blot was developed by using the ECL chemilumenescence method (detailed in the text).

1A: monoclonal antibody and 1B: phosphate buffer

2A, 4A and 6A: peptides resulting from the digestion of Lansing lung elastin from lung 1, 2 and 3 respectively with HNE.

3A, 5A and 7A: peptides resulting from the digestion of collagenase-treated lung elastin from lung 1, 2 and 3 respectively with HNE.

8A: peptides resulting from the digestion of aortic elastin with HNE.

2B, 4B and 6B: peptides resulting from the digestion of Lansing lung elastin from lung 1, 2 and 3 respectively with HNCG.

3B, 5B and 7B: peptides resulting from the digestion of collagenase-treated elastin from lung 1, 2 and 3 respectively with HNCG.

8B: peptides resulting from the digestion of aortic elastin with HNCG.

2C-8C and 2D-8D: peptides resulting from the digestion of the preparations of elastin with both HNE and HNCG.

Soluble peptides (100µl) resulting from 24 hours of digestion with HNE and/or HNCG (10mU/ml) were transferred onto nitrocellulose (13cm x 9cm; Amersham) using a Dot-Blot apparatus (Bio-Rad). The nitrocellulose was probed with the mouse monoclonal antibody directed against Val-Gly-Val-Ala-Pro-Gly (1:500 dilution) and then with sheep anti-mouse antibody conjugated to horseradish peroxidase (1:100 dilution; Serotec) according to the method of Wrenn et al (1986). The blot was developed by using the ECL chemiluminescence method (Amersham) according to the manufacturer's guidelines, with exposure of the film (Hyperfilm, Amersham) for 5 seconds (Figure 9.2). It appeared that some of the peptides resulting from digestion with the HNCG alone, but not with HNE were recognised by the monoclonal antibody directed against Val-Gly-Val-Ala-Pro-Gly, which may suggest that some of the peptides resulting from the digestion of Lansing lung elastin by HNCG do indeed differ from those resulting from the digestion by HNE. This is not surprising since HNE can potentially cleave the peptide bonds to the carbonyl side of the valyl- and alanyl-residues of the hexapeptide, but the hexapeptide does not have any potential sites of cleavage by HNCG (see Section 1.2).

9.1.2 Ion-exchange chromatography

Attempts were made to separate the elastin-derived peptides by cationic ion-exchange chromatography using a Mono S column (Pharmacia) developed with 50mM sodium acetate buffer, pH 4.6 using a gradient of 0.15-1.0M NaCl; and by anionic ion-exchange chromatography using a Mono Q column (Pharmacia) developed with 20mM Tris-HCl, pH 8.0 using a

gradient of 0.15-1.0M NaCl. The peptides eluted from the column were detected by monitoring the absorbance at 215nm of the eluate. Neither procedure separated the peptides satisfactorily; so ion-exchange chromatography was considered to be inappropriate.

9.2 Reverse-phase chromatography

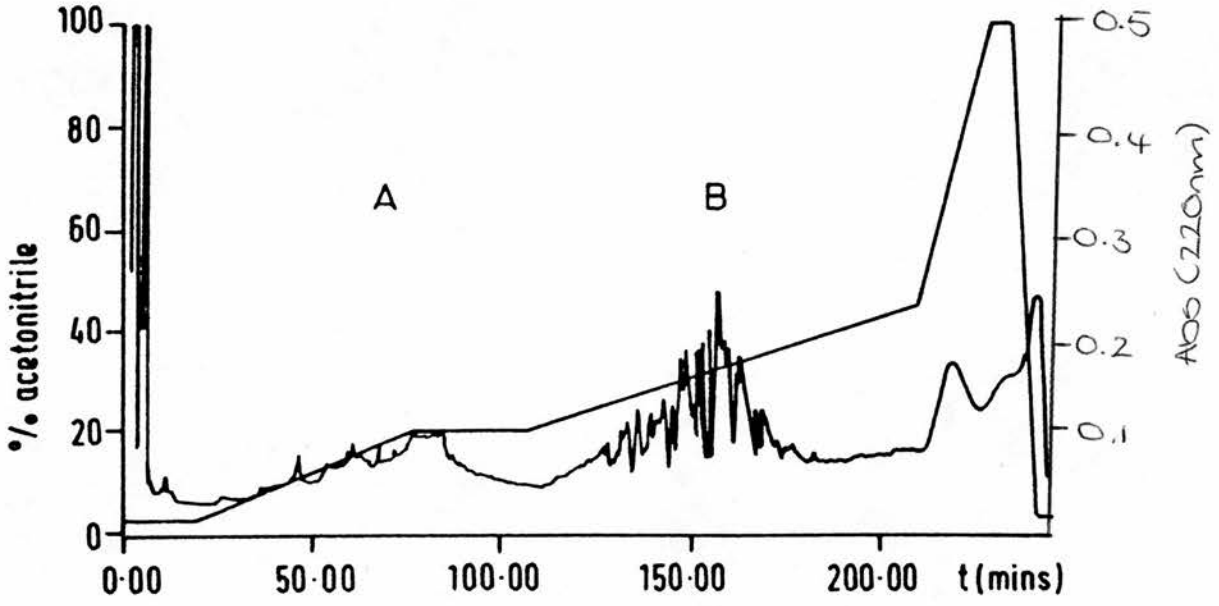
The separation of elastin-derived peptides by other authors have usually exploited the hydrophobicity of the peptides by using reverse-phase chromatography (Smyraki et al, 1987; Manning et al, 1989; Sandberg et al, 1990). Reverse-phase chromatography involves the partition of molecules between a stationary phase attached to a silica matrix and a mobile phase of increasing hydrophobicity.

9.2.1 The choice of mobile phase

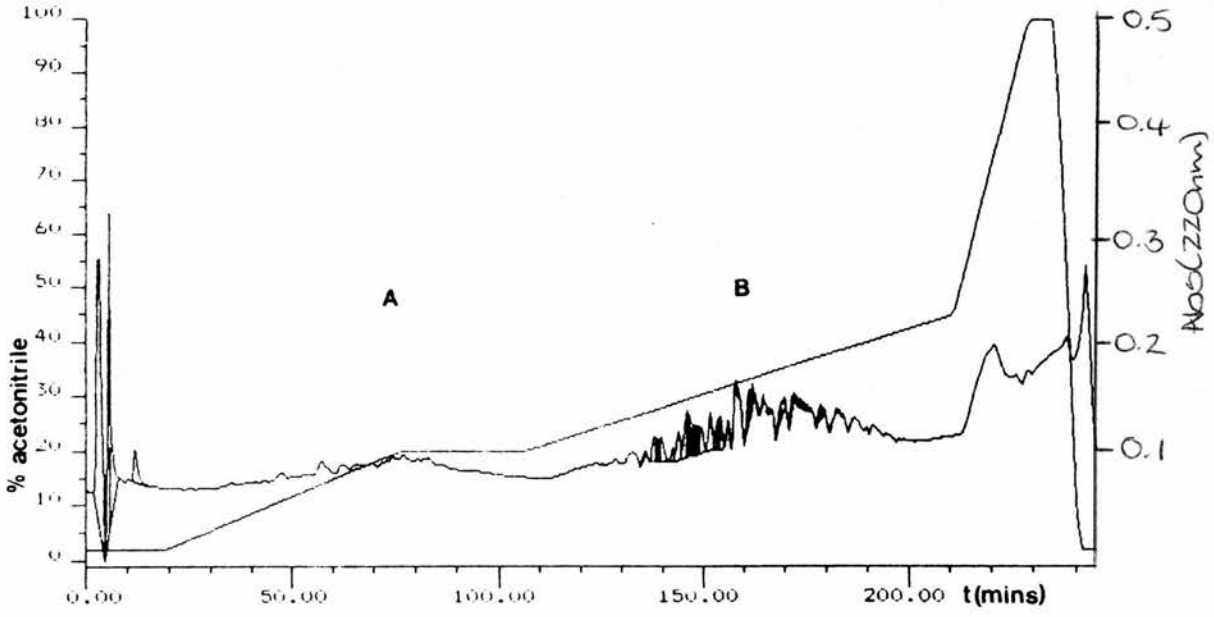
The initial separations of elastin-derived peptides were performed on a C_2/C_{18} PepRPC HR 5/5 column (Pharmacia) which was designed for the separation of peptides. Many of the elastin-derived peptides were not expected to be detectible at 280nm so their elution was monitored at 220nm. This influenced the choice of mobile phase because the chosen solvent was required to have a minimal absorbance at 220nm but to be sufficiently hydrophobic to elute the peptides from the column. The separation of the peptides was attempted with a number of solvents e.g., methanol, acetonitrile and propan-2-ol, before opting for a gradient of acetonitrile containing 0.1% TFA (detailed in Section 2.4.2) which is similar to the mobile phase used by Sandberg et al (1990) to separate elastin-derived peptides

Figure 9.3 The separation of elastin-derived peptides by reverse-phase chromatography

a) The products of digestion with HNE for 48 hours.



b) The products of digestion with HNCG for 48 hours



(a) The elastin-derived peptides (0.3ml) resulting from the digestion of Lansing lung elastin with HNE (10mU/ml) for 48 hours, and (b) the elastin-derived peptides resulting from the digestion of Lansing lung elastin with HNCG (10mU/ml) for 48 hours were separated by reverse-phase chromatography. 100% corresponds to an absorbance at 220nm of 0.5.

resulting from digestion with thermolysin. TFA is routinely used in high pressure liquid chromatography of amphipathic molecules to arrange that all species have a positive charge.

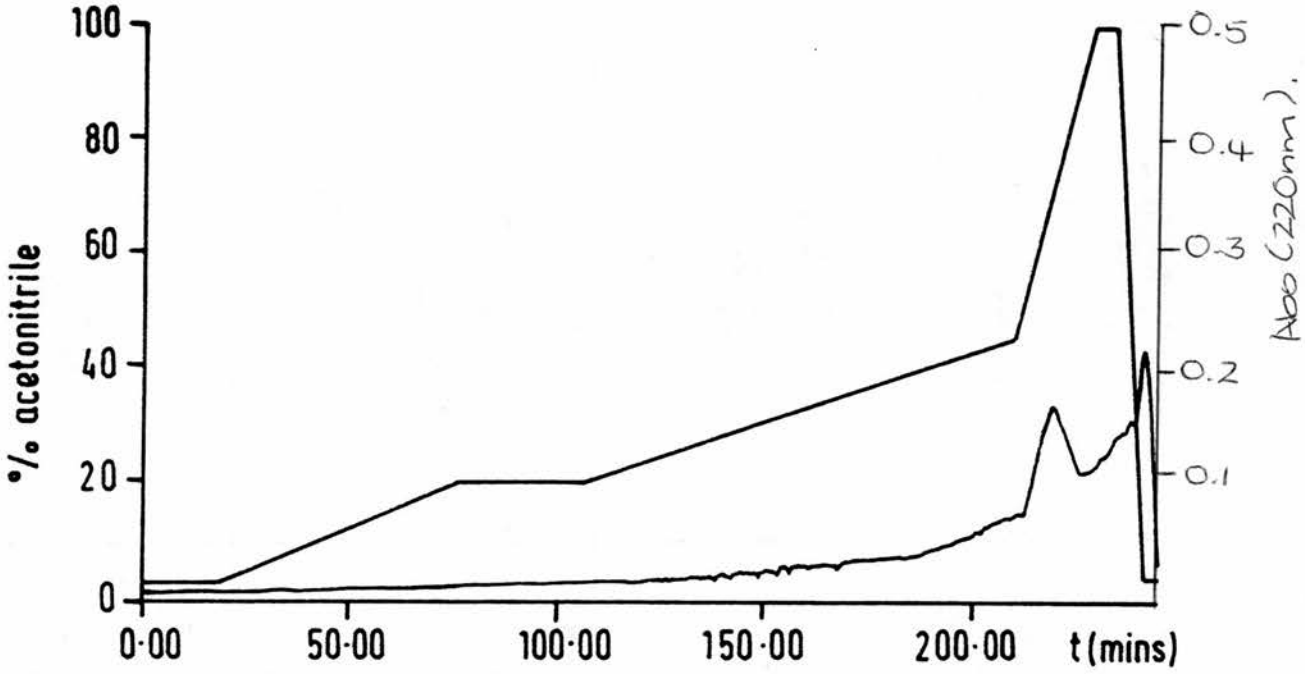
Kham et al (1982) suggested that the pH of the mobile phase used for the separation of elastin-derived peptides should be above pH 7 or below pH 4 to prevent the aggregation of the elastin peptides. Partridge et al (1955a) observed that coacervation at room temperature and low ionic strength is minimal below pH 4 therefore the presence of 0.1% TFA (pH 2.0) should discourage any aggregate formation during the peptide separation.

9.2.2 The separation of the elastin-derived peptides

When the elastin-derived peptides were separated as detailed in Section 2.4.2 they were eluted as three main groups regardless of the digesting enzyme (Figure 9.3). The first group was the unretained material in which the buffer salts, unretained peptides and the relatively hydrophilic peptides and enzymes elute. The unretained material was not further characterised because it would be difficult to separate some peptides from any buffer salts.

The remaining peptides were eluted in two groups which will be referred to as groups A and B. Manning et al (1989) also separated elastin-derived products resulting from the digestion of an elastin (from bovine ligamentum nuchae) into two main groups. Smyrlaki et al (1987) achieved their greatest success when separating elastin-derived peptides by using a multi-step elution procedure. This approach was exploited to clarify the separation between groups A and B; an isocratic step was incorporated into

Figure 9.4 Demonstration of 'ghost peaks' eluted from the reverse-phase column



A gradient of acetonitrile was applied to the PepRPC column (Pharmacia) as detailed in section 2.4.2; but no sample was loaded. Therefore the peaks eluted represented the impurities in the buffer system. The absorbance was monitored at 220nm with a full-scale deflection of 0.5 absorbance units (path length = 1cm).

the gradient (Figure 9.3) at 20% acetonitrile for 30 minutes (between 75-105 minutes after injection).

A fourth group of peaks was evident at the end of the gradient (between 40%-100% acetonitrile). These "ghost" peaks also appeared during a blank run (Figure 9.4) in which no sample was loaded and increased in size with an increasing number of runs. The "ghost" peaks probably result from the elution of some UV absorbing contaminants in the solvents which are eluted at high concentrations of the organic solvent (Corran, 1988).

The total yield of peptides in the unretained material and groups A and B was approximately 50%, as determined by the BCA method (see Section 2.3.2), and no more than 65% when measured by the TNBS method (see Section 2.3.3). The proportion of detected material eluted as the unretained material, or group A or group B peptides was typically 22%, 8% and 70% respectively when measured by the BCA method, or 7%, 50% or 43% respectively, if measured by the TNBS method. These data suggest that the unretained material contains the enzyme, which responds well to the BCA reagent because it probably has a larger number of peptide bonds than the peptides. The peptides of group A respond poorly to the BCA reagent but have more amino groups than the peptides of group B, which display a better response to the BCA reagent. Therefore, the peptides in group A are probably smaller than the peptides in group B.

The BCA method is known to be ineffective in detecting small peptides (see Section 4.1.2). Therefore, the quoted yield in response to the BCA method may be an underestimate of the total yield which actually

represents the yield of the large peptides. The inadequate yield of large peptides is probably not surprising because they are potentially more hydrophobic than smaller peptides particularly if they are denatured by the acetonitrile to expose internal hydrophobic groups. This suggests the large, retained peptides may accumulate on the column. This would eventually affect the efficiency of the column.

The high retention of elastin-derived peptides on reverse-phase columns was also observed by Smyrlaki et al (1987) and Manning et al (1989). Attempts were made to increase the yield of the elastin-derived peptides by developing the column with a gradient of 0%-60% propan-2-ol/0.1% TFA after the development with acetonitrile. Propan-2-ol is more hydrophobic than acetonitrile and should theoretically, produce a greater yield of large hydrophobic peptides. Unfortunately the propan-2-ol had a high background absorbance at 220nm so the detection of peptides was not possible. So, the use of propan-2-ol was rejected because far UV-FPLC grade propan-2-ol is not commercially available.

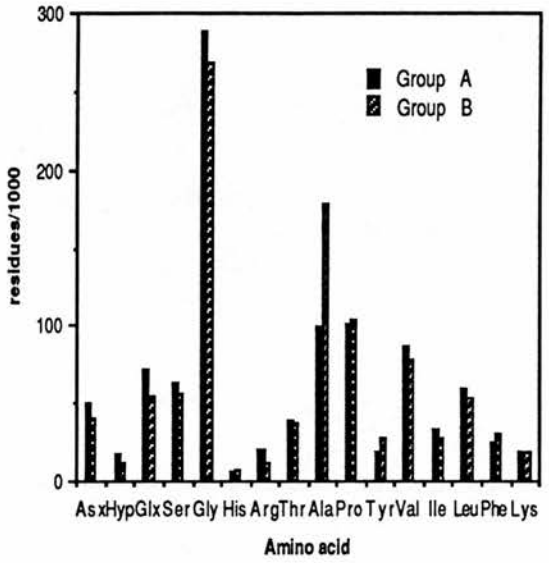
9.3 Characterisation of peptide groups A and B

9.3.1 Amino acid composition of groups A and B

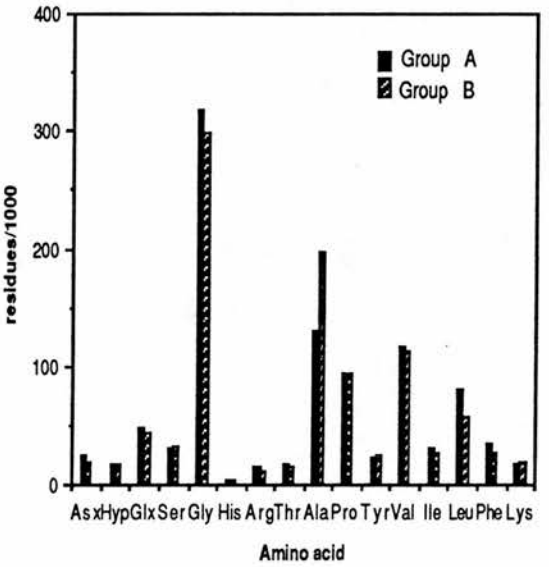
Lansing lung elastin was digested with either HNE (10mU/ml) or HNCG (10mU/ml) for 24 hours at pH 7.4 and 37°C as detailed in Section 2.3.1. The soluble peptides were isolated and separated by reverse-phase chromatography (detailed in Section 2.4.2). The peptides eluted in groups A and B were pooled as the two groups, concentrated by evaporation, and

Figure 9.5 Amino acid composition of groups A and B

a) Elastin-derived peptides resulting from digestion with HNE

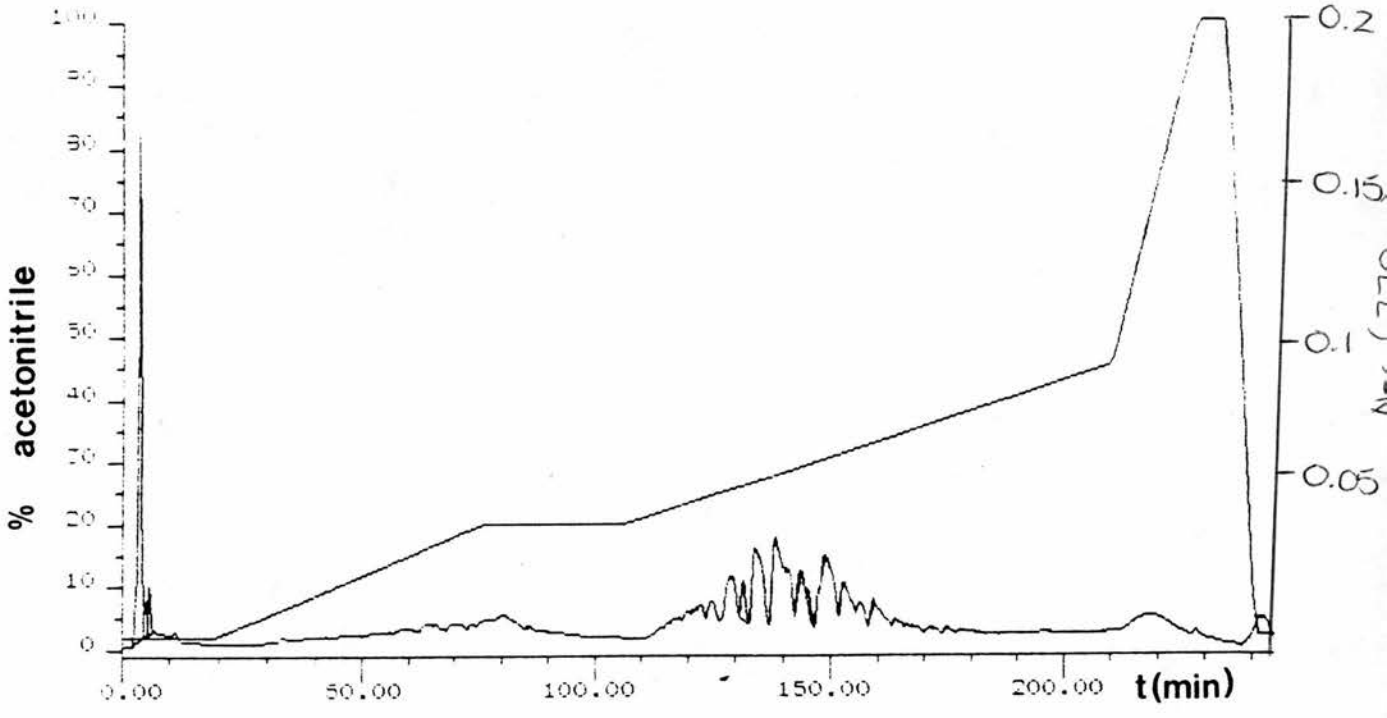


b) Elastin-derived peptides resulting from digestion with HNCG



Elastin-derived peptides were prepared and separated by reverse-phase chromatography as detailed in the text. The peptides of groups A and B were collected and their compositions were determined as detailed in section 2.2.3.

Figure 9.6 The detection of the absorbance at 270nm of elastin-derived peptides during their separation by reverse-phase chromatography



Elastin-derived peptides (0.4ml) resulting from the digestion of Lansing lung elastin with HNE for 48 hours were separate by reverse-phase chromatography. 100% corresponds to an absorbance at 270nm of 0.2.

their amino acid compositions were determined as detailed in Section 2.2.3 (Figure 9.5). The compositions of the peptides obtained by digestion with HNE or with HNCG and which were eluted as group A were similar. The same was true of group B. However, group A contained a high proportion of glycine and quite large amounts of alanine, valine and proline, which suggests that these peptides were cleaved from the non-cross-linked elastic region of the elastin. The proportions of glycine and valine were lower in group B as compared with group A and there was a large increase of alanine, but the proportion of alanine was still less than the proportion of glycine. Since poly-alanine sequences are found in the amino acid sequence close to the cross-link regions of elastin it seemed possible that some of them contain desmosine cross-links.

It can be seen from Figure 9.5 that there is little difference between the amount of tyrosine in the peptides of group A and those in group B. So it can be assumed that if one group of peptides contains a greater amount of peptides capable of absorbing at 270nm than the other group then the absorbance at 270nm represents peptides which have desmosine in their sequence. Attempts were made to locate the desmosine-containing peptides by repeating the separation and measuring the absorbance of the eluate at 270nm (Figure 9.6). As expected, the peptides of group B but not group A were detected at this wavelength. Unfortunately the yield of peptide material was insufficient to allow an accurate quantitation of the amount of desmosine by the methodology detailed in Section 3.2.2.

Table 9.1 Fractionation of the peptides by size

Retention size of filter	% of peptides passing through the filter (measured with BCA reagent)
2kDa	20
10kDa	42
30kDa	56

Samples of soluble elastin-derived peptides were passed through filters with different retention sizes as detailed in the text. The percentage of the peptides which passed through the filters was measured by the BCA method as detailed in section 2.3.2.

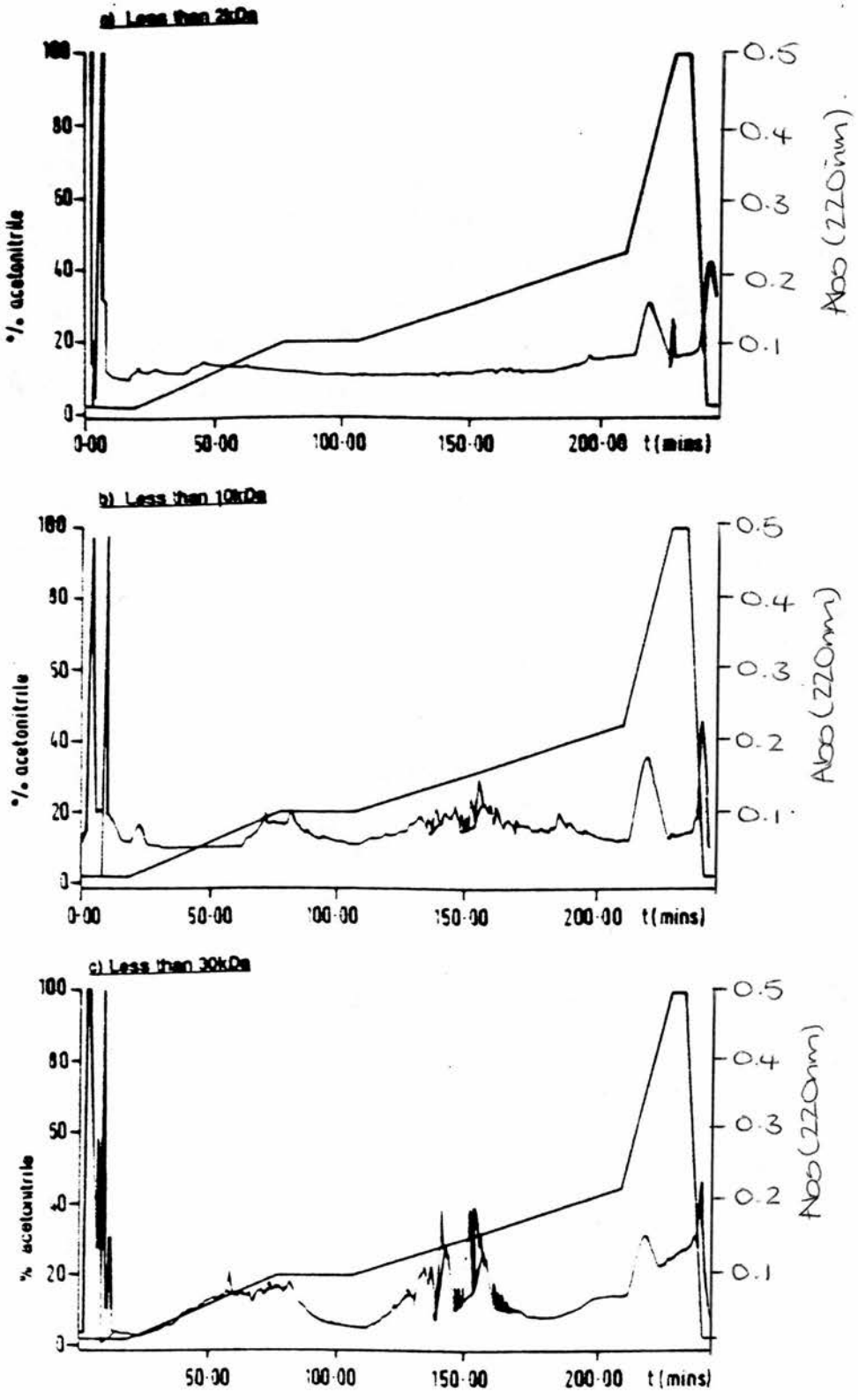
9.3.2 Determination of the relative size of the peptides in groups A and B

The elution pattern observed in Section 9.3.1 was rather surprising because the elastic region of elastin is relatively more hydrophobic than the inelastic cross-linked region; but the peptides derived from the elastic region appeared to be relatively hydrophilic and were eluted first from the column.

Manning et al (1989) observed that the elastin-derived peptides which were eluted with 7%-20% acetonitrile were small. Therefore a possible explanation for the elution pattern observed in Section 9.3.1 is that the peptides in group A are smaller than the peptides in group B. So, see also p. 161 although some of the peptides in group B contain the hydrophilic sequences associated with the cross-link, they may be sufficiently large to make the whole peptide relatively more hydrophobic than smaller peptides. A small peptide will generally appear more hydrophilic than a large peptide which is made up with the same amino acids because of the relatively greater contribution to hydrophilicity made by the hydrophilic amino- and carboxyl-termini in the small peptide.

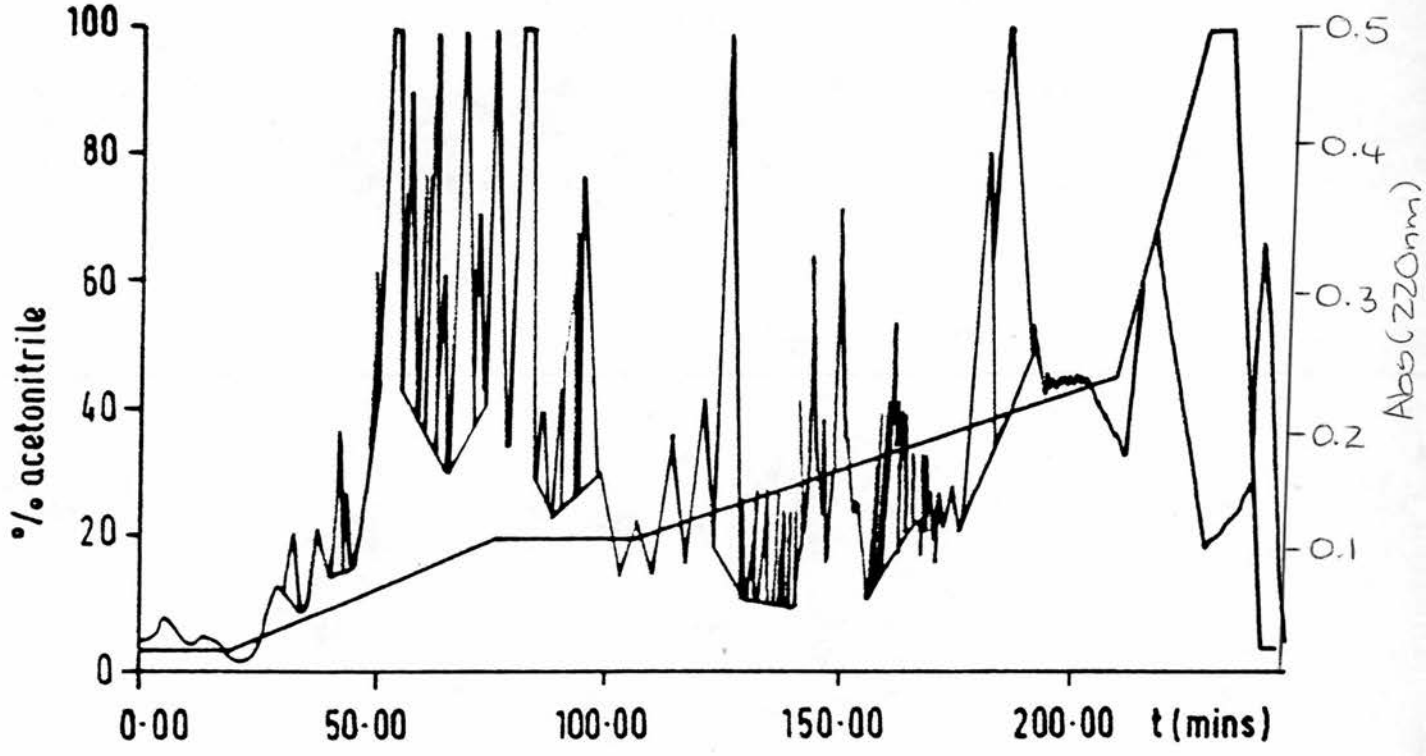
Lansing lung elastin was digested with HNE (10mU/ml) for 24 hours at 37°C and pH 7.4, after which the soluble peptides were isolated (see Section 2.3.1). Samples (450μg by BCA) of these elastin-derived peptides were fractionated by ultrafiltration through a YM2 or a YM30 membrane (Amicon) under nitrogen (10 psi) using a 5ml ultrafiltration cell (Amicon), or through a 10kDa Millipore filter. The amount of peptides which passed through each membrane was measured (Table 9.1) before they were separated by reverse-phase chromatography as detailed in Section 2.4.2

Figure 9.7 Reverse-phase separation of fractionated peptides



Elastin-derived peptides (450µg by BCA assay) were prepared as detailed in the text, and passed through either a 2kDa, 10kDa or 30kDa membrane. The peptides which passed through the membranes were separated as detailed in section 2.4.2. 100% corresponds to an absorbance at 220nm of 0.5.

Figure 9.8 The removal of elastin-derived peptides from the reverse-phase column by digestion with pepsin



The reverse-phase column was treated with pepsin as detailed in the text. 100% corresponds to an absorbance at 220nm of 0.5.

Table 9.2 The composition of elastin-derived peptides digested from the reverse-phase column with pepsin

Amino acid	Number of residues/1000 residues		
	pepsin digestion fragments	Pepsin (a)	Elastin (b)
Asx	135	116	7
Glx	100	75.4	22
Ser	121	124.6	7
Gly	115	98.6	335
His	4	2.9	6
Arg	6	5.8	6
Thr	81	72.5	7
Ala	68	46.4	267
Pro	70	46.4	109
Tyr	49	46.4	23
Val	47	58	100
Met	12	11.6	4
Cys	9	17.4	0
Ile	52	66.7	19
Leu	76	81.2	55
Phe	46	127.5	20
Lys	8	2.9	1

a) Based on the compostion of pepsin quoted by Rajagopalan et al (1966).

b) The composition of Lansing lung elastin quoted by Reilly and Travis (1980).

(Figure 9.7). Both groups A and B appeared to contain a heterogeneous mixture of peptides, some of which may co-elute. The resulting profiles tentatively suggest that the peptides in group B are relatively larger than the peptides of group A.

9.3.3 Examination of fragments of the retained peptides

It was stated in Section 9.2.2 that some of the large elastin-derived peptides are retained on the reverse-phase column. After several runs the initial pressure through the column increased slightly, which may confirm that some material was retained on the column. If some peptides remained on the column they would eventually impair the performance of the column. Attempts were made to remove fragments of the retained elastin-derived peptides from the column after several runs by digesting the material with pepsin.

Pepsin (Worthington; 20μg in 2ml of 0.1% TFA) was loaded onto the reverse-phase column (1.1ml) which had been equilibrated with 0.1% TFA. The flow of the mobile phase was stopped for an hour after which the enzyme was washed off the column with 0.1% TFA (10ml). The fragments of the retained elastin-derived peptides were eluted from the column using a gradient of acetonitrile as detailed in Section 2.4.2 (Figure 9.8). The fragments were pooled, concentrated by rotary evaporation and their amino acid composition was determined as detailed in Section 2.2.3 (Table 9.2). The composition was similar to that of pepsin. However, the amount of protein washed off the column after digestion was always greater than the amount of pepsin initially loaded onto the column when measured by the

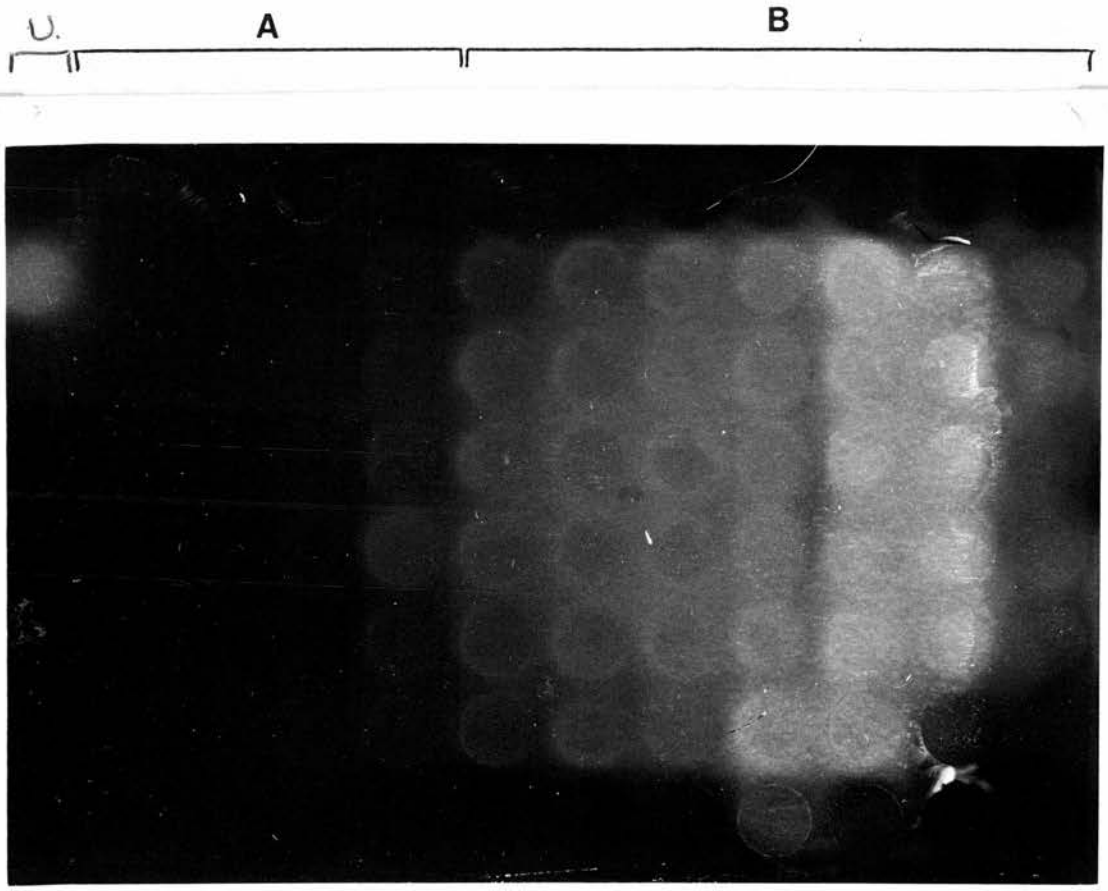
BCA method (Section 2.3.2); and the pepsin treatment was sufficient to return the initial pressure to normal suggesting that the treatment had removed some of the retained material.

9.3.4 Identification of borohydride reducible peptides

If the peptides of group A were smaller than the peptides of group B it would be reasonable to expect an endopeptidase to hydrolyse the peptides of group B further, so that the proportion of small peptides (group A) would be greater after 48 hours of digestion as compared with the proportion after 24 hours of digestion. However, in Section 6.3 it was suggested that some of the peptides resulting from digestion by HNE are capable of reacting together in 0.1M sodium phosphate buffer/0.15M NaCl, pH 7.4 to form larger aggregates and that the formation of the aggregates was prevented by reducing the peptides with sodium borohydride (Section 6.4.1); so it seemed possible that the small peptides involved in aggregate formation could be identified by examining the peptide separation profiles produced after 48 hours of digestion with HNE (10mU/ml) with and without sodium borohydride reduction.

Suspensions of Lansing lung elastin were digested with HNE (10mU/ml) as detailed in Section 2.3.1. A suspension was reduced with sodium borohydride after 24 hours of digestion, as detailed in Section 6.4.1; but on this occasion, tritiated sodium borohydride (Amersham; 350Ci/mol) was used at a ratio of 1:15 (w/w) of labelled to unlabelled sodium borohydride (this represented the addition of 4mCi of tritium/ml of elastin suspension). Samples (1ml) of the tritiated material were removed after 44

Figure 9.9 Autoradiographic study of the tritium incorporation into elastin-derived peptides



The peptides resulting from 44 hours of digestion with HNE, which had been reduced after 24 hours with tritiated sodium borohydride (as detailed in the text) were separated by reverse-phase chromatography as detailed in section 2.4.2. A piece of autoradiography film (Amersham) was placed over the top of the fraction collection rack and left in the dark for 5 days after which the film was developed.

U: fractions corresponding to the unretained material.
A: fractions corresponding to the peptides in group A.
B: fractions corresponding to the peptides in group B.

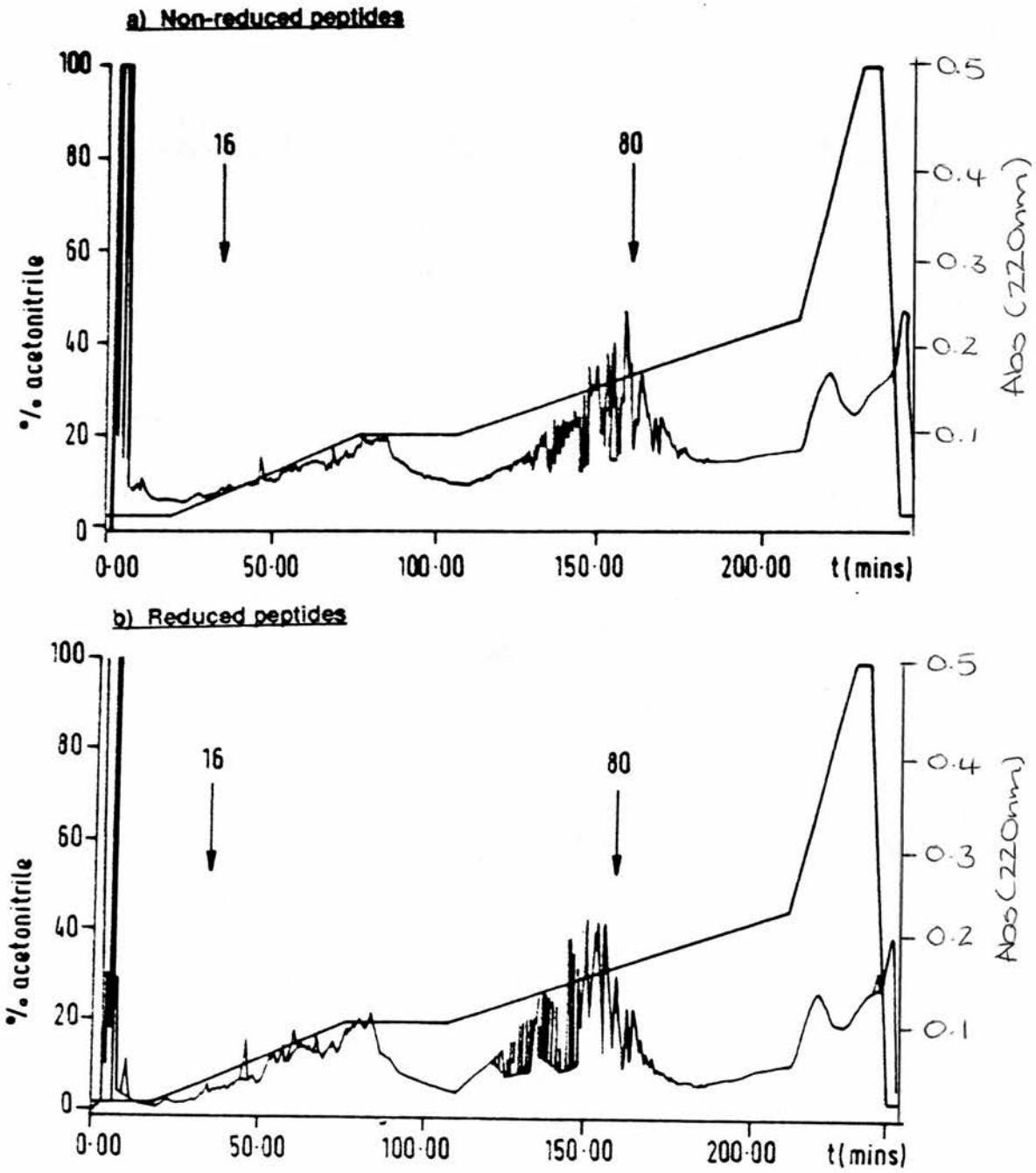
The experiment was performed on a single occasion.

and 48 hours of digestion. The insoluble and soluble material were separated by centrifugation as detailed in Section 2.3.3. The soluble peptides 0.2ml) were made up to 0.4ml with 0.1M sodium phosphate buffer/0.15M NaCl pH 7.4, and the insoluble material was suspended in 0.4ml of 0.1M sodium phosphate buffer/0.15M NaCl pH 7.4. Each sample was treated with 3.6ml of Cocktail T (BDH) and the tritium was measured using a 1900 CA Tri-carb liquid scintillation analyzer (Packard). Any tritium which was not incorporated into peptides would be detected in the soluble fraction so it was not surprising to find that the majority of the detectible tritium was located in the soluble fraction, and only 4% and 3% was located in the insoluble material after 44 and 48 hours of digestion respectively.

The soluble peptides (0.3ml) were separated by reverse-phase chromatography as detailed in Section 2.4.2. The location of the tritium after 44 hours of digestion was measured qualitatively by autoradiography (Figure 9.9). The excess of tritium was eluted with the unretained material, probably as a by-product of hydrolysed borohydride; and some of the peptides in group B appeared to be labelled. It was suggested in Sections 9.3.1-9.3.2 that some of the peptides in group B may be derived from the cross-linked regions of elastin. So, the peptides in group B are most likely to contain aldehyde cross-link intermediates which would be the borohydride-reducible groups implicated in coacervate stabilisation (Section 6.4), and could be expected to be labelled with the tritium.

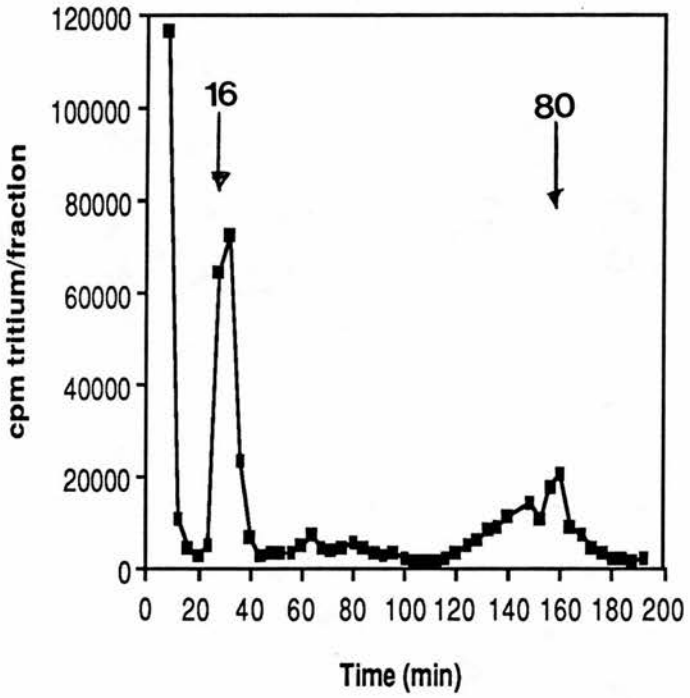
It was hoped that the elution pattern of the peptides in group B which had been reduced would differ from the elution pattern of those which had not been reduced. This would allow the identification of the peptides with

Figure 9.10 The comparison of the reverse-phase separation profiles of reduced and non-reduced peptides



Elastin-derived peptides resulting from 48 hours of digestion with HNE (10mU/ml) were separated by reverse-phase chromatography as detailed in section 2.4.2. In the case of profile (b) the peptides were reduced with sodium borohydride after 24 hours of digestion.

Figure 9.11 Measurement of tritium incorporation into peptides derived from elastin by digestion with HNE



An elastin suspension (3mg/ml) was digested with HNE (10mU/ml; 1:100 w/w). After 24 hours the suspension was treated with sodium borohydride (4:! w/w of elastin:sodium borohydride) which was composed of a 1:15 w/w ratio of tritiated borohydride (4mCi/ml elastin peptide suspension) to cold borohydride. The digestion was allowed to continue as before. Fractions were removed after a further 24 hours of digestion i.e. a total of 48 hours of digestion. The soluble peptides were separated by reverse-phase chromatography as detailed in section 2.4.4, and the amount of radiolabel eluted from the column was measured by counting the tritium in each alternate 1ml fraction as detailed in the text.

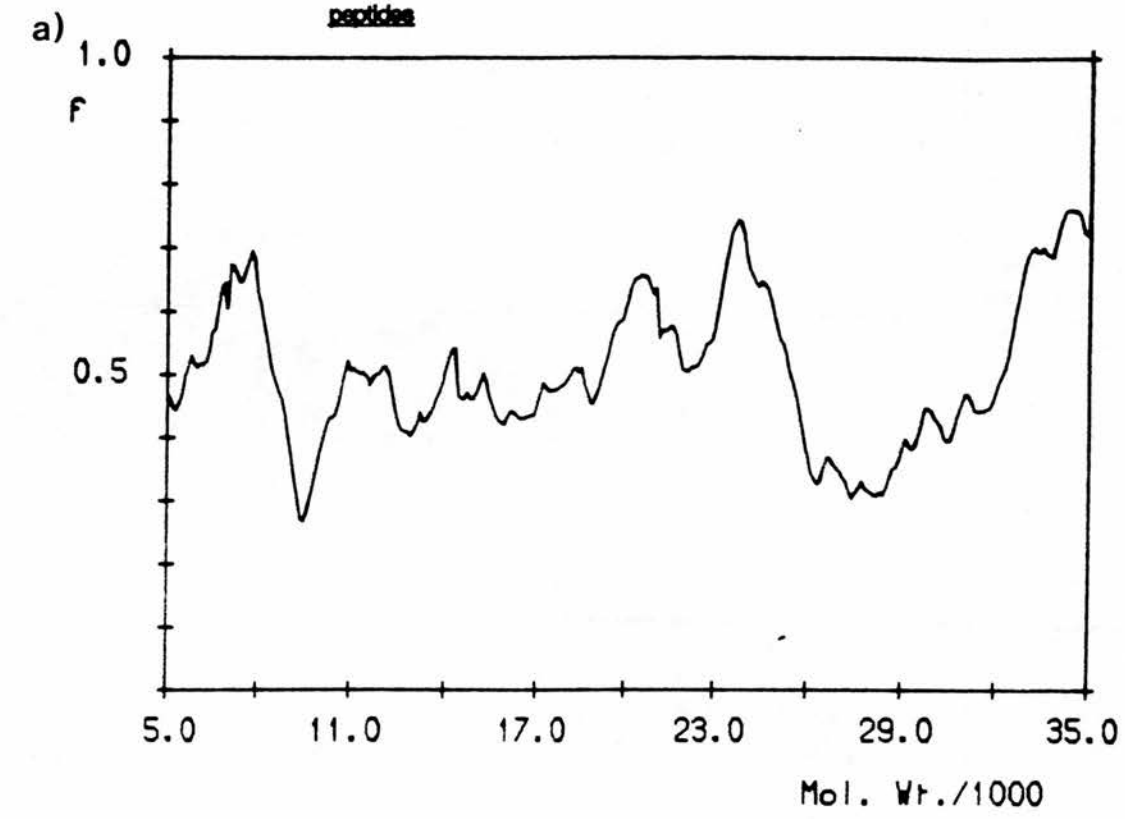
The experiment was performed on a single occasion.

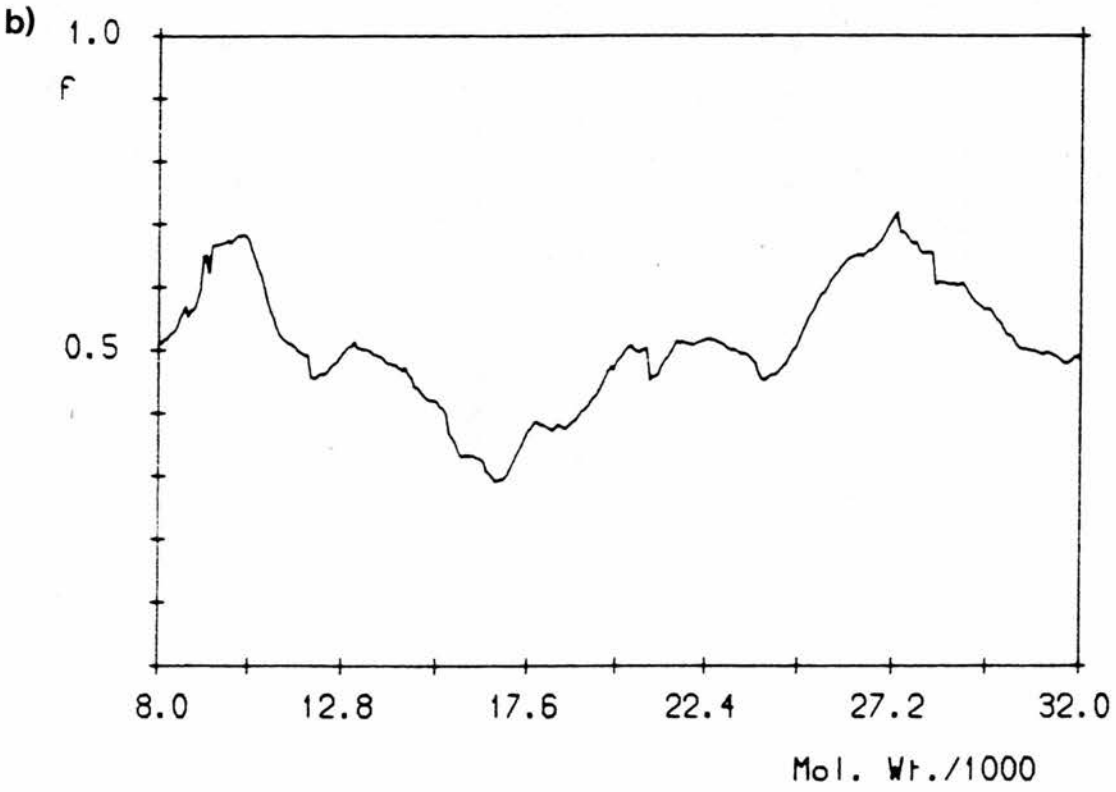
reducible residues. Unfortunately, when the profiles produced by reduced and non-reduced peptides were compared (Figure 9.10) there was no more difference than would be expected when comparing successive runs of the same peptide mixture. This is probably because the profiles are too complex to allow the observation of subtle changes.

An alternative way of identifying the reduced peptides produced after 48 hours of digestion with HNE was to quantitatively locate the tritium within the eluted fractions from the reverse-phase column. Cocktail T (3.6ml) was added to 0.4ml of each alternate fraction of the separated, tritiated peptides, and the tritium was measured in a 1900 CA tri-carb liquid scintillation analyzer (Packard) (Figure 9.11). The yield of tritium eluted from the column in the fractions was 100% of that which was loaded onto the column. So it was concluded that all the tritiated peptides were eluted from the column. It can be seen from Figure 9.11 that many of the tritiated peptides present after 48 hours of digestion with HNE were still evident in the group B peptides, but some tritium was also present in the early members of the group A peptides. This suggests that tritiated members of group B present after 44 hours of digestion may have been digested further between 44 and 48 hours to fragments which elute as early members of the group A peptides after 48 hours of digestion. Fraction 16, from group A, and fraction 80, from group B, were examined further. Samples (0.3ml) of the two fractions were subjected to acid hydrolyses and their amino acid composition was determined as detailed in Section 2.2.3.

The integer composition and the minimum mass of a peptide can be estimated by the method of Black and Hogness (1969). If the peptide was

Figure 9.12 Determination of the fraction of maximum deviation of the tritiated peptides





The amino acid composition of the tritiated peptides in fractions (a) 16 and (b) 80 were analysed by the method of Black and Hogness (1969) as detailed in the text.

assigned a molecular weight M then the minimum number of residues of amino acid i (n_i) in the peptide can be calculated according to the formula:-

$$n_i = F_i\ [(M-18)/\Sigma\ F_i \times R_i]$$

where F_i is the average number of residue i found by amino acid analysis and R_i is the molecular weight of residue i. After calculating the values of n_i the fraction of maximum deviation (f) from the molecular weight M may be calculated:-

$$f = \frac{\Sigma\ \Delta_i / I_i}{0.5\ \Sigma 1 / I_i}$$

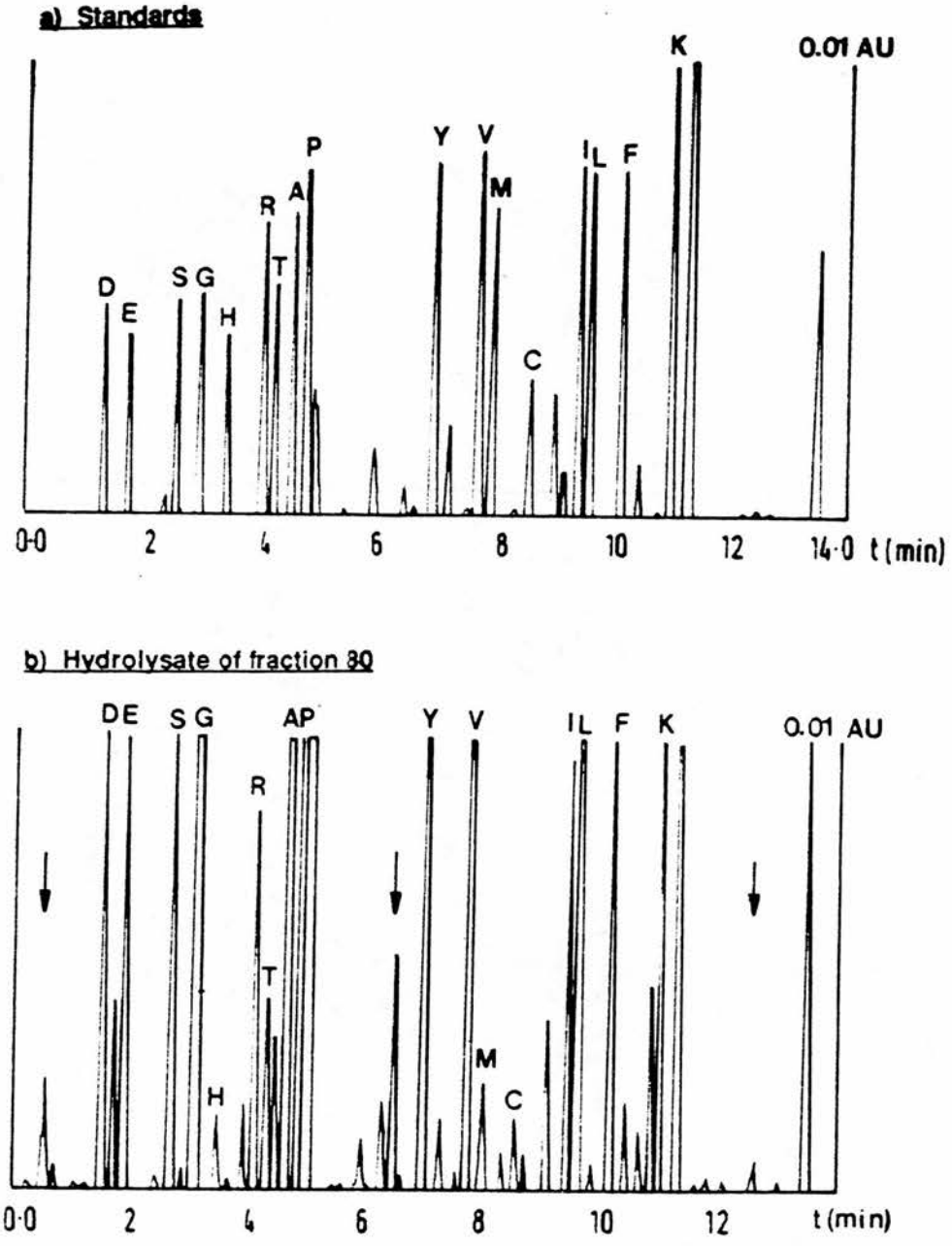
where Δ_i is the difference between n_i and the nearest integer I_i, regardless of sign. The minimum molecular weight of the peptide is taken to be the molecular weight at which (f) reaches a minimum. A computer programme was created by Dr. A.P. Ryle based on the method of Black and Hogness (1969). The computer programme calculates the value of (f) over a range of molecular weights from an amino acid composition of a protein or peptide and the integer composition of the protein or peptide at the molecular weight at which the value of (f) is minimal. The computer programme was used to calculate the value of (f) over a range of molecular weight from the amino acid compositions of the peptides in fractions 16 and 80 (Figure 9.12). The minimum values of (f) for the peptides in fraction 16 and 80 corresponded to

Table 9.3 The integer composition and calculated mass of the tritiated peptides of fractions 16 and 80

Amino acid	Number of residues in integer composition	
	Fraction 16 from group A	Fraction 80 from group B
Asx	(0) 0	(6.04) 6
Glx	(9.01) 9	(8.90) 9
Ser	(9.10) 9	(5.61) 6
Gly	(56.81) 57	(69.10) 69
His	(0.68) 1	(0.92) 1
Arg	(1.79) 2	(3.18) 3
Thr	(3.04) 3	(2.11) 2
Ala	(19.09) 19	(53.98) 54
Pro	(0) 0	(4.23) 4
Tyr	(1.79) 1	(7.02) 7
Val	(5.91) 6	(13.77) 14
Met	(0) 0	(0.89) 1
Cys	(0) 0	(1.21) 1
Ile	(2.06) 2	(2.86) 3
Leu	(5.06) 5	(11.90) 12
Phe	(2.91) 3	(4.07) 4
Lys	(2.18) 2	(2.71) 3
Mr by summation	9570.5	16804.4
min f value	0.267	0.291
Mr at min f value	9500	16733

The compositions of the tritiated peptides at the molecular weight corresponding to the minimum value of f are presented in brackets. The nearest integers are presented to the right of the brackets.

Figure 9.13 Comparison of the separation profiles from the amino acid analyzer of a standard mixture of PTC-amino acids and PTC-residues from the hydrolysed peptides of fraction 80



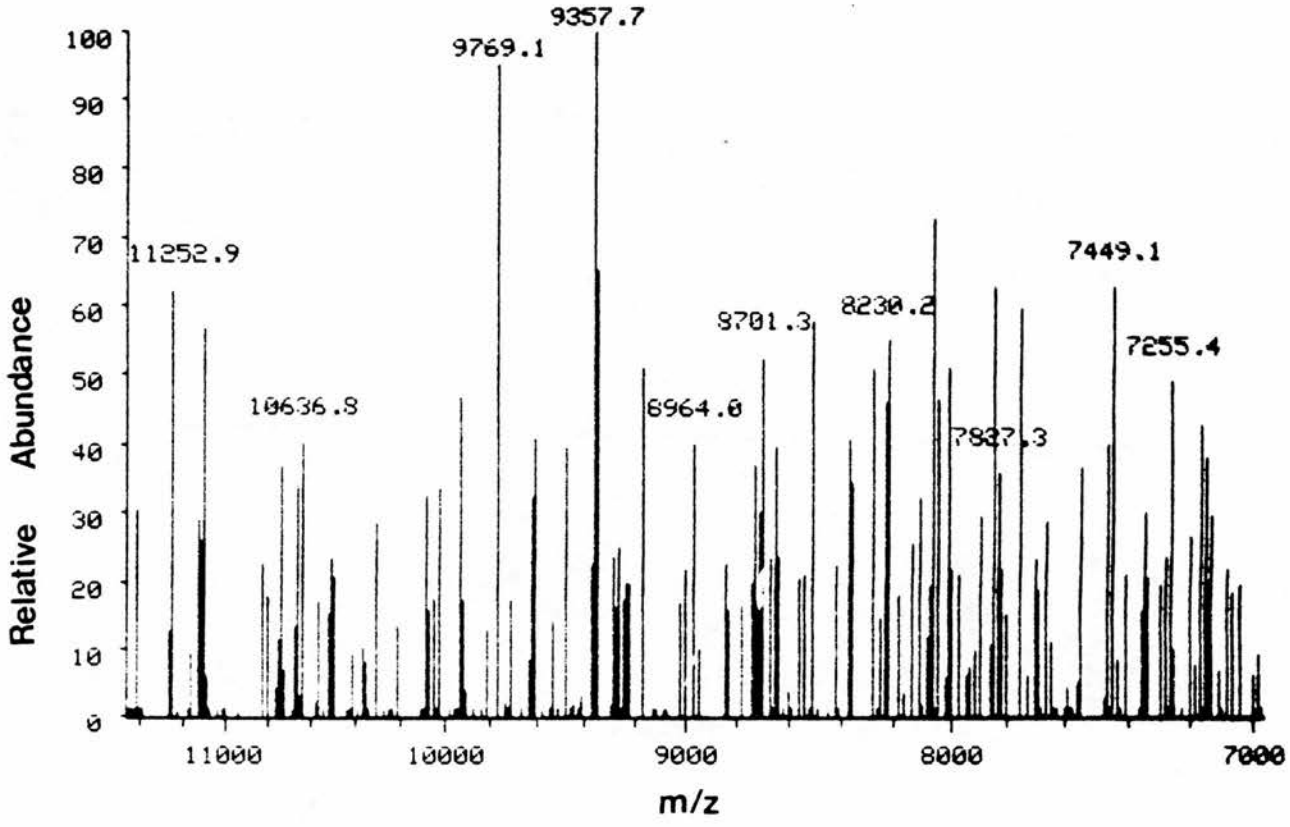
↓ represents unusual, unidentified peaks which are not present in the standard profile, and may represent unusual residues such as cross-links or cross-link intermediates found in the tritiated peptides. The single letter abbreviation is used for each amino acid.

molecular weights of 9500 and 16733 respectively. The integer compositions of the peptides at these molecular weights are presented in Table 9.3.

The peptide in fraction 80 (estimated molecular mass 16733) had similar proportions of alanine and glycine, which would be expected for a peptide from the cross-linking region of elastin which had some sequence associated with the elastic region. When the separation profile of the acid hydrolysed and phenylthiocarbamylated-amino acids (PTC-amino acid) of fraction 80 was compared with the separation profile of a mixture of standard derivatised amino acids, there appeared to be a number of unidentified peaks (Figure 9.13) which could represent either incompletely hydrolysed fragments of the peptides or reduced cross-link intermediates with the potential to cause aggregation (see Sections 1.3 and 6.3.4). Paz et al (1976) separated the amino acids of acid-hydrolysed elastin by ion-exchange chromatography and noticed the presence of some unusual residues, which they identified as reduced aldol condensation products and reduced desmosines (Robins, 1982). The reverse-phase chromatogram (Figure 9.13) cannot be compared directly with the chromatograms obtained by Paz et al (1976). However, desmosine and isodesmosine standards were shown by the Wel-Met Protein Characterisation Service to be eluted in their system after approximately 12 minutes. Therefore, the extra peaks eluted after 12-13 minutes may represent desmosine and isodesmosines.

As expected the peptide in fraction 16 (estimated molecular mass 9570.5) were smaller than the peptide in fraction 80 (Table 9.3). It has already been suggested that the peptide of fraction 16 could be a fragment

Figure 9.14 Mass spectrometry of fraction 16



Fraction 16 was examined by continuous flow fast atom bombardment mass spectrometry by Mr A. Taylor of the Department of Chemistry, University of Edinburgh, as detailed in the text. 100% corresponds to 1387mV.

from the inelastic, reducible region of a larger peptide. If this was so the proportion of alanine to glycine would be at least equal; but surprisingly the proportion of glycine was much greater than the proportion of alanine (Table 9.3). This may suggest that the peptide was not derived from the cross-link region, or there was more than one peptide in the fraction. Mass spectrometry was used to deduce the exact mass of the peptide(s) of fraction 16, and to attempt to deduce some information about the tritiated residues of the peptide(s).

A sample of fraction 16 (0.3ml corresponding to approximately 75pmol) was concentrated by evaporation, dissolved in a small amount of glycerol and examined by continuous flow fast atom bombardment mass spectrometry (courtesy of the Department of Chemistry, University of Edinburgh) (Figure 9.14). Two major peaks were seen to approximate to the estimated molecular mass of 9570.5 ion masses 9769.1 and 9357.7), but they were possibly fragments of two larger peptides with ion masses of 11252.9 and approximately 11100. In both these pairs of peaks the second peaks, but not the first, appeared to have a number of isotopic forms which would be expected for a tritiated peptide. The peaks with smaller ion masses correspond to the ion masses of fragments of the parent peptide(s). The information presented by the mass spectrum may suggest that fraction 16 contained two co-eluting peptides of which only one was the tritiated peptide of interest. This is not altogether surprising as a peptide peak was seen to elute as fraction 16 in both the reduced and unreduced reverse-phase chromatogram (Figure 9.10), suggesting that the tritiated peptide of fraction 16 may be co-eluted with another peptide. Unfortunately, it was not

possible to obtain any information about the tritiated residue of the peptide from the spectrum, or to determine if the tritium had reduced a cross-link intermediate.

Any further examination and isolation of reducible cross-link intermediates would require the separation of a hydrolysate by reverse-phase chromatography followed by scintillation counting and mass spectrometry of any tritiated residues. This would require the harvesting of larger amounts of the peptides, and although potentially very interesting, the procedure would be extremely laborious and time consuming. So this work was not pursued any further since the identification of cross-link intermediates was outwith the original aims of the project.

9.4 The isolation of a specific peptide

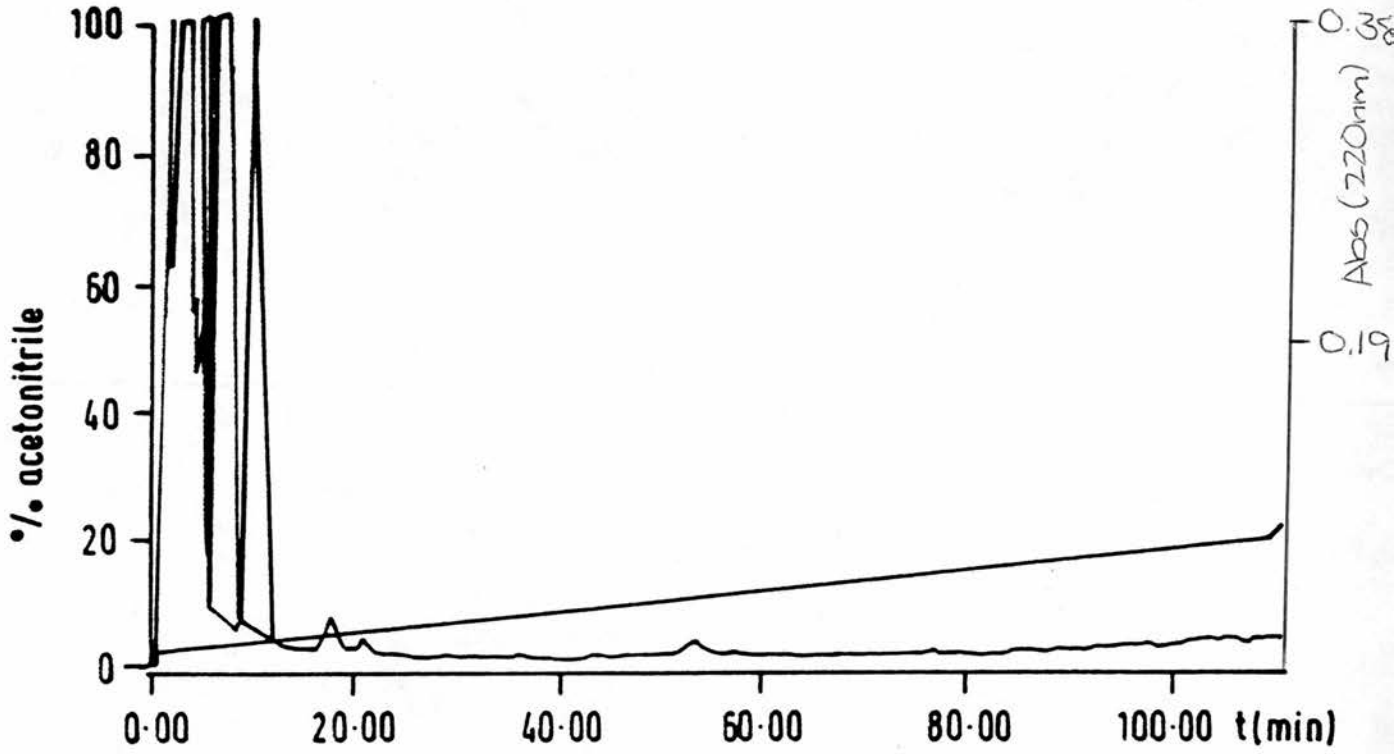
Each group of peptides was examined in turn in an attempt to determine if any peptides specific for a particular type of digestion could be identified.

9.4.1 Examination of peptide group A

Fraction 16 eluted as an early member of group A and appeared to contain two co-eluting peptides (see Section 9.3.4). So other members of group A could be co-eluted when separated by the gradient conditions detailed in Section 2.4.2, and their separation may be improved by re-applying them to the PepRPC reverse-phase column and developing the column with a shallow gradient of acetonitrile.

Peptides from group A were concentrated to dryness by rotary

Figure 9.15 Elution profile produced when peptides from group A were re-loaded onto the reverse-phase column



Peptides from group A were concentrated then dissolved in 0.1% TFA (19ug of peptides by BCA assay in 0.5ml). The peptides were re-applied to the PepRPC column which was developed with a gradient of 2-20% acetonitrile/0.1% TFA over 110 minutes. The peptides were expected to be eluted at 10-20% acetonitrile. 100% corresponds to an absorbance at 220nm of 0.38.

evaporation, then 19μg of peptides by the BCA method were dissolved in 0.5ml of 0.1% aqueous TFA. The peptides were re-applied to the reverse-phase column which had been equilibrated with 2% acetonitrile/0.1% TFA, and the column was developed with a gradient of 2%-20% acetonitrile/0.1% TFA over 110 minutes. It was expected that the peptides would be eluted from the column at the same percentage of acetonitrile (10%-20%) as previously observed in Figure 9.3. However, when the peptides of group A were re-applied to the reverse-phase column they were seen to elute earlier than expected (Figure 9.15). Corran (1988) stated that a change in the retention of a peptide or protein is a result of an irreversible change to the peptide or protein such as denaturation and aggregation, or more likely, dissociation.

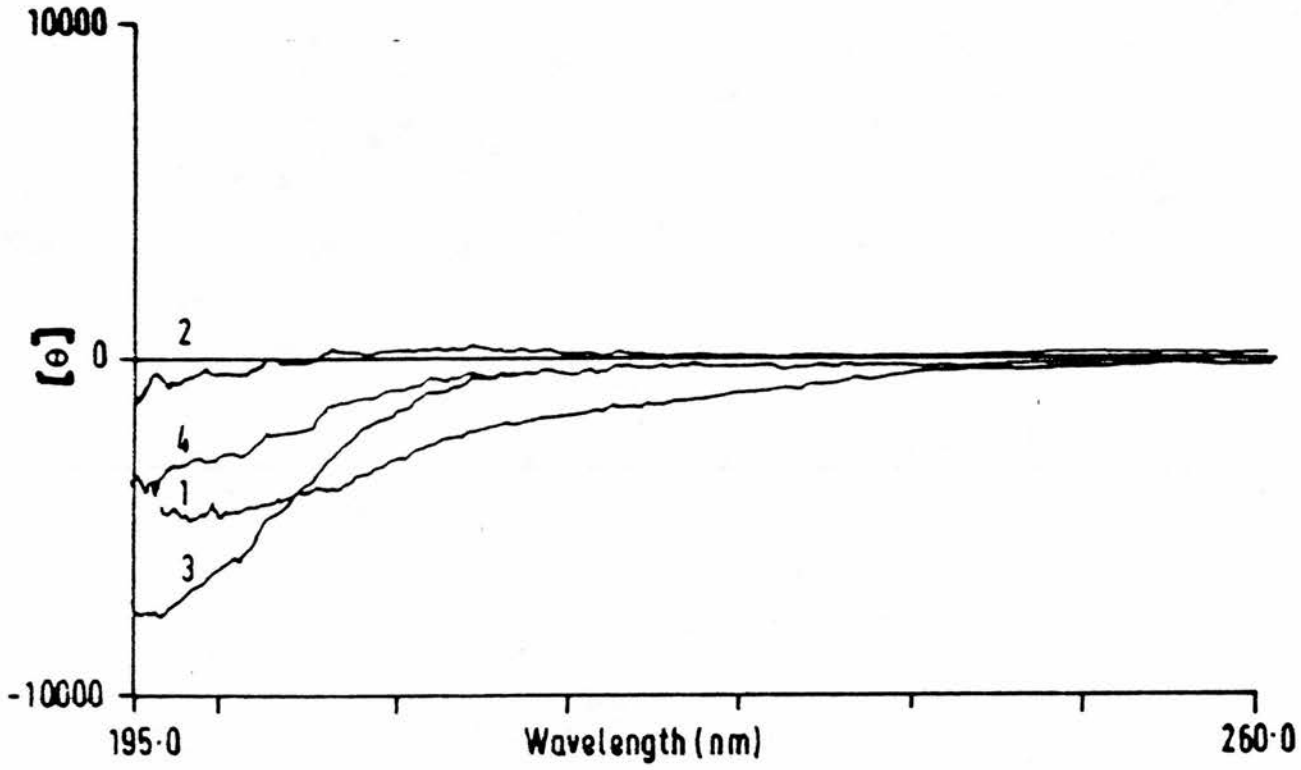
Urry (1983) stated that the polypentapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ and the polyhexapeptide $(Ala\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly\text{-}Val)_n$ repeating sequences in the elastic region of tropoelastin can form β-spirals. A β-spiral is a helical repeat of β-turns. A β-turn is a 10-atom hydrogen bonded ring. The β-spiral of the polypentapeptide has 2.8 pentapeptide repeats per turn and the polyhexapeptide has 2 hexapeptide repeats per turn. Urry (1983) suggested that during coacervation molecules of the polypentapeptide or molecules of the polyhexapeptides are capable of associating together to form a twisted filament. Similar associations between molecules of tropoelastin may be necessary to correctly align the molecules prior to cross-link formation (Cox et al, 1974). Cross-links are formed between short, α-helical, alanine-rich sequences which are contiguous with the elastic

repeating sequences (see Section 1.3). It is possible that some of the peptides from human lung elastin in 0.1M sodium phosphate buffer/0.15M NaCl, pH 7.4, had structural features which were changed during reverse-phase chromatography and rotary evaporation. Any change of secondary structure could presumably be investigated by circular dichroism spectroscopy, which is the technique used by Mammi et al (1968) and Urry et al (1969) to examine coacervates of α-elastin.

Circular dichroism involves the measurement the molar ellipticity of an optically active solution at a given wavelength. A linearly polarised beam of light may be considered to consist of a right- and a left-handed circularly polarised component which are in phase. When the linearly polarised beam of light is passed through an optically active solution the two components of the light may be absorbed to different extents. The resulting difference of absorbance of the two components is proportional to the molar ellipticity and concentration of the optically active compound. When the molar ellipticity is plotted against the wavelength of the polarised light the resulting spectrum will show maxima and minima which may be characteristic of certain structural features. For example, a β-pleated sheet has a minimum molar ellipticity near 220nm and a maximum molar ellipticity between 195 and 200nm.

Circular dichroism was used to examine a mixture of elastin-derived peptides before and after they were separated by reverse-phase chromatography and concentrated by rotary evaporation to establish whether or not these procedures caused a change of the secondary

Figure 9.16 Circular dichroism examination of the peptides before and after reverse-phase separation



The molar ellipicity (measured in deg.cm^2.dmol^{-1} of amino acids) of (1) elastin-derived peptides resulting from 48 hours of digestion with HNE (10mU/ml) and (2) the unretained material, (3) group A and (4) group B peptides produced when mixture (1) was separated by reverse-phase chromatography, were measured in a JASCO J-600 spectropolarimeter with a path length of 0.1cm at 20°C.

structure of the peptide mixture.

Mixtures of elastin-derived peptides resulting from 48 hours of digestion with HNE (10mU/ml) were separated by reverse-phase chromatography as detailed in Section 2.4.2. The unretained material and group A peptides and group B peptides were collected separately and concentrated to dryness by rotary evaporation. After concentration they were dissolved in sufficient water to give solutions which had an absorbance at 215nm of approximately 1.0. In general, the unretained material from a single fractionation had a very small absorbance at 215nm in comparison with the peptides of group A or the peptides of group B resulting from the same fractionation. The concentration of amino acids in each solution of peptides was calculated by assuming that a solution containing 1mg of peptides per ml has an absorbance at 215nm of 15 (Harris, 1987) and the average residue weight of elastin is 85. The concentration of amino acids in the unfractionated material was 1.5mM, and the concentrations of amino acids in the solution of unretained material, group A peptides and group B peptides were 0.995mM, 0.94mM and 0.851mM respectively. The majority of the absorbance at 215nm of the unretained material was due to the presence of the phosphate from the digestion buffer, so the concentration of peptides in the unretained material is over-estimated.

Miss S Kelly and Dr. N Price (Department of Biological and Molecular Sciences, University of Stirling) kindly examined 1ml samples of the mixture of elastin-derived peptides and the groups of separated peptides in a JASCO J-600 spectropolarimeter with a path length of 0.1cm at 20°C (Figure 9.16). The amount of secondary structure of each group of peptides

Table 9.4 Estimation of the amount of secondary structure of the peptides

Peptides	α -helix (%)	β -sheet (%)	Remaining (%)
Peptide mixture	2	49 + 1.3	49 + 1.3
Group A	0	55 + 2.6	45 + 2.6
Group B	0	58 + 2.9	42 + 2.9

The percentage of α -helix and β-sheet was calculated by Miss S Kelly from the data in figure 9.16 over 196-240nm using the CONTIN computer programme (Provercher and Glockner, 1981). There was no evidence to suggest that the unretained material had any structural features.

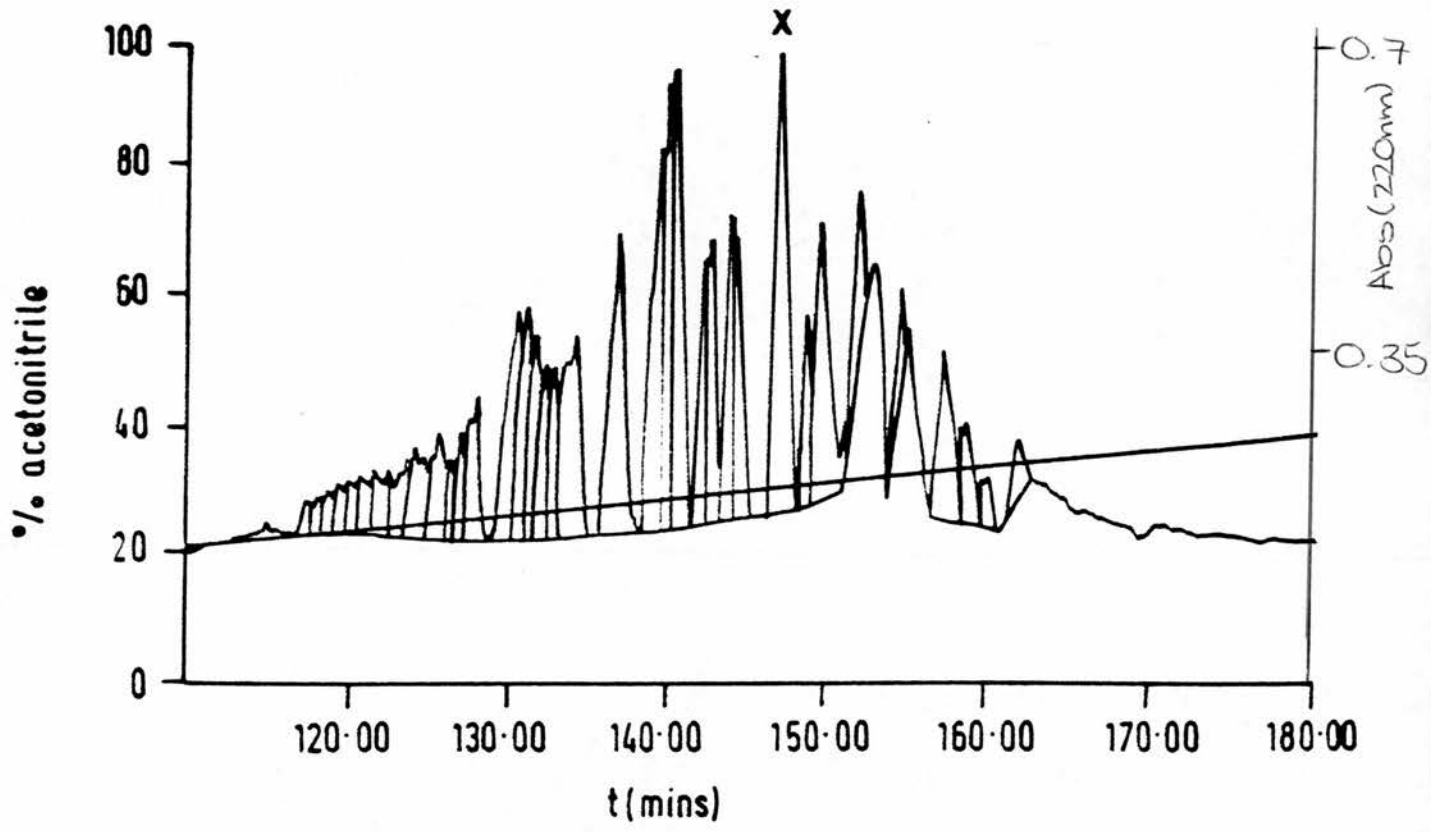
was estimated from the spectra in Figure 9.16 using the CONTIN computer programme which is based on the method of Provencher and Glockner (1981) (Table 9.4). The CONTIN computer programme calculates the percentage of α-helix and β-sheet in a protein by reference to the circular dichroism spectra of 16 reference proteins whose structural features had been determined from X-ray diffraction data. The results of the investigation did not provide any evidence to suggest that the peptides in group A or in group B had undergone any great secondary structural changes as a result of their passage through the reverse-phase column and subsequent concentration. A possible explanation for the change of behaviour of the peptides of group A when re-applied to the reverse-phase column is that they were initially associated together by hydrophobic attraction, possibly as the twisted filament-type of structure described by Urry (1983); but their elution from the column in 10%-20% acetonitrile/0.1% TFA caused a dissociation of the peptide mass to the less hydrophobic, individual peptides.

The peptides of group A were not examined any further as a source of a specific peptide because of their apparent lack of stability and lack of desmosine-containing peptides.

9.4.2 Examination of peptide group B

In Section 9.3.1 it was suggested that some of the peptides of group B may contain desmosine, so the search for a specific peptide was concentrated on this group. First, because desmosine is a marker of elastin

Figure 9.17 A typical chromatogram of the group B peptides



A typical chromatogram of elastin-derived peptides which elute in group B. Peptide X was reproducibly produced from several different digestion samples. 100% corresponds to an absorbance at 220nm of 0.7.

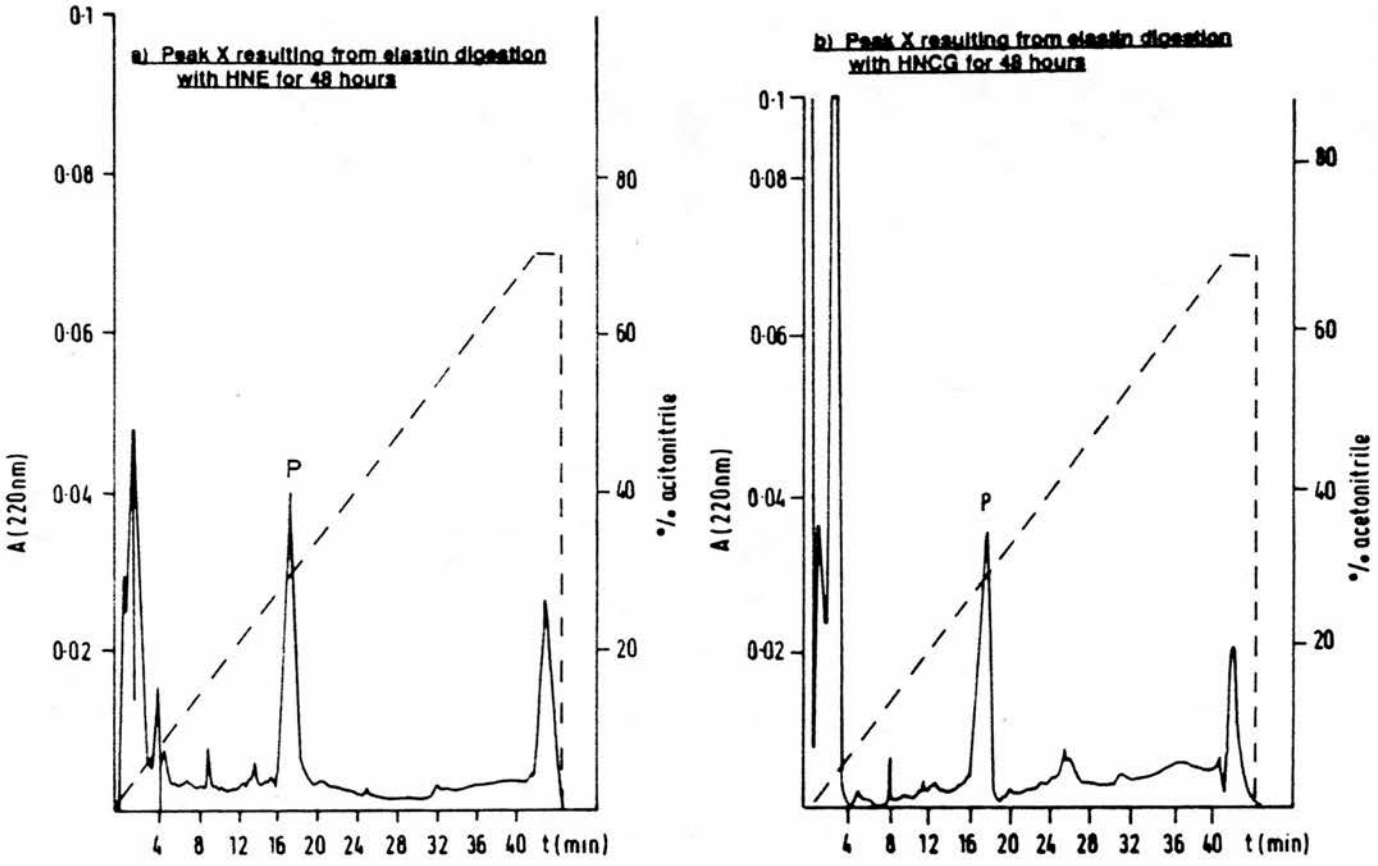
which is not found in collagen or other matrix proteins, and so would not be found in contaminant peptides. Secondly, because desmosine is not re-used by the body (Harel et al, 1980) so desmosine-containing peptides are more likely to be evident in plasma, urine and bronchoalveolar lavage fluid than peptides derived from the elastic region of elastin. The peptides from the elastic region of elastin have been shown to bind to fibroblast receptors (Wrenn et al, 1988) and to neutrophil receptors (Varga et al, 1989), and they are chemotactic for these cell types. Therefore, peptides from the elastic region of elastin may play a role within damaged elastic tissue by attracting the cells necessary for tissue repair. Furthermore, as speculated in Section 6.5 they may be retained in the area of damage by forming Schiff's bases with the damaged fibre (Stone et al, 1988).

When the elution profile of the peptides in group B was expanded and examined it appeared that the constituent peptide peaks were not all reproducible. The peptide profiles of several chromatograms were compared, and a single peak (X) was selected because it appeared to be reproduced in all the chromatograms when the absorbance at 220nm (Figure 9.17) and at 270nm of the eluate was monitored. Peptide (X) was quite well separated from the neighbouring peptides, so it was isolated and subjected to further analysis.

9.4.3 Examination of peptide X

Suspensions of Lansing lung elastin from lung 1 and 2 were digested with HNE and/or HNCG (10mU/ml) for 24 or 48 hours as detailed in Section 2.3.1. The soluble peptides were isolated and fractionated by reverse-

Figure 9.18 Examination of peak X by reverse-phase chromatography on an Aquapore RP-300 column



Peaks X resulting from a) the digestion of Lansing elastin by HNE for 48 hours and b) the digestion of Lansing elastin by HNCG for 48 hours were isolated as detailed in the text and concentrated. The peptides were dissolved in 0.5ml of 0.1% TFA and 0.2ml portions were fractionated by reverse-phase chromatography as detailed in section 2.4.3.

Table 9.5 The amino acid composition of the selected peptides resulting from 24 hours of digestion of Lansing lung elastin

Amino acid	residues/1000			
	lung 1/HNE	lung 2/HNE	lung 2/HNCG	lung2/HNE +HNCG
Asx	107.85	106.91	79.40	53.58
Glx	117.45	101.08	143.74	66.42
Ser	116.30	100.00	81.46	44.86
Gly	302.00	262.88	192.09	248.62
His	0	0	96.70	0
Arg	27.31	0	7.97	15.60
Thr	0	0	61.19	20.41
Ala	134.47	201.59	152.79	215.35
Pro	0	0	0	115.03
Tyr	0	47.31	24.89	32.36
Val	46.14	81.31	58.63	77.21
Met	0	0	0	0
Cys	0	0	0	0
Ile	31.10	0	27.71	15.49
Leu	61.08	66.67	61.72	55.80
Phe	26.85	0	11.70	20.91
Lys	28.46	32.46	0	18.26

phase chromatography according to Section 2.4.2. The peak eluting at position X was isolated, concentrated and applied to a microbore Aquapore RP-300 HPLC column (as detailed in Section 2.4.3).

Typical chromatograms are shown in Figure 9.18. Some of the material was not retained and was not subject to further attention. A major peak was eluted at approximately the same percentage of acetonitrile as peak X. The major peptide peak (P) in all cases was collected, hydrolysed and the amino acid composition was determined as detailed in Section 2.2.3. These peptides will be referred to as selected peptides for the purpose of further investigation. The peaks produced at the end of the gradient are solvent peaks which result from the change-over of the solvent from 70% to 0% acetonitrile.

9.4.4. Comparison of the compositions of the selected peptides eluted from the Aquapore RP-300 column

The amino acid compositions of the selected elastin-derived peptides resulting from 24 hours of digestion of Lansing lung elastin from lung 1 with HNE and Lansing lung elastin 2 with HNE and/or HNCG are presented in Table 9.5. At first sight, the data does not provide any evidence to suggest that the selected peptide resulting from the digestion of Lansing lung elastin from lung 2 with HNE is related to the peptide resulting from the digestion of Lansing lung elastin 1 with HNE, or that the selected peptide resulting from the digestion of Lansing lung elastin 2 with HNE is not related to the peptide produced from the same elastin after digestion with HNCG alone or HNE and HNCG.

Table 9.6 Integer composition of the selected peptides resulting from the digestion of elastin from Lung 1 and Lung 2 with HNE for 48 hours

Amino acid	Number of residues in integer composition		
	Lung 1 peptide	Lung 2 peptide	Lung 1 peptide/187 amino acids
Asx	(23.56) 24	(14.87) 15	12.36
Glx	(45.52) 46	(23.04) 23	23.70
Ser	(26.99) 27	(28.31) 28	13.91
Gly	(79.73) 80	(47.46) 47	41.21
His	(0) 0	(2.92) 3	0
Arg	(10.88) 11	(6.59) 7	5.67
Thr	(0) 0	(7.45) 7	0
Ala	(62.03) 62	(18.89) 19	31.94
Pro	(9.04) 9	(0) 0	4.64
Tyr	(7.00) 7	(5.52) 6	3.61
Val	(30.12) 30	(6.09) 6	15.45
Met	(0) 0	(0) 0	0
Cys	(0) 0	(0) 0	0
Ile	(14.01) 14	(5.02) 5	7.21
Leu	(28.06) 28	(11.91) 12	14.42
Phe	(11.26) 11	(4.09) 4	5.67
Lys	(14.35) 14	(5.12) 5	7.21
Mr by summation	34922.8 (363 residues)	18127.9 (187 residues)	
minimum f value	0.269	0.357	
Mr at min. f value	34869	18067	

The integer composition of the specific peptides resulting from the digestion of elastins from Lung 1 and Lung 2 with HNE for 48 hours as determined by the method of Black and Hogness (1969). The calculated number of residues to 2 decimal places are given in brackets and the nearest integers are presented to the right of the brackets.

The data presented in Section 6.2 suggests that the digestion of Lansing lung elastin with HNE has reached completion by 48 hours, so any peptides present after 48 hours of digestion can be assumed to be end products. The selected peptides resulting from 48 hours of digestion of Lansing lung elastin from lung 1 and the Lansing lung elastin from lung 2 with HNE should theoretically be identical. Samples (100µl) of each selected peptide were hydrolysed as detailed in Section 2.2.3 then made up to 40µl in 0.025% EDTA solution. An aliquot (10µl, corresponding to 25µl of the original peptide sample) of the hydrolysate was analysed. The amino acid composition of both the peptides was deduced, and the integer composition was determined using the method of Black and Hogness (1969) which was detailed in Section 9.3.4 (Table 9.6). The analyses suggested that the peptide from lung 1 had a molecular weight of 34923 which is twice the predicted size of the peptide from lung 2 (18128). Although both peptides are smaller than the peptides of α-elastin (60000-84000; see Section 6.3.1) examined by Partridge et al (1955b) it seems unlikely that HNE would not have cleaved the peptides further at any of their several alanyl- and valyl- residues of both peptides. So, the size of both peptides may have been over-estimated by the method of Black and Hogness (1969), because the method is designed for the analysis of the composition of a pure, single-chain peptide and not for peptides which contain heterogeneous chains joined together by a desmosine cross-link. In both peptides the proportion of aspartic acid and glutamic acid accounts for 20% of the composition. Assuming the peptides contain desmosine, the

composition suggests that at least one of the peptide chains in both lung elastin peptides must have been encoded by exon 10A (Indik et al, 1987) which codes for the only relatively acidic sequence of tropoelastin (exon 10A encoded a 33 amino acid sequence of which 2 are residues of aspartic acid and 4 are residues of glutamic acid). Indik et al (1989) stated that the sequence encoded by exon 10A is rarely found in foetal aortic elastin and so the expression of exon 10A may be age-related. Therefore, it would not be surprising to find the sequence encoded by exon 10A in a peptide derived from the lung elastin of an elderly subject. Indik et al (1989) also state that exon 10A appears to be a 5' donor and alternative splicing may occur within this exon. So, it is not possible to deduce how much of the compositions of the peptides from the lung elastins are derived from exon 10A because the position within exon 10A at which it is spliced is not provided by Indik et al (1989).

When the number of amino acids of the peptide from lung 1 was equated to that of the peptide from lung 2 there were several discrepancies, particularly in the number of residues of serine, glycine, alanine, valine and proline. These residues collectively account for 78% of the sequence of elastin (Indik et al, 1987) so they are likely to be found in most elastin-derived peptides and it is important that their presence is accurately measured. The discrepancy between the peptides may simply suggest that the peptides are different and so unrelated, or that the peptide from lung 1 consists of the peptide from lung 2 linked by a Schiff's base to a second peptide (see Section 6.3.4). A third explanation is that one of the peptides was impure. However, it seems rather surprising that peptides which have

Table 9.7 The comparison of three analyses of the same peptide

Amino acid	residues/189.28 residues (the number of residues in the original analysis)		
	original analysis	same hydrolysate same peptide	different hydrolysate same peptide
Asx	14.87	14.94	6.895
Glx	23.04	22.41	12.17
Ser	28.31	27.40	17.03
Gly	47.46	51.05	55.62
His	2.92	3.73	0
Arg	6.59	6.22	0
Thr	7.45	7.47	0
Ala	18.89	18.67	38.93
Pro	0	0	14.48
Tyr	5.52	4.98	7.53
Val	6.09	6.22	11.59
Met	0	0	0
Cys	0	0	0
Ile	5.02	4.98	16.23
Leu	11.91	12.45	8.75
Phe	4.09	4.98	0
Lys	5.12	3.73	0
f from original analysis		1.202	11.37

apparently different compositions should have the same retention time from the reverse-phase columns, and therefore the same hydrophobicity.

The inconsistency between the behaviour of the two peptides on reverse-phase chromatography and their difference of composition may indicate that the analyses were unreliable. This possibility was tested. The hydrolysate (10μl) of the peptide from lung 2 was analysed for a second time, and another sample of the same selected peptide from Lansing lung elastin 2 was subjected to hydrolysis. In this case 0.3ml of the peptide was hydrolysed then made up to 0.1ml in 0.025% EDTA solution. An aliquot (10μl, corresponding to 30μl of the original peptide solution) was analysed. From these analyses it was possible to examine the variation between two analyses of the same hydrolysate, and the variation between different hydrolysates of the same peptide. The number of amino acids in each of the compositions was equated to 187 (Table 9.7), which was the number of amino acids in the integer composition of the original analysis of the peptide from lung 2 (Table 9.6), and the deviation (f) between the analyses was calculated as detailed in Section 9.3.4. The values were 1.202 for the deviation between samples of the same hydrolysate and 11.37 for samples of different hydrolysates of the same peptide. This suggests that there is considerable variation between analyses which should be identical. Interestingly, the analyser detected 1057 and 1505 pmol of amino acids during the analysis of the same hydrolysate (equivalent to the amount of amino acid in 25μl of the original peptide solution), but only 615.8 pmol of amino acids in the sample of the second hydrolysate (equivalent to the

Table 9.8 The comparison of the amino acid composition of β-lactoglobulin determined by amino acid analysis with the known composition of the protein

Amino acid	Composition from sequence (Braunitzer et al, 1973)	Composition by analysis (residues/155 residues)
Asx	15.5	14.68
Glx	25	26.59
Ser	7	8.27
Gly	3.5	5.70
His	2	1.93
Arg	3	3.73
Thr	8	8.49
Ala	14.5	13.11
Pro	8	13.44
Tyr	4	3.72
Val	9.5	7.71
Met	4	2.71
Ile	10	6.24
Leu	22	21.62
Phe	4	3.95
Lys	15	13.10

The composition of β-lactoglobulin as determined by amino acid analysis was equated to 155 residues, which is the number of residues in the sequence of β-lactoglobulin minus cysteine and tryptophan (Braunitzer et al, 1973). Cysteine and tryptophan were not measured. The deviation (f) between the two data sets was 2.29.

amount of amino acids in 30μl of the original peptide solution).

The purity and actual expected composition of the elastin-derived peptide was not known so it was necessary to determine if the apparent inadequacies of the composition were due solely to a lack of purity. This would seem unlikely since a mixture of peptides should still give consistent analyses. A sample of pure β-lactoglobulin (provided by the Wel-Met Protein Characterisation Service) was hydrolysed and analysed as detailed in Section 2.2.3. The resulting composition was compared with the known composition of β-lactoglobulin (Braunitzer et al, 1973) and the value of (f) between the two compositions was calculated (Table 9.8). The amino acid composition of β-lactoglobulin determined by analysis did not accurately resemble the known composition of the protein. These data tend to support the opinion that the analyses are not adequate enough to allow an accurate comparison between peptides, and therefore it was not possible to state whether or not peptides produced from different samples of Lansing lung elastin by the same method could be shown to be identical in composition. This may be a result of inadequate hydrolysis or an inadequacy in the analysis itself. One source of error introduced during the analysis was the inadequate separation of the proline from a solvent peak (PTU) which causes either an over-estimation of the proline or no estimation of proline. It may be concluded that a very complicated mixture of peptides was produced by the enzymic digestion of lung elastin. The peptides were fractionated by reverse-phase chromatography but it was not possible to determine if peptides resulting from different conditions of digestion had different but

consistent compositions even though they exhibited the same behaviour on reverse-phase chromatography.

9.5 Concluding remarks about the investigation of specific elastin-derived peptides

The use of reverse-phase chromatography to fractionate elastin-derived peptides has been the focus of much work (Smyrlaki et al, 1987; Manning et al, 1989; Sandberg et al, 1990) but the yields of peptide material from the reverse-phase columns are poor, presumably because of irreversible binding of some of the peptides. An alternative technique allowing the separation of hydrophobic molecules is hydrophobic interaction chromatography. This involves using a column which is very similar to a reverse-phase column except that the concentration of the hydrophobic side chains is less dense. It would be expected that very hydrophobic peptides would not bind as tightly to the column and so the yield of material would improve. The major disadvantage is that small mildly hydrophobic peptides, such as those in group A may not bind to the column at all, so they would have to be separated by reverse-phase chromatography. This inability to retain small mildly hydrophobic peptides may explain why the technique has received very little attention (It was used by Ryan et al, 1983, but was later replaced with reverse-phase chromatography). The peptide profiles produced when the peptides were separated by reverse-phase chromatography using a gradient of 2%-100% acetonitrile/0.1% TFA were very similar regardless of which enzyme was used for their production; and many of the peptides were poorly resolved. Smyrlaki et al (1987) also

observed that elastin-derived peptides from adult lung-elastin were only partially resolved by reverse-phase chromatography.

When a single peptide was studied further it was evident that peptides which should theoretically have been identical because they were subject to identical digestion conditions and had the same hydrophobicity could not be shown to have the same composition because of inaccuracies in the amino acid analysis. These problems may in part be due to the fact that very little peptide material was available for analysis so the analyser was running at maximum sensitivity and any background peaks may be mistakenly interpreted to be amino acids causing an over-estimation of some amino acids.

In the case of a simple peptide the amino acid analysis could be confirmed by sequencing the peptide. However, a desmosine-containing peptide would have at least 2 free amino termini and 2 equimolar sequences (see Chapter 10 and Gray et al, 1973) so that no one sequence could be identified. It would not be possible to differentiate between a peptide with 2 termini and a mixture of 2 peptides of approximately equal amounts.

The fact that the peptide products resulting from the digestion with HNCG but, not with HNE, seemed to be recognised by a monoclonal antibody against an elastin hexapeptide suggests that some of the peptides do differ in composition (see Section 9.1.1); but this was not obvious from the elution patterns. So although peptides produced by the digestion with HNCG may differ compositionally from peptides produced by digestion with HNE, the differences do not stand out on reverse-phase chromatography.

CHAPTER 10

GENERAL DISCUSSION

CHAPTER 10

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The proteinase:antiproteinase theory of emphysema in its most primitive state suggests that HNE in excess of the inhibitory capacity of the lungs digests the elastin of the extracellular matrix. So the isolation and identification of a marker of elastin digestion by HNE has been a cause of concern for many years, and was the aim of this project. Antibodies against the marker peptide(s) could be used as a diagnostic tool to prove whether or not the elastin-derived peptides detected in the body fluids of emphysematous patients (Harel et al, 1980; Kucich et al, 1985) are the products of elastin digestion by HNE. Unfortunately such specific peptides have proved to be elusive.

Many of the problems which arise during any study on elastin and elastin-derived peptides are due to a lack of fundamental knowledge about elastin itself. The purification methods available for the isolation of elastin are crude and exploit the fact that elastin is more insoluble than the other proteins of the extracellular matrix. The purification of elastin involves solubilising all other proteins in the hope of leaving pure elastin as the insoluble residual material. There are few criteria for assessing the purity of the product (Soskel and Sandberg, 1983). The insoluble protein cannot be completely sequenced because of the complications introduced by the presence of the tetrafunctional desmosine and isodesmosine cross-links. It is generally accepted that desmosine and isodesmosine probably link 2

molecules of tropoelastin rather than 3 or 4 (Gray et al, 1973). This means that 2 peptide chains per cross-link will be sequenced simultaneously. Much of the sequence around the cross-link is similar in all chains so it would be difficult to assign each residue to a particular chain. Several authors have given compositions for elastin (Lansing et al, 1952; Ross and Bornstein, 1969; Richmond, 1974; Reilly and Travis, 1980); but these authors isolated the elastin from different tissues using different methods of isolation. In order to clarify the discrepancies in composition due to species variation and determine the relative merit of each isolation method, Soskel and Sandberg (1983) isolated elastin from hamster lung by 6 different method and came to the conclusion that the composition of the product was variable both when isolated by the same method and when comparing the product of different methods of isolation.

The first conclusive information about the composition and sequence of insoluble elastin was provided by Indik et al (1987) when they published the sequence of the cDNA of human foetal aortic tropoelastin, which is the soluble precursor of elastin. Tropoelastin is converted to the insoluble elastin after secretion by cross-link formation. Indik et al (1989) also provided an explanation for some of the disagreement over the composition of elastin by suggesting that sequence differences between the elastins of different organs and subjects of different ages could be a consequence of alternative splicing of the exons of a single gene (Indik et al, 1989). Alternative splicing of the elastin gene explains why previous investigations had led to the isolation of mRNA encoding at least 2 different forms of tropoelastin (Foster et al, 1980). The study of the molecular biology of

elastin has provided evidence to suggest that different exons encode the lysine-rich potentially cross-linking hydrophilic domains and the hydrophobic domains of the protein (Indik et al, 1989).

Elastin is a natural substrate for HNE which digests the insoluble protein to a mixture of soluble and insoluble products (Janoff and Scherer, 1968). The soluble products liberated during the digestion of elastin are under-estimated by standard protein assays (see Section 4.1.2) so they were measured spectrophotometrically with TNBS as an increase of free amino groups. If we assume that the 6-amino groups of the lysine residues in elastin are modified to cross-links and cross-link intermediates then any increase of free amino groups represents an increase of amino termini. The insoluble products of digestion cannot be measured with TNBS alone because the insoluble Tnp-peptides will sediment and will not be detected by the spectrophotometer. The TNBS reaction with amino groups is known to liberate sulphite as a by-product. Sulphite reacts with DCPIP to cause a decline of the absorbance at 605nm of the DCPIP (see Chapter 5 and the Appendix). By adding TNBS and DCPIP to a mixture of peptides it was possible to measure the sulphite by-product liberated from the TNBS as a decrease of absorbance at 605nm of DCPIP. This method was used to measure the total amount of peptides and the amount of insoluble products of digestion was calculated by subtracting the amount of soluble amino groups measured with TNBS form the amount of total amino groups measured with TNBS and DCPIP.

When Lansing lung elastin was digested with HNE the initial amount of soluble and insoluble products was approximately equal. After

approximately 2 hours of digestion the amount of soluble peptides began to exceed the amount of insoluble products (see Section 6.2.1). These data can be explained in terms of the proposed model of Robert et al (1974) which suggested that after HNE has bound to the elastin there is a slow limited hydrolysis of some peptide bonds. This exposes previously unavailable sites of proteolysis. This stage could be expected to yield predominantly insoluble termini together with some soluble peptides. The availability of new sites of proteolysis induces an increase in the amount of proteolysis to liberate predominantly soluble peptides; at this stage the amount of soluble peptides would be expected to exceed the amount of insoluble products.

It was expected that the digestion would eventually reach a plateau at which no further digestion occurred because all the susceptible bonds had been hydrolysed. However, between 24 and 48 hours of the digestion of Lansing lung elastin with HNE there appeared to be a decline of the amount of detectible free amino groups (see Section 6.2.1). This occurred regardless of whether the lung had been fixed in acetone or ethanol prior to the isolation of the elastin (see Sections 2.2 and 3.2.1). Subsequent investigations suggested that some of the peptides were capable of associating together. Elastin-derived peptides are known to associate together hydrophobically to form coacervates (Partridge et al. 1955a). However, the evidence presented in Section 6.3 suggest that coacervates of the peptides may be stabilised by the formation of Schiff's bases. Schiff's bases are formed when aldehyde groups react with amino groups. Some of the intermediates in the formation of desmosine and isodesmosine are

known to be aldol condensation products (Lent et al, 1969) which would presumably be capable of reacting with the amino termini of the peptide products of digestion to form Schiff's bases. In general, the formation of Schiff's bases may be prevented by reducing the aldehyde to an alcohol with sodium borohydride. When peptides were treated with sodium borohydride after 18 or 24 hours of digestion (Section 6.3.5) the apparent decline of the amino groups was prevented. Attempts were made to isolate the groups which were reduced by using tritium-labelled borohydride and then separating the peptides by reverse-phase chromatography. These experiments met with little success. Any future work on the formation of Schiff's bases between elastin-derived peptides could involve the development of a reliable spectrophotometric assay for the presence of aldehyde cross-link intermediates. Elastin is known to bind basic fuchsin (Weigert, 1898; McCallum, 1973) which is used routinely to detect aldehydes. If the fuchsin was shown to bind to the elastin via aldehyde groups then any decline of the absorbance of the basic fuchsin after reaction with the aldehyde cross-link intermediates of elastin-derived peptides could be used as a method of measuring the amount of aldehydes. If the decline of free amino groups could be correlated with a decline of detectible aldehyde intermediates it would tend to suggest that Schiff's base formation is responsible for the stabilisation of coacervating peptides. The significance of the formation of Schiff's bases will be discussed later in this Chapter.

The extent of digestion of elastin by HNCG, another serine proteinase of neutrophils, was less than the amount of extent by HNE (see Chapter 7).

The actions of HNCG were of some interest because HNCG was suggested by Boudier et al (1981) to act synergistically with HNE, although this was disputed by Reilly et al (1984). The data presented in Chapter 8 suggests that the pre-digestion of elastin with HNCG may aid HNE digestion simply because HNCG performs the initial limited proteolysis of the elastin. Therefore subsequent digestion by HNE would appear to be faster than expected simply because the susceptible, interior bonds are available for hydrolysis as soon as the enzyme is added to the substrate.

The soluble peptides produced as a result of the digestion of elastin with HNE and/or HNCG are potential diagnostic tools which which could be used to confirm the alleged role of HNE in emphysema. The cross-links desmosine and isodesmosine are unique to elastin, so peptides containing the cross-links are natural candidates in the search for marker peptides. The elastin-derived peptides resulting from the enzymic digestion of elastin could be fractionated by reverse-phase chromatography (see Chapter 9). This project was concerned primarily with all peptides regardless of whether they contained desmosine. If the study had been limited to the identification of desmosine-containing peptides it would have been advantageous to fractionate the peptides by affinity chromatography using either antibodies raised against desmosine-containing peptides or anti-elastin peptide antibodies which have been detected in the circulatory system of emphysematous patients (Fulop et al, 1989) as the ligand. The desmosine-containing peptides in the eluate from the affinity-column may be selected by monitoring the absorbance at 270nm. Finally, these peptides could be separated from one another by using reverse-phase chromatography.

The peptides produced by digestion of elastin with HNE and with HNCG did not differ on the basis of hydrophobicity, and it was not possible to see any difference when they were separated by reverse-phase chromatography. Many of the peptides appeared to co-elute when the reverse-phase column was developed with an acetonitrile gradient. Any future work could involve the separation of co-eluting peptides using different reverse-phase columns developed with different solvents. There are an infinite number of combinations of columns and solvents. Alternatively the peptides could be examined by hydrophobic interaction chromatography.

It was not possible to determine if the peptides produced as a result of digestion by different enzymes were compositionally different as the amino acid analyses of hydrolysates of the peptides were inconsistent and unreliable. There was insufficient material available to allow analysis on a less sensitive, but possibly more reliable amino acid analyser, so the investigation of the peptides could not be pursued any further.

HNE is an endoproteinase which is capable of cleaving a large number of bonds within the elastin and has a specificity which over-laps with the specificity of other enzymes such as proteinase-3 and HNCG, so it seems unlikely that peptides specific for HNE or HNCG digestion could be shown to be produced consistently from elastin, which had been isolated by a crude method which gave a product of unknown purity. Even if peptides produced by HNE digestion but not HNCG digestion and vice-versa were identified it would be naive to assume that they could be used as marker peptides. Before any such assumptions can be made experimental

evidence is required to prove that other elastinolytic enzymes are incapable of generating these peptides. This is particularly important when comparing the action of HNE and proteinase-3, which is also found in the azurophil granules of neutrophils (Kao et al, 1988). Although the enzymes differ in their specificity with synthetic substrates, they are both capable of cleaving bonds adjacent to small amino acids such as alanine and valine in large proteins such as elastin (Rao et al, 1991). Similar consideration should also be given to the elastinolytic enzymes of other inflammatory cells, such as macrophages (Werb and Gordon, 1975), and the elastases which can be secreted by micro-organisms such as *Pseudomonas aeruginosa* (Morihara et al, 1965; Hamdaoui et al, 1987) and *Aspergillus fumigatus* (Reichard et al, 1990) which are commonly found in the respiratory tracts of immunosuppressed and cystic fibrosis patients. So the detection of elastin-derived peptides resulting from the attack of excessive enzymes from the inflammatory cells will be complicated if the emphysematous patient is infected with an opportunistic micro-organism which is capable of secreting elastolytic enzymes.

Even if it was established that a particular enzyme digested elastin to give a characteristic peptide *in vitro*; it would be unrealistic to assume the same elastin-derived peptide would be produced *in vivo*. This is simply because each enzyme is not found in isolation, but as a member of a cocktail of elastinolytic enzymes. The consequence of the co-operation between the enzymes will lead to the production of elastin-derived peptides *in vivo* which were not found *in vitro*. Since the enzymes are assumed to reach excessive levels because of inadequate inhibitory mechanisms it is

important to consider the role played by the different inhibitors in controlling excesses of enzyme. Historically emphysema has been associated with a deficiency of α-1-PI; a correlation between a deficiency of the low molecular weight inhibitors and emphysema has not yet been shown. Similarly, attention should be paid to the fact that the majority of patients with emphysema are not deficient in α-1-PI but are heavy smokers, so the implications of the oxidant : antioxidant theory should be examined because excess oxidants may oxidatively inactivate the inhibitors allowing the enzymes to reach excessive levels.

Having established a realistic view of the proteinase:antiproteinase balance, the methods discussed in this Thesis could be employed to isolate groups of specific elastin-derived peptides which could then be used to study imbalances; but such peptides are probably of little use. First, their detection would be confined to bronchoalveolar lavage fluids and the blood in the pulmonary vein. Pelham et al (1985) were unable to detect any significant difference between the concentration of urinary desmosine of adults with interstitial lung disease or emphysematous adults with α-1-PI deficiency and Pi_{zz} individuals with no symptoms of emphysema. Secondly, elastin-derived peptides will have a great deal of sequence similarity so antibodies raised against a single peptide may well recognise several other peptides which are not specific to a particular type of enzymic-digestion. Thirdly, elastin may be subject to age-related changes, so the peptides which are found in the bloodstream of a child with a genetic deficiency of α-1-PI will not necessarily be the same as those in the bloodstream of an

emphysematous 70 year old who is a heavy smoker (Smyrlaki et al, 1987). Finally, elastin is present within all tissues subjected to high pressure differentials. The sequence of the elastin is unlikely to alter dramatically from tissue to tissue, even allowing for the contribution of alternative splicing (Indik et al, 1989). This is confirmed by Starcher and Galione (1976) who showed that the amino acid composition of elastin in all tissues was constant, although they did suggest there is some species variation. Similarly, Kucich et al (1981) demonstrated that antibodies raised against elastin-derived peptides from lung cross-reacted with elastin-derived peptides from aorta. So any differences between the peptides from different tissues is insufficient in themselves to prove conclusively that the source of the peptides was the elastin of the lungs rather than arterial elastin.

Although there is a great deal of evidence to suggest that elevated levels of unspecific elastin-derived peptides are found in the urine and plasma of patients with emphysema it is concluded that the use of elastin-derived peptides containing desmosine as markers of a specific enzyme imbalance is probably a little optimistic because large proportions of elastin contain similar sequences and the active enzymes capable of causing the greatest harm have an overlapping specificity so it is unlikely that such specific peptides exist. Weinbaum et al (1991) recently used domain-specific antibodies to illustrate some interesting points about unspecific elastin-derived peptides. They found that circulating antigens reacted with an antibody directed against the carboxy terminus of tropoelastin, but did not react with an antibody directed against a tyrosine-rich region of tropoelastin (200-221). Both antibodies recognised the intact elastin within the alveolar

interstitium but the antibody which recognised the carboxy terminus of the protein did not react with the urinary elastin-derived peptides. The use of domain-specific antibodies to screen plasma and bronchoalveolar lavage fluid can potentially provide information about the areas of the tropoelastin molecule which are most susceptible to degradation. The relevant antigens could be affinity purified from the body fluids and sequenced. If the sequence of the peptide was compared with the sequence of tropoelastin (Indik et al, 1987) it may be possible to identify the enzyme(s) which could have hydrolysed the peptide from elastin.

Recent research on the elastin-derived peptides from the elastic region of the macromolecule has suggested they have some potentially very exciting roles in the repair of the fibres and in cellular regulatory events. These peptides and their roles have received little attention in the past because they are difficult to distinguish from peptides of collagen or of the protein core of proteoglycans or glycoproteins, which are all potential substrates of HNE (see Section 1.3). When elastolytically damaged tissue is repaired the newly formed elastic network is disorganised (Karlinksy and Snider, 1978). This tissue will eventually be destroyed producing emphysematous lesions, the production of which may be exacerbated by a lysyl oxidase deficiency (Tinker et al, 1990). Therefore, inadequate repair mechanisms may contribute to the progression of emphysema. Hornebeck et al (1986) showed that elastin-derived peptides are chemotactic and are capable of binding to a 120kDa cell-membrane complex designated elastonectin which is found on the membrane of fibroblasts. The complex is involved in cell adhesion and receptor-mediated signal transduction. So it

is possible that the cell adheres to damaged fibres via the complex, after which a receptor-mediated stimulation of protein synthesis occurs. Foster et al (1990) have shown that fibroblasts in culture which have been subjected to proteolytic attack will synthesise tropoelastin in response to elastin-derived peptides. In contrast, the production of tropoelastin is down-regulated by fibroblasts which have not been subjected to proteolysis in response to the same elastin-derived peptides. The protein of the elastonectin complex which acts as the receptor was isolated by Wrenn et al (1988) and is a 67kDa peripheral membrane protein which specifically recognises the hexapeptide, Val-Gly-Val-Ala-Pro-Gly, found in the elastic region of elastin (see Section 1.3 for a discussion about the role of the receptor in fibre assembly). This hexapeptide is also recognised by receptors on the surface of a highly metastatic lung carcinoma cell-line (Blood et al, 1988). Interestingly, a 67kDa membrane receptor for laminin peptides has been isolated from A2058 melanoma cells which is structurally and functionally similar to a 67kDa receptor for elastin peptides on the surface of chondroblasts (Mecham et al, 1989). So a family of receptors may be involved in the repair of the different components of the matrix.

Fibroblasts are not the only cell-type which may be influenced by elastin-derived peptides. Neutrophils are also chemotactically attracted by elastin peptides which, again, bind to the cells and initiate signal transduction mechanisms which lead to the breakdown of phosphatidylinositol (Varga et al, 1989). Activation of the neutrophils could theoretically produce an increase in HNE synthesis and, consequently, further damage to the matrix. However, it should be noted that not all of the

products of HNE digestion are capable of activating neutrophil cellular events. Recent research has suggested that the elastase-generated Fc fragment of immunoglobulin G can actually inhibit neutrophil chemotaxis (Eckle et al, 1991).

It is possible that when an elastic fibre is damaged some of the small "elastic" peptides either bind hydrophobically to the damaged fibre or act as signals causing a chemotactic response from fibroblasts. The fibroblasts then attach to the damaged fibre and synthesise tropoelastin and lysyl oxidase. When the tropoelastin is secreted into the region of damage it would be expected to form desmosine cross-links. However, in the presence of a higher-than-normal concentration of amino termini, the modified lysine residues of the new tropoelastin and any cross-link intermediates of the damaged fibre may form Schiff's bases which will be significantly weaker than desmosine and isodesmosine (discussed further in Chapter 8). The formation of Schiff's bases requires the presence of an aldehyde and amino group whereas desmosine formation requires three aldehydes and an amino group. So, it is possible that during repair "salvage" mechanisms such as Schiff's base formation are used to maintain an intact fibre regardless of the quality of cross-linking. This would produce a disorganised fibre of low quality, which after several years of damage-repair cycles would break down completely to form an emphysematous lesion. If this theory was correct it would follow that fibrosis of the tissue occurs prior to the formation of lesions; but fibrosis and emphysema have generally been considered to be mutually exclusive (Snider et al, 1985). Recent electron microscopy studies (Nagai and Thurbeck, 1991) have questioned this

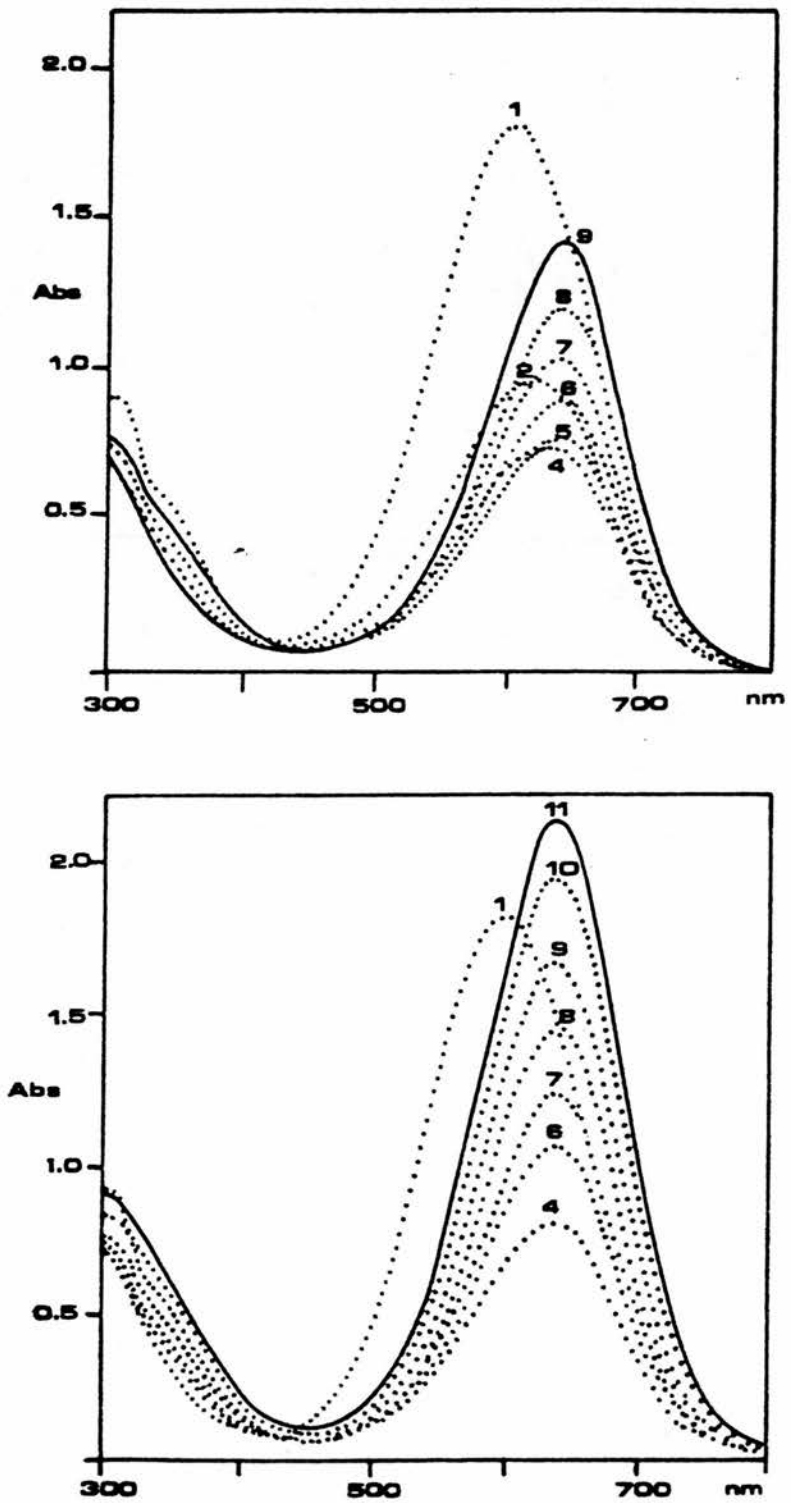
assumption by showing that fibrosis and emphysema have been detected within the same lung. So further research to clarify whether or not the diseased lung has a smaller amount of desmosine cross-linking and a greater amount of "salvage repaired" fibres than normal could provide a very interesting insight into the disease processes.

Although the damage inflicted on the matrix during the production of emphysema is a consequence of proteinase:antiproteinase and oxidant:antioxidant imbalances, the repair of the tissue and progression of the disease may be dictated by cell-matrix interactions. So it would be advantageous to study the balance between neutrophil and fibroblast activation in response to the chemotactic influence of elastin-derived peptides.

In conclusion it is highly unlikely that reproducible marker elastin-derived peptides specific for digestion by a particular enzyme are produced during degenerative lung disease. So, more fruitful studies on elastin-derived peptides are possible by considering their role in cellular regulation and cell-matrix interactions. By studying the fundamental biochemical regulation processes associated with the extracellular matrix it may become apparent which cellular disturbances provoke symptoms such as proteinase:antiproteinase and oxidant:antioxidant imbalances which will ultimately lead to matrix damage.

APPENDIX

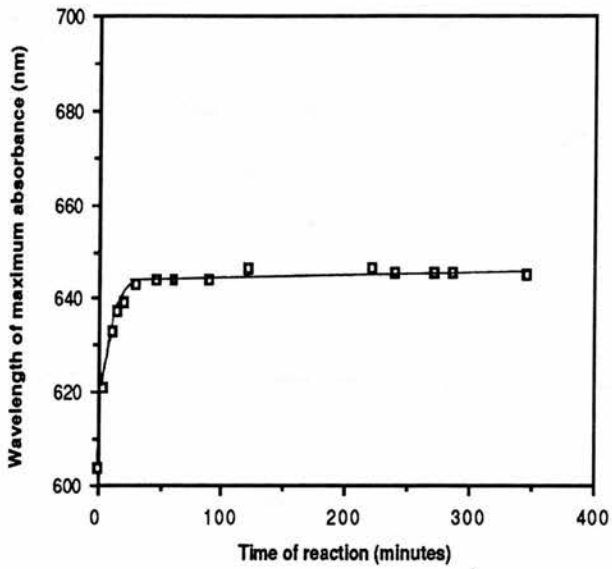
Figure A.1 The reaction between DCPIP and sulphite for a variable amount of time



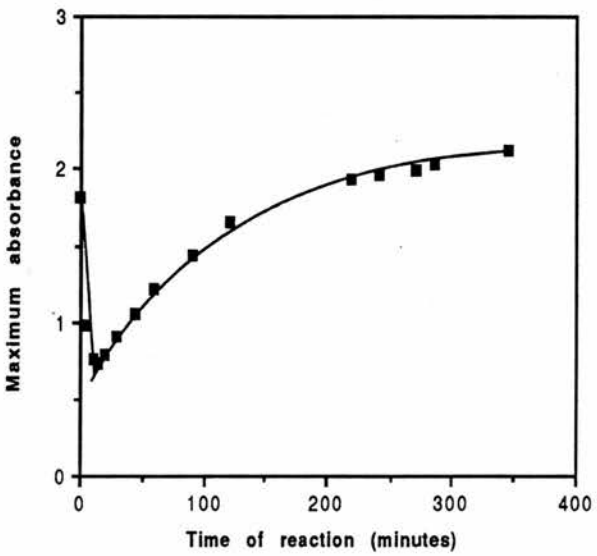
0.2µmol of DCPIP was treated with equimolar sodium sulphite at pH 8.0 and 37°C (total volume 2ml). The absorption spectrum of the solution was determined after several time intervals, as follows:-
(1) 0, (2) 5, (3) 10, (4) 15, (5) 20, (6) 30, (7) 60, (8) 90, (9) 120, (10) 240 and (11) 340 minutes after the addition of sulphite.

Figure A.2 The change of the maximum absorbance and the corresponding wavelength during the course of the reaction between DCPIP and sulphite

a) The change of the wavelength of the maximum absorbance



b) The change of the maximum absorbance



0.2µmol of DCPIP in 0.05M sodium phosphate buffer pH 8.0 was reacted with an equimolar amount of sodium sulphite in a solution with a total volume of 2ml. The λ_{max} and the maximal absorbance were measured at intervals during the reaction.

APPENDIX

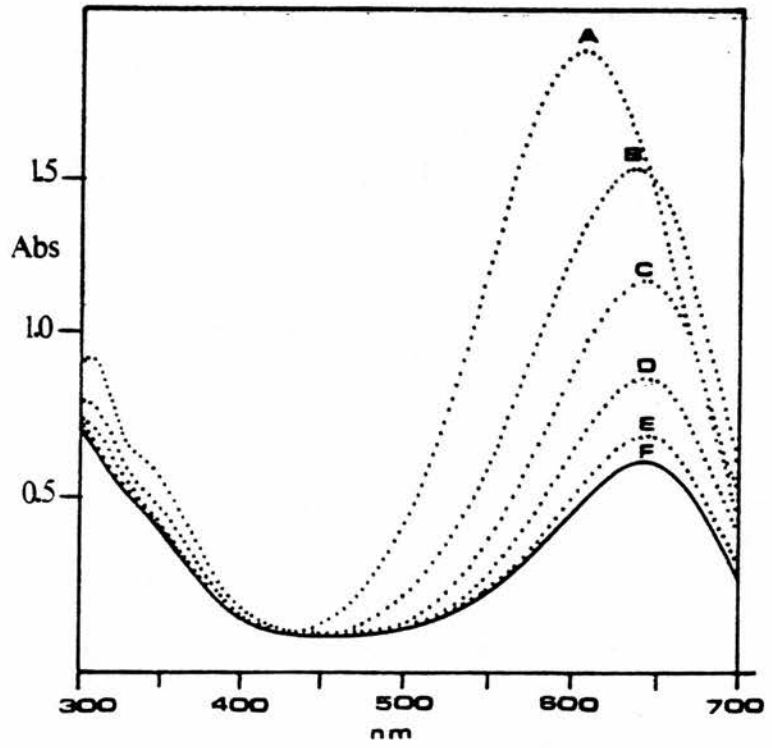
In Chapter 5 it was observed that DCPIP was not reduced by equimolar amounts of sulphite, and the reaction actually appeared to have a 1:2 stoichiometry of DCPIP:sulphite. This was surprising and worthy of further investigation, although it was not relevant to the original aims of the project.

A.1 Further investigation of the reaction between DCPIP and sulphite

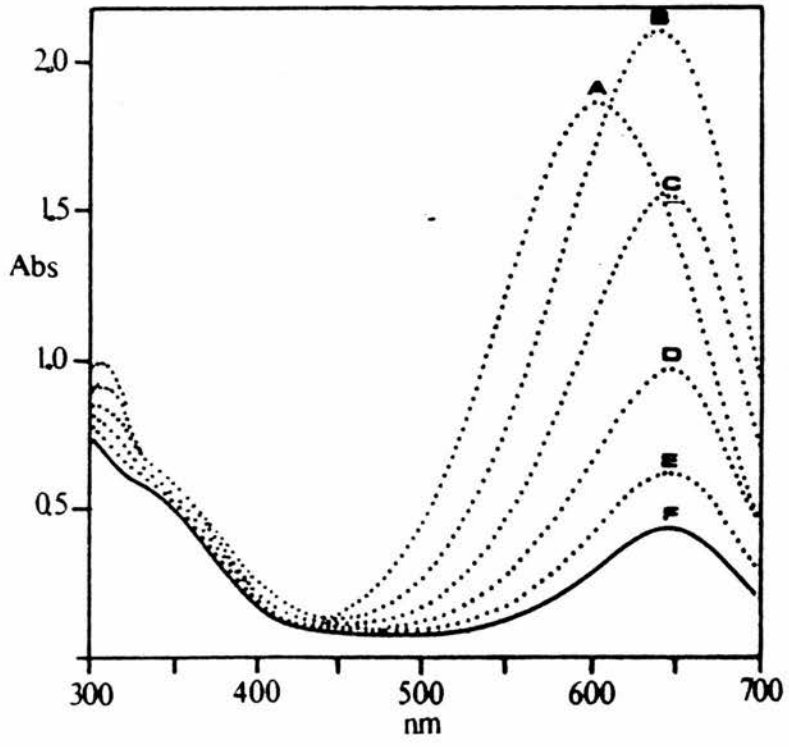
The reaction between DCPIP and a reducing agent was expected to result in a colourless product, showing a decrease of absorbance at 605nm and no apparent change of the wavelength of the maximum absorbance (λmax). When DCPIP was reduced with sodium borohydride, a simple decrease of absorbance at 605nm without a shift of the λmax was seen. The absorbance at 605nm of the reduced DCPIP was restored by oxidising it with iodine. When the absorption spectra of DCPIP after reduction with one mol of sulphite for a varied amount of time (Figure A.1) was examined it appeared that the reduction of the DCPIP by sodium sulphite was not as simple as expected. When the change of λmax and the absorbance at λmax were compared to the time of reaction (Figures A.2a and b) it appeared that during first 30 minutes of the reaction a decrease of the absorbance at λmax occurred, but this was followed by a rapid increase of the absorbance until approximately 2 hours of reaction after which the absorbance continued to increase very gradually. The shift in the wavelength of the maximum

Figure A.3 Absorption spectra of the reaction between DCPIP and varied sulphite

a) 30 minutes incubation

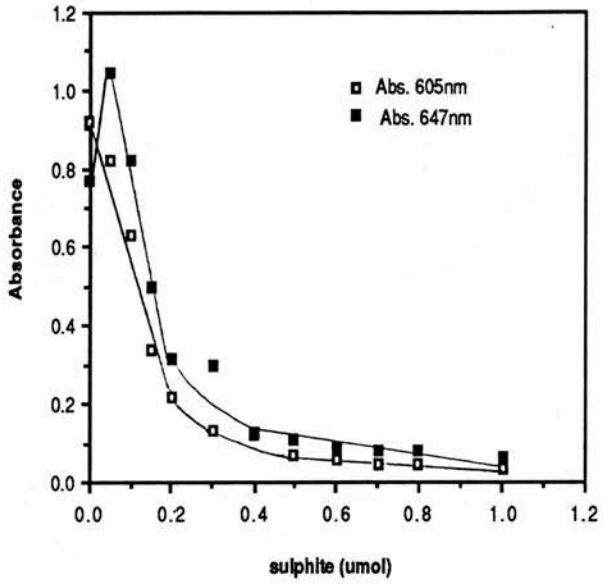


b) 2 hours of incubation



0.2µmol of DCPIP was treated with (A) 0 µmol, (B) 0.2 µmol, (C) 0.4 µmol, (D) 0.6 µmol, (E) 0.8 µmol or (F) 1.0 µmol of sulphite at pH 8.0 in a final volume of 2ml. After 30 minutes or 2 hours of incubation at 37°C the absorption spectra were measured. The above spectra represent a typical result.

Figure A.4 The change of absorbance at 605nm and 647nm of DCPIP after reaction with varied sulphite



0.1μmol of DCPIP in 0.05M sodium phosphate buffer pH 8.0 was reduced with sodium sulphite (0-1.0μmol) at 37°C for 4 hours after which the absorbances at 650nm and 647nm of each solution (final volume 2ml) against the phosphate buffer were measured.

absorbance occurred during the first hour of reaction. Absorption spectra showing the reaction between 0.2μmol of DCPIP and a variable amount of sulphite (0-1.0μmol) in 2ml of solution were recorded after 30 and 120 minutes of reaction (Figure A.3a and b). These spectra supported the conclusion that over a range of concentrations of sulphite the initial reaction may be observed as a shift in the λmax accompanied by a decline of the maximal absorbance. After 2 hours of reaction, the absorbance of the product of the reaction between DCPIP and one mole of sulphite had increased to a value greater than the absorbance of the original DCPIP solution, while the absorbance of the products of the reaction between DCPIP and excess of sulphite had continued to decline. This suggests that the first mole of sodium sulphite reacted with the DCPIP at pH 8.0 to cause a shift of the λmax to 647nm and an increase of absorbance at this new λmax; further sodium sulphite caused a decrease in this absorbance (Figures A.1-A.4). It seems that the lack of response of DCPIP to small amounts of sulphite (Figure 5.3) is due to the fact that the decrease of absorbance at 605nm in response to small amounts of sulphite (less than equimolar amounts) is obscured by the increasing absorbance at 647nm due to the formation of a new compound.

It seemed possible that the shift of λmax was an artifact of the pH dependence of the absorbance of DCPIP. A 0.2mM DCPIP solution was prepared in 0.05M sodium pyrophosphate buffer, pH 8.5, at which the molar absorbance of DCPIP is maximal (Armstrong, 1964). The λmax of the

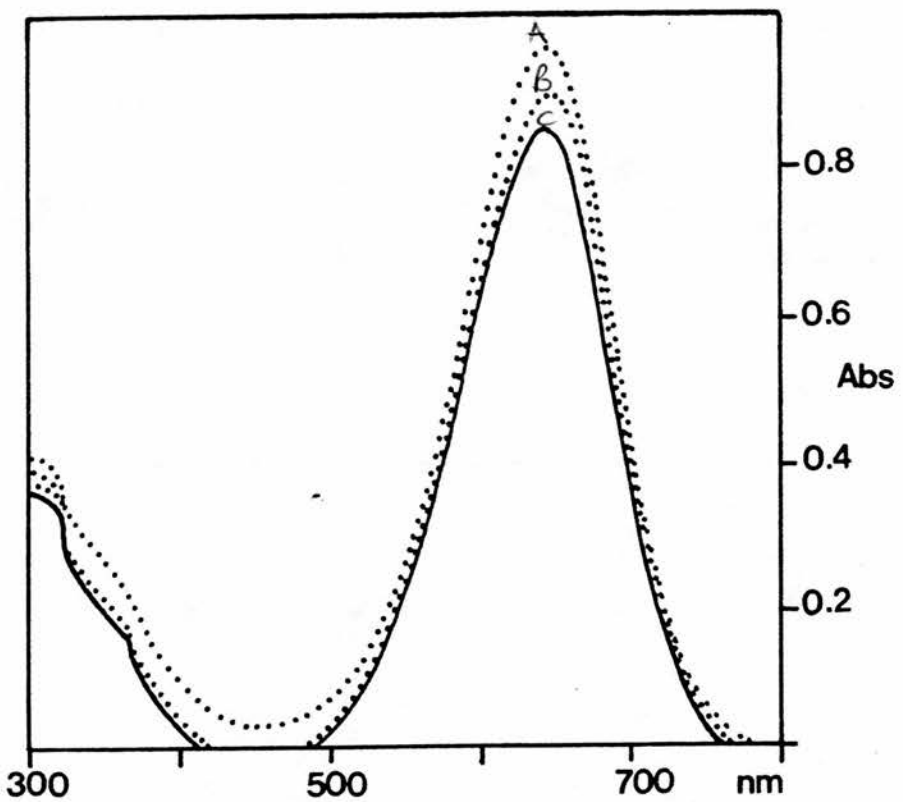
solution was 605nm. When an equimolar amount of sodium sulphite was added to the DCPIP solution a shift of the λmax to 647nm was observed after 2 hours of incubation at 37°C. The buffering capacity of 0.05M sodium pyrophosphate buffer pH 8.5 was considered sufficient to counteract any effect on pH induced by the addition of 20µl of 10mM sodium sulphite to 1ml of 0.2mM DCPIP diluted to 2ml with the 0.05M sodium pyrophosphate buffer, pH 8.5. So the shift of the λmax of DCPIP during the reaction with sodium sulphite is not an artifact induced by a change of pH.

It was concluded that the first mole of sulphite reacts in some way with the DCPIP, giving a shift in the λmax, and that further sulphite reduces the new compound. The first reaction could be a simple addition reaction in which one mole of DCPIP reacts completely with one mole of sulphite to give one mole of a new compound (P), or alternatively the reactants may be in equilibrium with the new compound:-

$$\underset{\lambda max\ 605nm}{DCPIP} + SO_3^{2-} \rightleftharpoons \underset{\lambda max\ 647nm}{P}$$

If an equilibrium was present then it could be assumed that the removal of the sulphite, by oxidation to sulphate with hydrogen peroxide, would force the equilibrium toward the left, to produce an increase of DCPIP which in turn would cause the λmax to shift back towards 605nm. Solutions

Figure A.5 The oxidation with hydrogen peroxide of the compound formed after the reaction between DCPIP and equimolar sulphite



Solutions containing 0.1 µmol of DCPIP were treated with 0.1 µmol of sodium sulphite and incubated for 2 hours at 37°C and pH 8.0. Each solution was treated with hydrogen peroxide (0-1.0 µmol) to give a final volume of 2ml. They were incubated for a further 2 hours after which the absorption spectrum of each solution was measured. The spectra did not appear to differ.

Solutions A, B and C were treated with 0 µmol, 1.0 µmol and 0.5 µmol of hydrogen peroxide respectively.

containing 0.1μmol of DCPIP and 0.1μmol of sulphite were incubated at 37°C for 2 hours, after which the λmax of the solutions was 645nm. The solutions were treated with hydrogen peroxide solution (0-1.0μmol) giving each one a final volume of 2ml, and the absorption spectra were measured after a further 5, 30 and 120 minutes. The spectra produced after 120 minutes of reaction with hydrogen peroxide are shown in Figure A.5. It was concluded that hydrogen peroxide treatment did not affect the λmax of the new compound. Similarly, the λmax of the new compound could not be changed from 645nm to 605nm by oxidation with an excess of iodine or an excess of potassium ferricyanide. So the reaction between DCPIP and equimolar sulphite was assumed to be irreversible.

By assuming that the first mole of sulphite adds to one mole of DCPIP (λmax 605nm) to give one mole of a compound (P; λmax 647nm) then the equilibrium constant for the reduction of P can be calculated using the decrease of absorbance at 647nm produced by the addition of excess sulphite (Figure A.4), such that:-

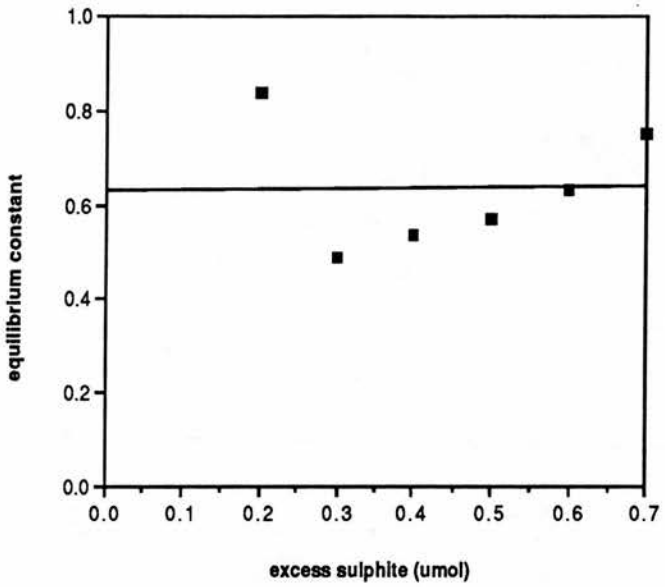
$$P + SO_3^{2-} \longrightarrow PH_2 + SO_4^{2-} \qquad (1)$$

λmax=647nm colourless

and

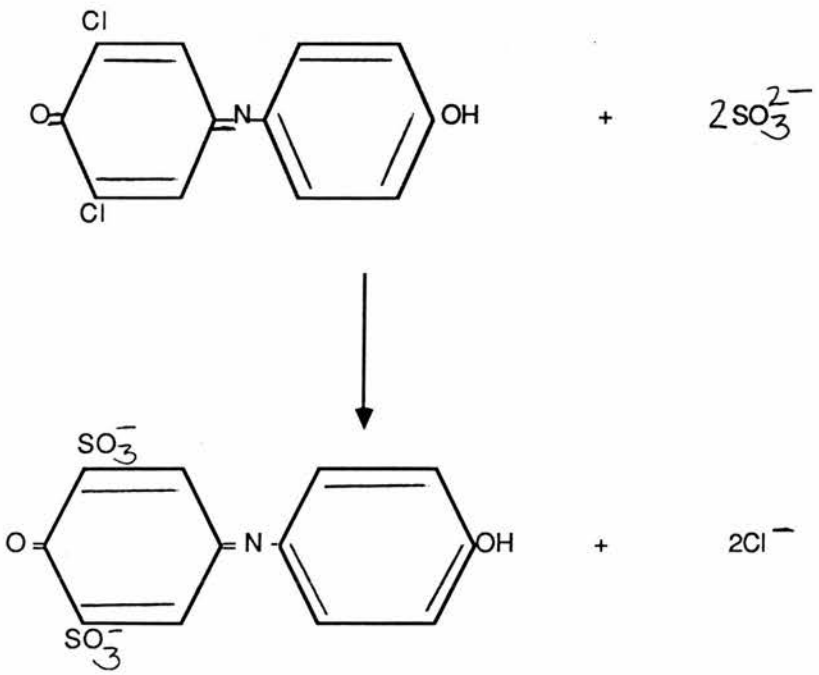
$$K_{eq} = \frac{[PH_2].[SO_4^{2-}]}{[P].[SO_3^{2-}]} \qquad (2)$$

Figure A.6 Determination of the equilibrium constant of the reduction with sulphite of the product of the reaction between DCPIP and equimolar sulphite



0.1μmol of DCPIP in 0.05M sodium phosphate buffer pH 8.0 was reduced with sulphite (0-0.8μmol) at 37°C for 4 hours. The final volume of each solution was 2ml. The decreases of absorbance at 647nm between the solution which had been treated with equimolar sulphite and each solution treated with excess of sulphite were used to determine the equilibrium constant of the reduction of the new compound (P) by sulphite, as detailed in the text.

Figure A.7 The displacement of the chloride groups of DCPIP with sulphite



If the equilibrium conforms to this simple reaction then the equilibrium constant (K_{eq}) can be determined graphically (Figure A.6) by plotting the Keq against varied sulphite. The K_{eq} for the reduction of 0.1μmol P with sodium sulphite (0.2μmol-0.7μmol) was 0.637± 0.134.

A.2 The mechanism of the reaction between DCPIP and sulphite

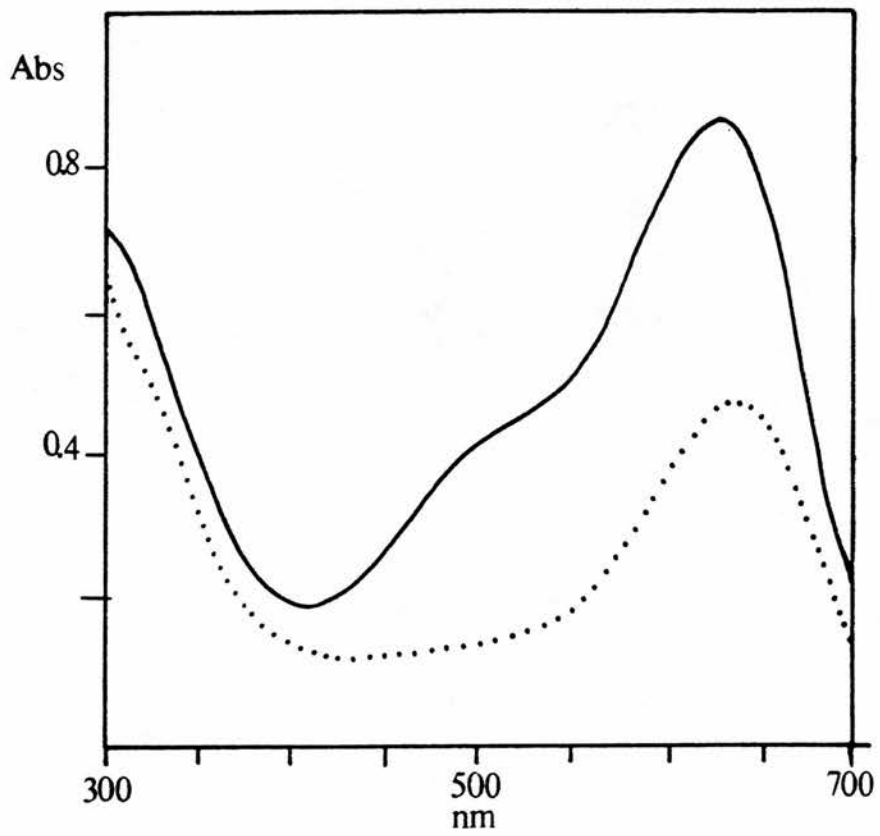
The actual mechanism of the apparently complicated reaction between DCPIP and sulphite was a cause of great interest. Three possible models were considered. First, the sulphite displaces the chloride of DCPIP, secondly a sulphite addition compound is formed and thirdly a single electron transfer reaction occurs.

A.2.1 Displacement of the chloride of DCPIP with sulphite

The reaction between DCPIP and sulphite could involve the displacement of the chloride of DCPIP as shown in Figure A.7. If this were the case then the displaced chloride could be detected by reaction with silver nitrate which gives a white precipitate of silver chloride.

Sodium sulphite (1.0μmol in 100μl) was added to 1.0μmol of DCPIP in 9.9ml of 0.05M sodium phosphate buffer pH 8.0 giving a total volume of 10ml. The solution was incubated at 37°C for 2 hours, after which it was acidified to approximately pH 4 by the addition of 15 drops of 1.5M HNO_3. Hydrogen peroxide (50μl of a 26% (v/v) solution) was added to the solution

Figure A.8 The reduction of phenolindophenol with sulphite



0.2 µmol of phenolindophenol was treated with 0.2 µmol of sodium sulphite at pH 8.0 (final volume 2ml) and incubated at 37°C for 2 hours after which the absorption spectra of the treated solution (– – –) and an untreated control (————) were recorded.

in order to oxidise any remaining sulphite to sulphate. Potassium chromate was added to the DCPIP reaction mixture as an indicator before the solution was titrated with 0.1mM silver nitrate solution. The end-point of the reaction was seen when a red precipitate of silver chromate was formed. DCPIP is pink at pH 4 so the end-point of the reaction between silver nitrate and any chloride in the solution was expected to be subtle. When silver nitrate was added to the reaction mixture, silver chromate was immediately formed. So it was concluded that chloride was not liberated during the reaction between DCPIP and sodium sulphite.

If the displacement of chloride by sulphite was not involved in the reaction, then it could be predicted that if phenolindophenol was reduced with sodium sulphite a shift of λmax would be evident even though the compound differs from DCPIP in that it has no chloride substituted at the 2- and 6- positions of the quinone ring. When 0.2μmol of phenolindophenol (Sigma) in 0.05M sodium phosphate buffer pH 8.0 was reduced with 0.2μmol of sodium sulphite (the final volume of the solution was 2ml) it seemed that phenolindophenol was incompletely reduced with one equivalent of sodium sulphite, and the shift of λmax, from 630nm to 637nm (Figure A.8), was very small when compared to the shift observed during the reaction between DCPIP and sodium sulphite (Figures 5.4-5.7). It was concluded that the presence of the chloride on the quinone ring of DCPIP may contribute indirectly to the mechanism of the reaction between DCPIP and sodium sulphite.

Figure A.9 The addition of sulphite as bisulphite to the oxo- group of DCPIP

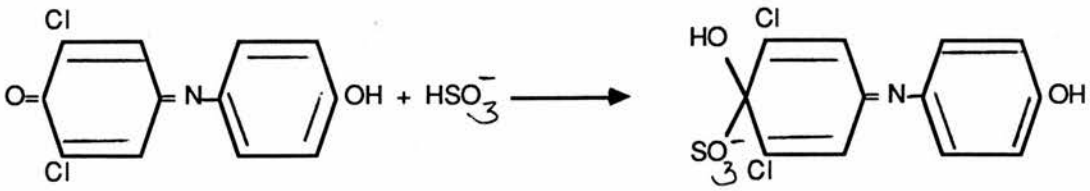
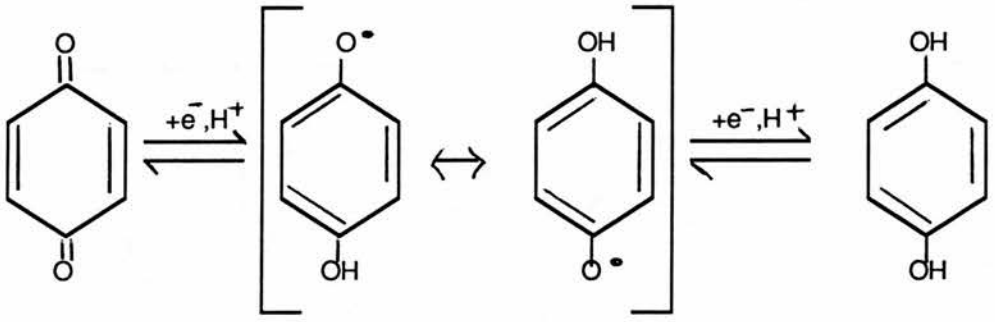


Figure A.10 The reduction of a quinone to a quinol



quinone semiquinone intermediates quinol

A.2.2. The formation of a sulphite addition compound

Chloride groups are electron-withdrawing. This would give the carbon of the oxo- group of the quinone group in DCPIP a δ^+ charge, which would in turn make the carbon more attractive to reaction with a negatively charged compound such as sulphite. So, it seemed possible that an addition reaction occurs between the first mole of sulphite and the keto-group on the quinone group as shown in Figure A.9. Any further sulphite would reduce the compound as previously predicted. This seemed a likely explanation for the reaction between DCPIP and sulphite; if a little surprising as the addition of sulphite was predicted to move λmax to a shorter wavelength because the double bonds will no longer be in resonance. In Future work will involve using nuclear magnetic resonance spectroscopy to investigate compound P, the product of the reaction between DCPIP and equimolar sulphite.

A.2.3 The formation of a charge transfer complex

Quinones are reduced by the addition of 2 electrons to a quinol via a semiquinone radical (Figure A.10). This occurs in photosynthesis when plastoquinone is reduced to plastoquinol, and in oxidative phosphorylation when ubiquinone is reduced to ubiquinol. Charge transfer complexes can form between semiquinones. The formation of a charge transfer complex causes a shift of the λmax of the quinone to a longer wavelength.

DCPIP has a quinone ring. It seemed possible that the first mole of sulphite is involved in a single electron transfer reduction to give

semiquinones capable of forming charge transfer complexes, which would explain the shift of λmax (Section A.1). However, this would seem unlikely because the formation of a charge transfer complex would be reversed by oxidation with potassium ferricyanide.

The ability of DCPIP to form charge transfer complexes was investigated. Sodium borohydride solution (approximately 20mM) was added dropwise to 5ml of 0.2mM DCPIP in 0.05M sodium phosphate buffer, pH 8.0 until the DCPIP had been completely bleached. A further 5ml of 0.2mM DCPIP solution was added to the bleached solution and incubated at 37°C for 2 hours. The λmax of the solution of DCPIP after 2 hours was 605nm therefore DCPIP does not appear to form charge transfer complexes.

A.3 Concluding remarks about the reaction between DCPIP and sulphite

The λmax of DCPIP is moved to a longer wavelength during reaction with an equimolar amount of sulphite. The addition of further sulphite to the reaction mixture causes a decline of the absorbance at the new λmax. The possible mechanisms for this reaction were investigated. The displacement of the chlorides of DCPIP with sulphite and the formation of charge transfer complexes were eliminated, but no satisfactory explanation was produced. Further investigations by nuclear magnetic resonance spectroscopy will be performed in the future in an attempt to provide a satisfactory explanation for the reaction between sulphite and DCPIP.

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