FRAGMENTATION OF ELASTIN BY SELECTIVE CHEMICAL CLEAVAGE

A Thesis presented by

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to

THE UNIVERSITY OF ST ANDREWS

in application for the

DEGREE OF MASTER OF SCIENCE

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1980

DECLARATION

I hereby declare that the following Thesis is based on work performed by me, that the Thesis is my own composition and that no part of it has previously been presented for a Higher Degree.

The research was conducted in the Department of Biochemistry of the University of St Andrews under the direction of Professor A Serafini-Fracassini.

C E R T I F I C A T E

I hereby certify that Ukazu C. Oluoha has spent seven terms as a matriculated postgraduate student under my direction and that he has fulfilled the conditions of Ordinance General No. 12 and Resolution of the University Court 1967, No. 1 and that he is qualified to submit the accompanying Thesis for the Degree of Master of Science.

A C K N O W L E D G E M E N T S

I would like to express my sincere gratitude to my supervisor, Professor A Serafini-Fracassini for his constant help and guidance during the course of this work. My thanks also go to Dr Giam-Batista Ventrella and Dr John Hinnie for their practical help; Mr JC Hunter for the operation of amino acid analyser; Mr G Armitt for the operation of ultracentrifuge; and to Mr W Blyth for photographic work.

CONTENTS

ABBREVIATIONS

The following abbreviations are used:

1.	EDTA	Ethylenediaminetetra acetic acid
2.	GUNCl	Guanidiniumhydrochloride
3.	DTT	Dithiothreitol
4.	NBS	N-Bromosuccinimide
5.	TosylF	p-Toluenesulphonylfluoride
6.	DPPCl	Diphenylphosphorylchloride
7.	Tris	Tris-(hydro-methyl)-amino-ethane
8.	SDS	Sodium dodecyl Sulphate
9.	TEMED	Tetramethylethylenediamine
10.	ANS	1-Anilino-8-naphthalene Sulphonate

A B S T R A C T

Elastin was isolated from bovine aorta using guanidine hydrochloride and collagenase prepared by affinity chromatography. Microfibrillin was extracted with dithiothreitol. The purified elastin was solubilized by specific chemical modification of serine residues using diphenylphosphoryl-chloride. Two peptides were isolated on column chromatography using Sephadex G series. Acrylamide gel electrophoresis of the isolated fractions revealed single bands. Apparent molecular weight of F_1 and F_2 on Sephadex G100 indicated 68,000 and 2,000-3,000 respectively. Molecular weight of F_1 from SDS acrylamide gel electrophoresis gave 42,000 daltons while the meniscus depletion revealed an apparent molecular weight of 10,000 daltons at vanishing concentration. Amino acid analyses of the purified elastin, supernatant and the isolated fragments were performed on Locarte amino acid analyser.

S E C T I O N 1

INTRODUCTION

1. INTRODUCTION

Elastin is the protein component of elastic fibres which confers on them rubber-like mechanical properties (Hoeve & Florey 1974). It can be seen under the electronmicroscope as an amorphous central core surrounded by an envelope of microfibrils about 110 A^{o} in diameter (Greenlee et al 1966; Ross et al 1969, 1970; Karrer 1960). Sandberg (1976) suggested that the microfibrillar component being the first to appear during fibrogenesis serves to determine the orientation of the elastic fibres and the alignment of newly synthesised elastin prior to its crosslinking. Elastin is a highly insoluble protein found in elastic connective tissues where it is in intimate association with collagen, glycoproteins and mucopolysaccarides. Examples of such tissues are aorta and ligumentum nuchae where it constitutes 51% and 70% respectively of the tissue dry weight (John & Thomas 1971). The insolubility of elastin is attributed to extensive crosslinking of protein chains by the polyfunctional amino acids, desmosine and isodesmosine, lysinonorleucine and merodesmosine (Partridge et al 1963; Thomas et al 1963; Franzblau et al 1965). These amino acids probably link two polypeptide chains (Figs 1 and 2).

The isolation of elastin has been made difficult because of its insolubility in those solvents which are normally employed for the extraction of tissue proteins. Consequently all isolation and purification methods have been geared to the removal of other components leaving insoluble elastin as the residue. These methods include use of 89% formic acid at 45^{o}C for 72 hours, extraction with boiling alkali (Lansing et al 1952) and repeated autoclaving (Partridge et al 1955). The latter two methods have been most commonly used since such preparations have reproducible amino acid composition. However, it should be noted that these methods involve extremes of temperature or pH that lead to extensive cleavage of labile peptide bonds without solubilizing

the protein (Franzblau 1971). Alteration in the physical and chemical properties of the protein could also result. Assessment of the N-terminal residues of such preparations has shown a high level of peptide bond cleavage (Spina et al 1975).

Attempts have been made to isolate elastin using enzyme procedures and thus avoiding drastic chemical treatment. Hospelhorn and Fitzpatrick (1961) employed trypsin followed by collagenase to remove contaminating proteins, while Miller and Fullmer (1966) introduced the use of repeated guanidine treatment followed by collagenase. The use of α-amylase and collagenase was employed by Steven and Jackson (1968). These enzymic methods yielded amino acid composition slightly different from those elastins prepared by hot alkali and autoclaving. Contaminating proteins were removed (Ross & Bernstein 1969) by extraction with guanidine followed by collagenase digestion while microfibril component was solubilized using dithiothreitol. This resulted in a residue with amino acid composition very similar to the one isolated using autoclaving and boiling NaOH (Lansing et al 1952; Partridge et al 1955). Since collagenase used by Ross and Bornstein (1969) has been shown to possess pronounced elastolytic activity (Robert & Robert 1969), Serafini-Fracassini et al (1975) and Spina et al (1975) used collagenase purified by affinity chromatography to isolate elastin. These preparations of elastin have amino acid composition identical to that prepared by Ross and Bornstein (1969) while the assessment of N-terminal amino acids indicated that this method yields the least degraded form of elastin yet isolated from mature tissue (Sandberg 1976).

The amino acid composition of elastin although unique varies with species, the tissue under investigation, and the age of the animal. At present it is not very clear whether these variations are due to age, tissue, and species-related difference or differences in the efficiency of the methods used in the removal of the contaminating proteins. However, the work of Field et al (1978) seems to indicate that there is tissue compositional variability. In spite of these variations all preparations show a high content of non polar

TABLE 1

AMINO ACID COMPOSITION OF MATURE ELASTINE AND TROPOELASTIN

SOURCE	COW (1)		PIG (1)	COW (2)		TROPOELASTIN (3)
	Ligamentum nuchae	Aortic	Aortic	Ligamentum nuchae	Aortic	from copper deficient pig aorta
1. Hydroxypro.	13.1	15.5	11.0	8.1	10.6	9.9
2. Aspartic acid	6.5	7.6	6.2	5.8	6.5	3.7
3. Threonine	9.4	9.6	13.6	9.3	9.6	14.4
4. Serine	9.1	10.0	11.4	8.7	9.2	11.6
5. Glutamic a.	15.7	16.2	19.1	15.5	16.0	16.9
6. Proline	117.0	122.0	117.0	116.1	112.7	104.0
7. Glycine	332.0	311.0	330.0	329.7	332.4	326.0
8. Alanine	228.0	223.0	234.0	228.2	223.9	230.0
9. Valine	138.0	137.0	120.0	132.2	131.3	132.0
10. Halfcystine	0.0	0.0	0.0	0.0	0.0	0.0
12. Methionine	0.0	0.0	0.0	0.0	0.0	0.0
13. Isoleucine	25.0	26.7	17.8	24.0	23.5	16.0
14. Leucine	59.9	64.8	54.2	59.7	58.2	45.2
15. Tyrosine	6.2	8.1	15.9	5.9	7.5	16.3
16. Phenylalanine	29.1	33.0	33.0	29.4	29.7	26.5
17. Histidine	0.5	0.5	0.5	0.5	0.5	0.0
18. Lysine	2.7	5.1	6.2	3.3	4.6	43.3
19. Arginine	4.6	6.2	6.1	5.8	5.9	4.3
20. Isodesmosine	0.9	1.2	1.2	1.6	1.9	0.0
21. Desmosine	1.4	2.0	1.8	2.2	2.4	0.0
23. Lysinonorleu.	1.2	0.9	0.9	1.1	1.0	0.0
24. Merodesmosine	0.0	0.0	0.0	0.2	0.2	0.0

(1) Rasmussen et al (1975) formic acid-cyanogen bromide method

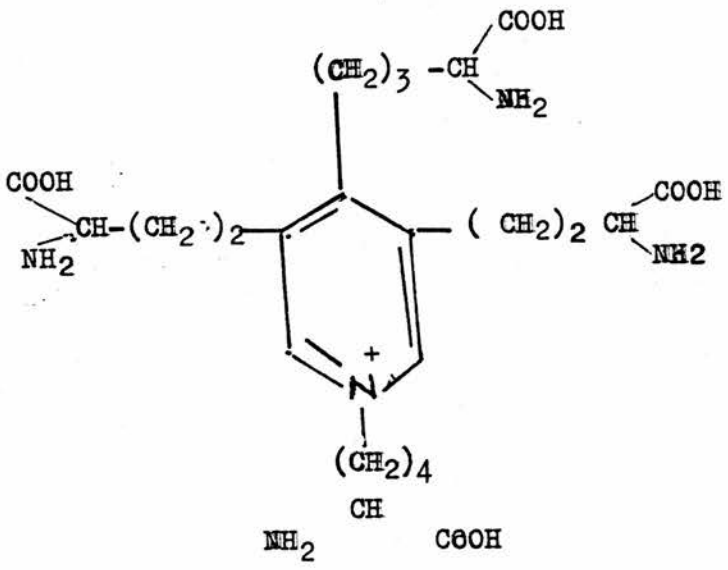
(2) Serafini-Fracassini et al (1975)

(3) Smith et al (1972)

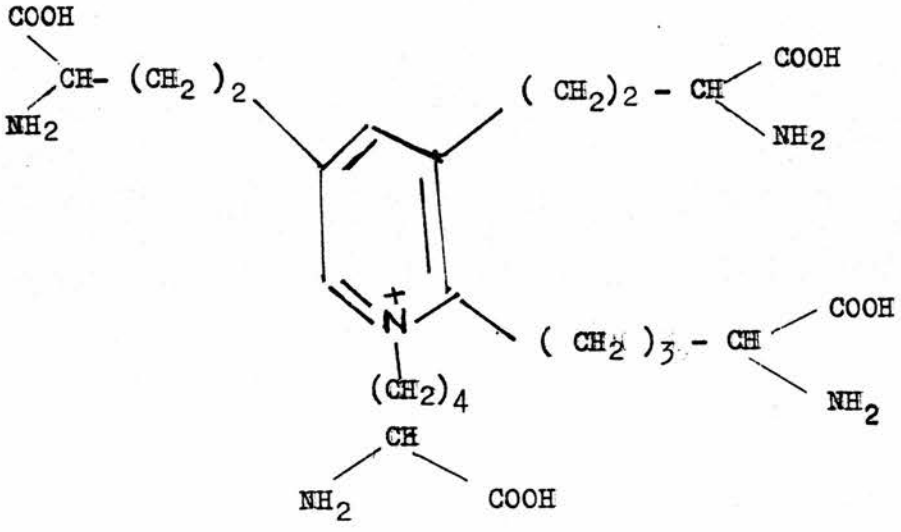
FIG 1

These are Desmosine and Isodesmosine polyfunctional amino acids. They can crosslink up to four polypeptide chains.

FIG 1



DESMOSINE



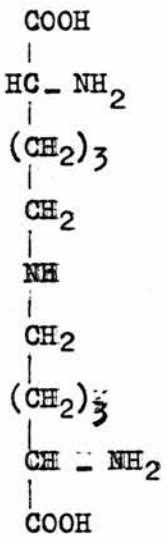
ISODESMOSINE

FIG 2

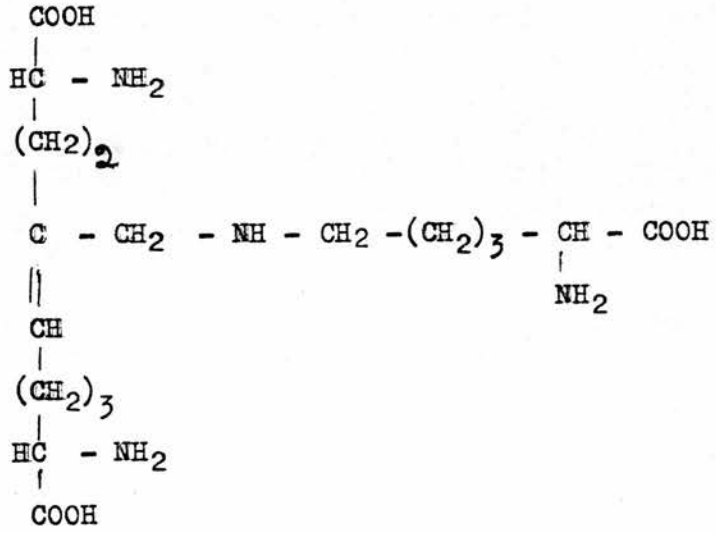
Lysinonorleucine is an intermediate in the biogenesis of (Iso) desmosine cross links. It can also act as cross link, connecting two subunit chains.

Merodesmosine: This amino acid does not in itself occur in elastin. It appears only as reduced form of an intermediate in the biosynthesis of Desmosine.

FIG 2



LYSINONORLEUCINE



MERODESMOSINE

amino acids (about 86% have uncharged side chains). Glycine, alanine, valine and proline predominate (glycine constitutes $^{1}/_{3}$ of the residues), while methionine, cysteine and tryptophan are absent (Table 1).

The amino acids desmosine and isodesmosine have been identified in elastin (Fig 1) (Patridge et al 1963; Thomas et al 1963). Because they possess four free amino groups and four free carboxyl groups they can link via peptide bonds up to four polypeptide chains. Elastin has been shown to contain dehydrolysinonorleucine and dehydromerodesmosine (Pax et al 1971) while their reduced natural analogues, lysinonorleucine (Franzblau et al 1965) and merodesmosine (Starcher et al 1967) (Fig 2) have also been islated. In addition Paz et al (1971,1974) have identified tetrahydrodesmosine and dihydrodesmosine in ligumentum nuchae. These amino acids have been shown to be intermediates in the synthesis of desmosines, although they can act as cross-links in their own right. Desmosines are formed, through a series of reactions, from four lysine residues. Lysyl oxidase catalyses the first of these reactions, which is the oxidative deamination of the ε-amino group of lysine (Gallop et al 1972; Francis et al 1973; Piez 1968; Franzblau 1971).

Many workers have tried different methods both chemical and enzymatic of solubilising elastin with theintention of elucidating its structure.

Patridge et al (1963) were the first to use chemical methods for the fragmentation of elastin. They used repeated one hour oxalic acid treatment at 100°C and produced two components which they named α-and β-elastins. Their intention was to produce a single polypeptide chain that could be sequenced. However, these α- and β-elastins turned out to be polydisperse. The α-elastin had a molecular weight range of 60,000 to 84,000 daltons and contained 17 polypeptide chains each of 35 amino acid residues. It formed aggregates (coacervates) when the colution temperature was raised abouve 25°C. Coacervation is pH dependent, pH 4-9, and the pH of aggregation varies with the method of preparation (Franzblau 1971). The β-elastin component was soluble at all temperatures and comprised of two polypeptide chains. It had

a molecular weight of 5,500 with 27 amino acid residues on average per chain.

Similar fragments have been produced from elastin by alkaline copper sulphate treatment at 37°C for 60 hours (Ioffe & Sorokin 1954) and hot ethanol-HCl or 40% Urea (Hall & Czerkawaski 1961). Elastin has also been solubilised with 0.1MKOH in 80% ethanol at room temperature and the product named K-elastin (Robert & Paullian 1963).

As chemical methods were not successful in producing a single polypeptide chain from elastin attention turned to the use of enzymic methods for solubilisation. Pancreatic elastase (EC 3.4.4.7) which was claimed to have high specificity for alanyl peptide bonds was employed (Loeven 1963; Visser et al 1969; Hall & Czerkawaski 1961; Lamy et al 1961). This enzyme has also been shown to attack other proteins (Grant & Ribbin 1957). Other mammalian enzymes like trypsin (EC 3.4.4.4), Chymotrypsin (EC 3.4.4.5) and pepsin (EC 3.4.4.7) were ineffective in solubilising elastin (Partridge 1962) while the action of pronase and papain (EC 3.4.4.10) was not specific (Robert & Robert 1969; Coulson 1971).

Since no effective method of elastin purification had been achieved the attention of many investigators turned to a search for elastin precursor which they hoped would help in the elucidation of elastin structure. A milestone in elastin research was reached when tropoelastin was isolated from copper deficient swine (Weissman et al 1963; Smith et al 1968) and characterised by Sandberg et al (1969). Other workers have since isolated precursor elastins from other copper deficient animals (Rucker & Goettlich-Riemann 1972; Ito 1973; Narayanom & Page 1974; Sykes & Patridge 1974; Whiting et al 1974). All isolated tropoelastins have been shown to have a molecular weight of between 72-74,000 daltons and are capable of coacervation like the α-elastin of Patridge et al (1955). The amino acid composition of tropoelastin

FIG 3

These are different cross links isolated from different animals and sequenced.

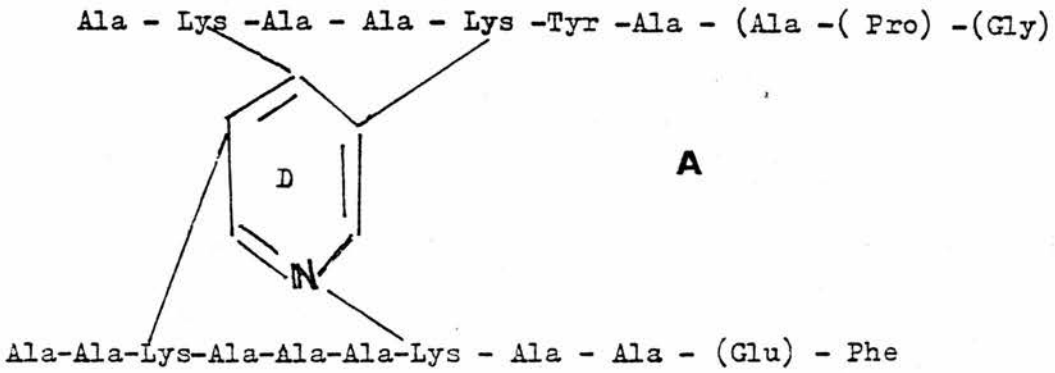
D = desmosine ISOD = Isodesmosine DES = Desmosine

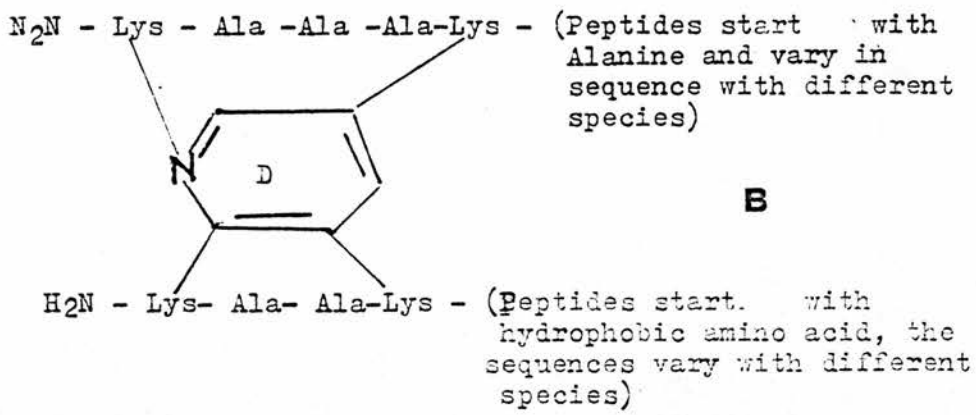
A. Cross link peptide isolated by Foster et al (1974) from Ligumentum nuchae of Bovine. The amino acids in parenthesis are tentatively assigned in that position.

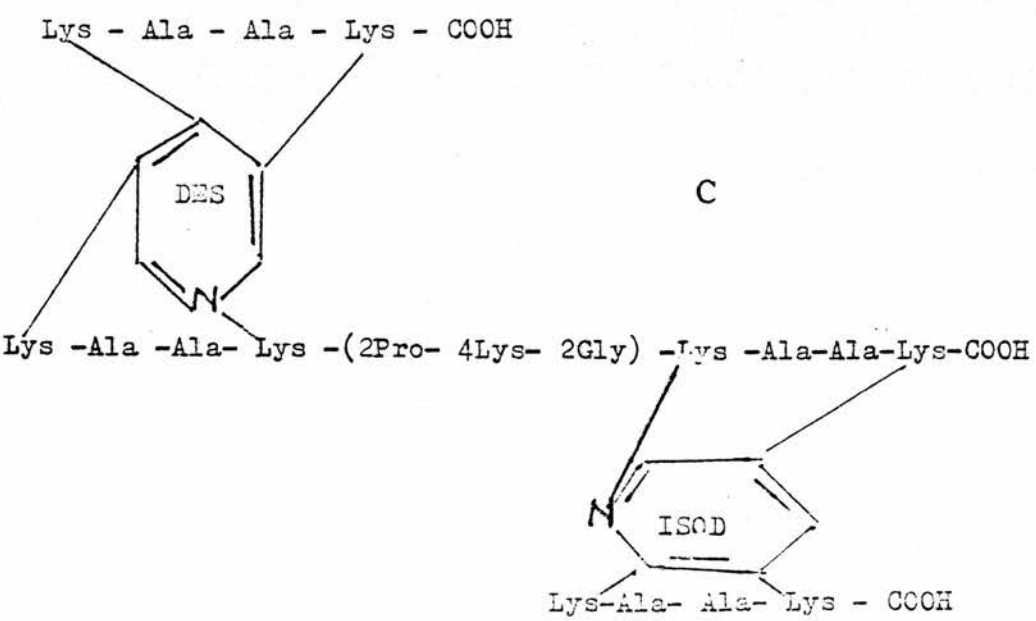
B. This peptide was isolated from Porcine aorta by Gerber and Anwar (1974), and is C-terminal portion aortic sequenced elastin cross linked peptide. The sequences at the COOH end of the peptide differ from one species to the other.

C. This is elastin cross link peptide from porcine aorta Davril and Han (1974). It shows that desmosine and isodesmosine are equally substituted in the same peptide chain.

FIG3


Ala - Lys -Ala - Ala - Lys -Tyr -Ala - (Ala -(Pro) -(Gly)
D
N
A
Ala-Ala-Lys-Ala-Ala-Ala-Lys - Ala - Ala - (Glu) - Phe


N2N - Lys - Ala -Ala -Ala-Lys - (Peptides start with Alanine and vary in sequence with different species)
N
D
B
H2N - Lys- Ala- Ala-Lys - (Peptides start. with hydrophobic amino acid, the sequences vary with different species)


Lys - Ala - Ala - Lys - COOH
DES
N
C
Lys -Ala -Ala- Lys -(2Pro- 4Lys- 2Gly) -Lys -Ala-Ala-Lys-COOH
N
ISOD
Lys-Ala- Ala- Lys - COOH

is the same as mature elastin in that 86% of its amino acid residues are non polar. However, it lacks desmosine and isodesmosine and has high lysine content (see Table 1). Sandberg et al (1969) determined its N-terminal sequence as Gly-Gly-Val-Pro.

The work of Sandberg et al (1971, 1972) on a small tryptic peptide and the large C-terminal fragment isolated from tropoelastin have demonstrated the clustering of alaninyl and lysyl residues in these regions and indeed the following sequences Lys-Ala-Ala-Lys, Lys-Ala-Ala-Ala-Lys have been found (Sandberg et al 1969, 1971). Since alanine-rich cross linked peptide have been isolated from mature elastin (Franzblau et al 1965; Keller et al 1969; Shimada et al 1969; Foster et al 1973a) (Fig 3) the areas of alanine and lysine clustering in tropoelastin give rise to crosslink regions in mature elastin. As it is also known that polyalanine sequences acquire an α-helical conformation it seems probable that these crosslink regions might contain α-helical structures.

The first extensive investigation on the primary structure of elastin was carried out by Foster et al (1973b). The results of their work on pro-elastin confirmed that hydroxyproline was in fact present in elastin, and revealed a primary structure possessing repeats of tetra-, penta-, and hexa-peptides Gly-Gly-Val-pro; Pro-Gly-Val-Gly-Val; Pro-Gly-Val-Gly-Val-Ala. These repeat peptides were used to prove that elastin binds calcium and that β-turns could exist in the protein (Urry 1974). These sequences showed that proline is usually followed by glycine but rarely in the reverse order. Gerber et al (1974) suggested that tropoelastin contained 12 pairs of lysine, 6 pairs in the sequence Lys-Ala-Ala-Lys and 6 in Lys-Ala-Ala-Ala-Lys and that two pairs, one from each of the two tropoelastin molecules, could give rise to desmosine or isodesmosine. An interesting feature of the desmosine peptides is that each peptide contains on a molar basis, one residue of serine, one of glutamic acid, one of tyrosine and two or three of phenylalanine, and

that these amino acids tend to cluster near the desmosine crosslink regions.

Tyrosine has been shown to be N-terminal in a large peptide produced from tropoelastin using trypsin. Since trypsin cleaves at the α-carboxyl of lysine, it has been suggested that tyrosine follows the Lys-Ala-Ala-Lys sequences (Sandberg 1976). It has been postulated that such could prevent oxidation of the adjacent lysine by lysyl oxidase. This residue could therefore now form a dehydrodesmosine ring by condensing with three oxidised lysinyl residues (allysine). It has also been suggested that the tyrosine lying adjacent to such a residue would allow interactions between its $\overline{11}$ electron systems and the pyridinium nucleus. This would provide a conducting path for electrons in the final oxidation step leading to formation of desmosine and isodesmosine. This view was supported by Francis et al (1973) and Foster et al (1974). However, Gerber and Anwar (1975) advocated that a hydrophobic interaction was responsible for the final oxidation step.

As a result of high insolubility of elastin, different structural models have been suggested using experimental data obtained from the chemical analysis of tropoelastin (Sandberg et al 1969; Foster et al 1973b) the mechanical and physical properties of the protein, the studies of its cross links, electronmicroscopic examination (Kawase 1959; Cox et al 1973; Gotte et al 1974) and proton magnetic resonance coupled with circular dichroism (Urry 1974). Below are some of the proposed models:

Network Model or Random Coil. Hoeve and Flory (1974) proposed that elastin is composed of a network of protein chains crosslinked loosely at intervals but free to take up an essentially random configuration. The protein chains swell in water and under thermal motion are distributed evenly in the spaces occupied by the solvent. This model was supported by Mistrali et al (1971) (Fig 4A).

FIG 4

The proposed models for Elastin structure

(A) Random Coil or Network model proposed by Hoeve and Florey (1974). The configuration of chains between cross linkages are much more coiled and irregular than shown.

(B) Globular model proposed by Patridge (1966,1970) and adopted by Weis-Fogh and Anderson (1970). B shows extended state and B_1 shows relaxed state. The straight lines joining the spherical bodies represent the cross links.

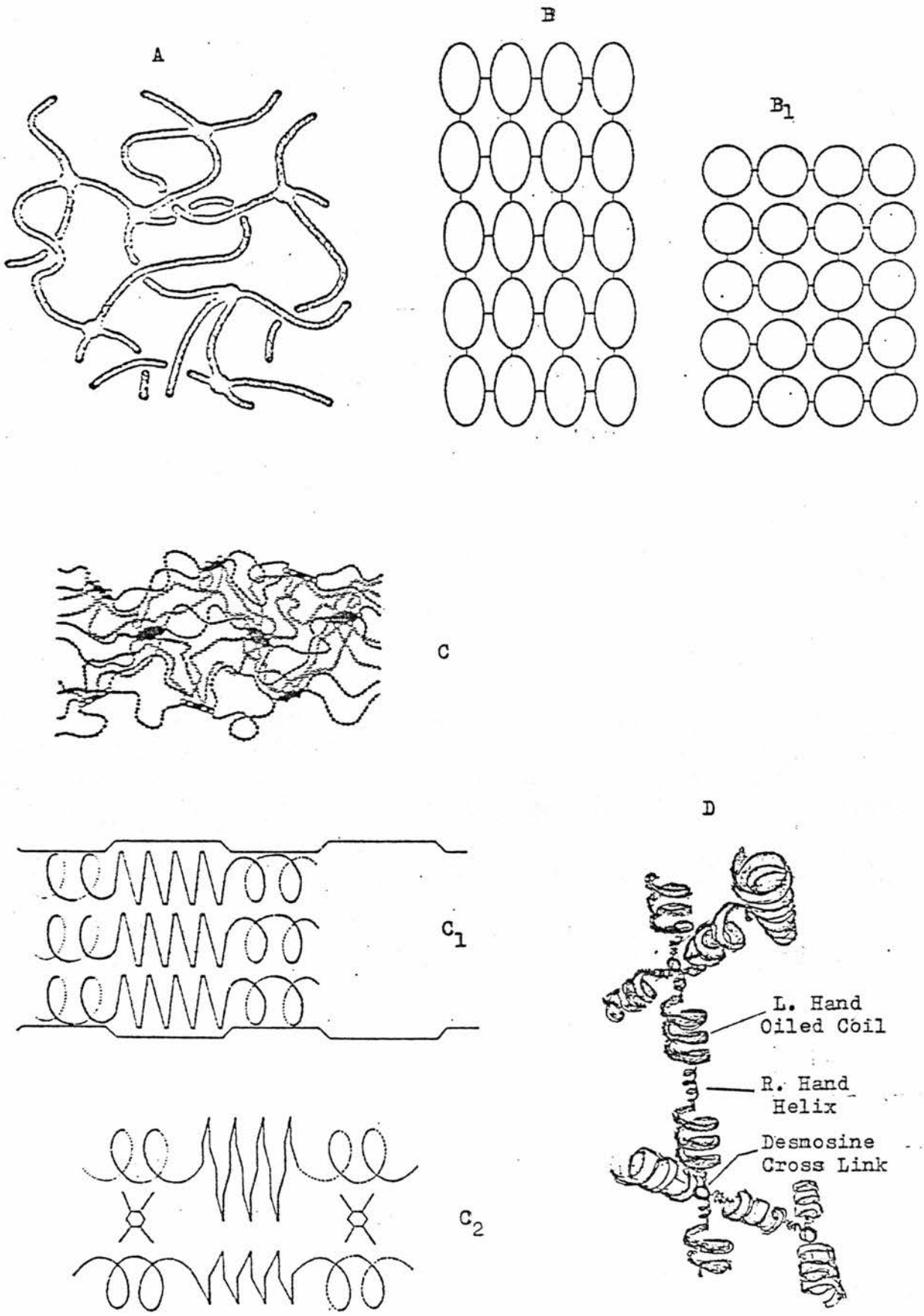
(C) The Fibrillar model as proposed by Donovan et al (1965). The black bodies are the cross links. It is in the relaxed position.

(C_1) The Fibrillar model of Urry (1974) consisting of alternating segments of α'-helix and β-spirals, along a single chain.

(C_2) Represents the association of single chains by cross linking and hydrophobic interaction, giving it a rope twisted appearance.

(D) Oiled Coil model suggested by Gray et al (1973). This contains alternating rigid and flexible areas. The rigid areas represent (iso)desmosine cross links.

FIG4


A
B
B1
C
C1
C2
D
L. Hand
Oiled Coil
R. Hand
Helix
Desmosine
Cross Link

Corpuscular Model (Fig 4B B_1). The corpuscular model was proposed by Patridge (1966, 1967, 1970) who suggested that insoluble elastin was formed from precusor elastin molecules joined together by crosslinks, the gross conformation attributed to tropoelastin was that of a globular protein with a hydrophobic interior and hydrophilic exterior. Patridge assumed that the precusor molecule contained two independent chains with the same or different amino acid composition. This dimer molecule carried on its surface four pairs of lysine residues which under suitable conditions were capable of interacting with other pairs situated in adjacent molecules to form a desmosine crosslinked system of corpuscles. These corpuscles are bound to each other at four points. Hoeve and Florey (1974) have indicated that problems would arise on the packing of such a system, while Torcia and Piez (1973) showed with ^{13}C nuclear magnetic resonance experiment that the elastin undergoes extensive random movements due to Brownian motion, a fact which is inconsistent with corpuscular model (Fig $4B_1$).

Fibrillar Model (Fig 4C,C_1,C_2). The fibrillar model was proposed by Donovan et al (1965) from the result of their work on elastase digest of elastin. He conceived the elastin molecule as a bundle of fibrils held together through hydrophobic forces in which each fibril was assembly of four filaments (Fig 4C). Each filament was made up of longitudinal series of polyfunctional cross-linking bodies joined together by two peptide chains. These crosslinks were connected to form a tubular structure. There was much coiling of the peptide chain which resulted in interaction between chains.

Urry (1974) extended this model by incorporating α-helical and β-configurations. He synthesised the repeating tetra-, penta-, and hexapeptides typical of tropoelastin and studied their conformations with proton magnetic resonance. He discovered that these can bind calcium and form β-turns. From his results and the run of alanine in the crosslinked region, he proposed that the elastin molecule consisted of β-spiral and α-helical segments along a single chain

(Figs 4C C_2). The β-turns occurred at the repeating penta- and hexapeptide sequences, while the α-helical regions occurred in the alanine clusters associated with the crosslinks. He suggested that the chains could associate by crosslinking or hydrophobic interaction (as Donovan et al, 1965, predicted) to give rise to a serial and parallel arrangment of elastin segments. In such a molecule, when the soft segment is extended, the stiffer one could begin to extend before the softer one passes out of dynamic range. This model was supported by electron microscopic findings (Gotte et al 1974; Cox et al 1974).

Liquid Drop Model. The rubber theory which was advanced by Hoeve et al (1958) was rejected by Weis-Fogh et al (1970). The latter authors proposed instead a liquid drop model. This was an extension of the globular model in which elastin was envisaged as spherical monomers which were crosslinked to form a three-dimensional aggregate. In this molecule a few hydrophilic amino acid residues were scattered over a hydrophobic surface with the result that any deformation of the subunits would lead to an inverse of the hydrocarbon-water interface. This model, like the globular model, will encounter the mechanical problems of packing (Fig 4B).

Oiled Coil Model. This model was basically derived from sequence data (Gray et al 1973) and like the liquid drop of Weis-Fogh (1970) visualized the elastic recoil to be generated by hydrophobic interactions. Each segment in the network was fibrillar and made up of a crosslink region (α-helical) and of an oiled coil domain. For the model each subunit is crosslinked to the others by desmosines forming a three-dimensional spring-like structure. The linking is at different angles and each desmosine crosslinks only two chains. The diameter of the coil would be able to accommodate non polar groups. The authors also advocated the presence of β-spirals. However, as Hoeve and Florey (1974) pointed out, this model exhibits the same mechanical deficiencies of the liquid drop (Fig 4D).

As none of the above models can account for all the properties associated with elastin molecule, it seems likely that the blending of some of the models might finally give a solution to the elusive structure of mature elastin.

It is the aim of this work (a) to try and isolate elastin from bovine aorta and solubilize it by selective specific chemical modification of its serine residues and (b) to attempt to elucidate the regularity of occurrence of its crosslinking pattern.

S E C T I O N 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Materials

All the reagents used were of analar grade and were obtained from BDH Chemical Ltd (London) unless otherwise stated. Guanidine hydrochloride was from Sigma Chemical Company and purified according to Nozaki (1972). Sodium dodecylsulphate, acrylamide and NN^1 methyl bisacrylamide were specially purified from BDH.1.Anilino-8-Naphthalene sulphonic acid was obtained as magnesium salt from Sigma Chemical Company and prepared and stored as .1% aquous stock at $4^{o}C$. N-bromosuccinimide was obtained as anhydrous crystals also from Sigma. Diphenylphosphorylchloride was obtained from BDH as solution of 1.3g per millilitre. p-Toluene-4-sulphonylchloride and sodium fluoride were from Hopkin and Williams Company (Essex, England). Sodium hydroxide, hydrochloric acid analytical grade and urea were all from May and Baker Ltd (Dagenham, England). β-mercapto-ethanol and Tris reagent grade were from Sigma. p-Toluene sulphonyl fluoride was prepared according to Weiner (1966).

Sephadex G25, G50, G100 were obtained from Pharmacia Fine Chemicals Company (London, England). Visking tubings were from the Scientific Instrument Centre Ltd.

Spectrophotometric measurements were made with Beckman-DB-GT Grating Spectrophotometer. All pH measurements were made on Radiometer Typ 25 PH meter, and fluorimetric measurements Aminco Bowman Spectrofluorimeter.

Standard proteins, Bovin albumen, ovalbamin, pepsin and myoglobin were from Sigma Chemical Company.

2.2 Purification of Guanidine hydrochloride

Guanidine hydrochloride is a powerful denaturing agent. The commercially available one has been shown to contain spectral impurities because of its association with by-products of the manufacture of melanine (Englebrechte et al, 1957) Ammeline (Fridovich 1965). Biguamide resulting from storage (Nozaki et al 1967) has been found among the impurities. It is therefore important to remove these contaminants when one is either determining molecular weight by meniscus depletion method or performing spectrophotometric measurements. Among the criteria used for the assessment of purity are absorption spectrum and the melting point (Iwong et al 1971). The method of purification is essentially that of Nozaki (1972) -

Procedure

125g of guanidinehydrochloride was dissolved in 500ml of cold ethanol and gradually heated up with constant stirring in a litre round bottomed flask with overhead stirrer and condenser. Benzene (250ml) was gradually added to the hot ethanol solution and the mixture kept at $4^{o}C$ overnight. Crystalline needles were collected and rinsed with a small amount of ethanol-benzene mixture. A yield of 69% was obtained which agrees with that of the authors. This was recrystalised in 160ml of hot methanol and cooled in dry ice-acetone mixture. The crystals were collected on a cooled Buchner funnel moistened with chilled methanol and drained. The residual methanol was removed by rotary vacuum evaporation at $37^{o}C$. The yield was 60% of the starting material.

2.3 Preparation of p-Toluenesulphonylfluoride (TosylF)

This was prepared by the modification of the procedure of Weiner et al (1966).

p-Toluenesulphonylchloride (10g) was dissolved in 16.7ml of dimethylformamide followed by the addition of 13.75g of sodium fluoride. The mixture was stirred for 10min at room temperature and heated in an oil bath set at 115°C for 3½hr with constant stirring. The orange coloured solution was allowed to cool and 291.7ml of water added and mixed. The mixture was extracted four times with 166.7ml of petroleum ether (boiling point 30°-60°C). The extract was dried over calcium chloride overnight. The ether extract was removed, left at -20°C for 24hr to allow the crystals of p-toluene sulphonylfluoride to form. The crystals were collected in a sintered glass filter and dried. The amount of crystals of p-toluenesulphonylfluoride was 5.4g, giving a yield of 54%. The melting point found was 41.5°C and this agrees with that of authentic p-toluenesulphonylfluoride (tosylfluoride) 41.5°-42°C.

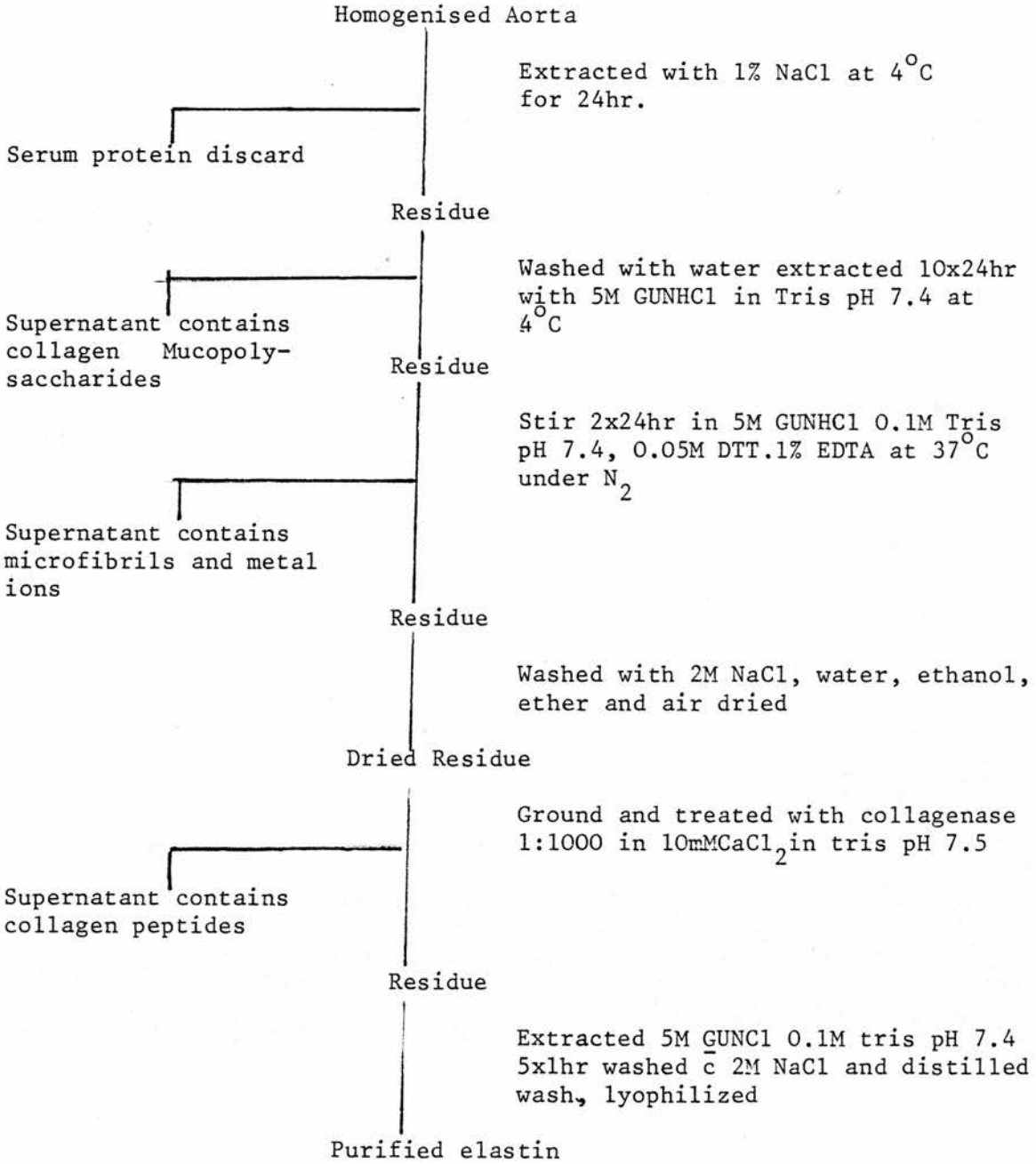
2.4 Isolation of aortic elastin (see Table 2)

Preparation: The procedure is as Serafini-Fracassini (1975). Aorta was stripped of the adhering tissues and cut into small slices and minced, and extracted with 1% sodium chloride at 4°C in the presence of toluene for 24hr. Toluene prevents bacterial growth. This first extraction removed serum protein and blood. The residue recovered by centrifugation was extracted for 10x24hr period in 5M guanidine hydrochloride buffered in 0.1M Tris pH 7.4 at 4°C. This treatment removes mucopolysaccharides and soluble collagen.

The extraction residue was then stirred for two further 24hr period in 5M guanidine Hydrochloride in 0.1M Tris pH 7.4 containing 0.05M dithiothreitol and 0.1% EDTA at 37°C under a blanket of nitrogen. The dithiothreitol breaks the disulphide bonds of microfibrils and solubilizes them, while

TABLE 2

SCHEME FOR ISOLATION OF BOVINE AORTIC ELASTIN



EDTA removes the metal ions that will interfere in the subsequent treatment with collagenase. The insoluble material was collected by centrifugation thoroughly washed with 2M sodium chloride, distilled water, ethanol and finally with ether and dried.

The dried material was ground to powder, treated with collagenase which was purified by affinity chromatography at a substrate:enzyme concentration of 1000:1 in 0.01M calcium chloride buffered in Tris 0.1M pH 7.5. The digestion was carried out at 37°C in a Radiometer pH stat, set at pH 7.5 and maintained at that pH with 0.01M sodium hydroxide.

At the completion of digestion, elastin was collected by centrifugation and washed in 5M guanidine in 0.1M Tris pH 7.4. This was followed by thorough washing with 2M sodium chloride and distilled water prior to lyophilisation.

2.5 Further purification of elastin

An aliquot (60mg) of elastin was further purified by suspending it in 20ml of 5M guanidine Hydrochloride in 0.1M tris/HCl buffer pH 7.4. This was stirred at 4°C for 24 hr, β-mercaptoethanol was added to make 3% and the mixture covered with nitrogen and stirred at room temperature overnight. The residue was recovered by centrifugation, washed four times to remove excess guanidine and mercaptoethanol. This was followed by 2 washes with acetone and the residue was left in fresh acetone overnight to remove water. Acetone was removed by washing with ether and air dried, and stored at -20°C.

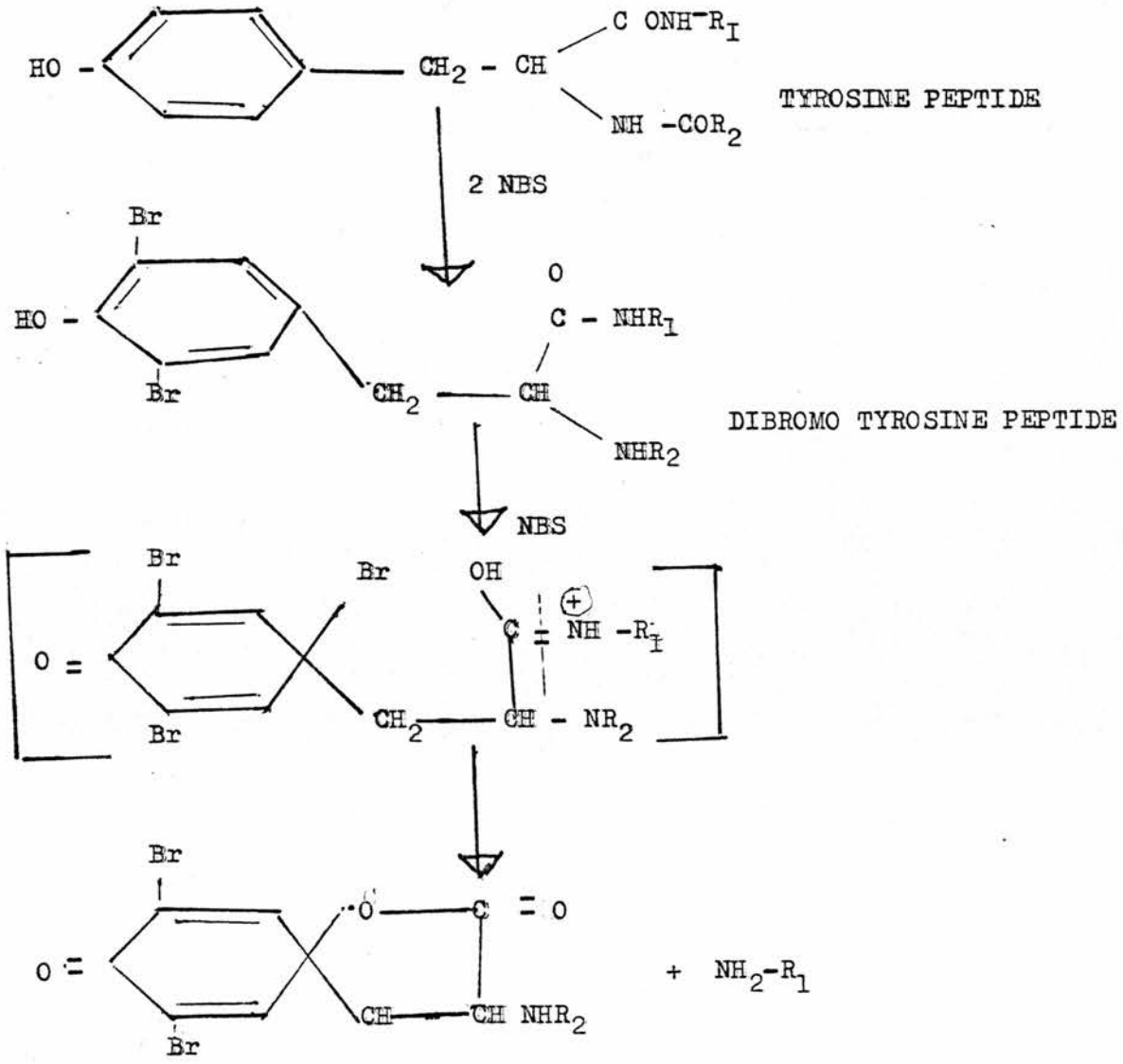
2.6 Modification of Tyrosine residues

2.6.1 N-Bromosuccinimide

This is a very reactive reagent introduced by Schmir et al (1959) as a source of bromine for the specific cleavage of tyrosyl residues. The effect of bromine was previously reported by Du Vigneaud and his group in 1953 when working on peptide hormones. The scission, he observed, occurred at the amide

linkage between the carboxyl group contributed by tyrosine and the amide nitrogen of the following amino acid. The mechanism of reaction is complex and the following has been proposed (Fig 5).

When 3 equivalents of NBS are used, a sequence of reactionstakes place in which the first two equivalents convert tyrosine to dibromotyrosine and the reaction with the third one causes the internal rearrangements that finally convert dibromotyrosine to spirodienone-lactone with subsequent cleavage as mentioned above.



SPIRODIENONE - LACTONE

FIG 5

NBS is used in 8M urea titrated to pH 4.6 with acetic acid (Schmir et al 1961). In this condition, provided that tryptophan is absent, NBS specifically cleaves at tyrosyl residues and the reaction with other amino acids is prevented.

Procedure: Elastin (30mg) was suspended in 10ml of 8M urea and left at room temperature for 24 hr with constant stirring. The pH was then adjusted to 4.6 with glacial acetic acid, noting the exact amount used, and stirred for several hours to ensure constant pH. Apart from the unfolding of protein by disruption of hydrogen bonding and hydrophobic interaction, urea forms bromourea which is the active reagent instead of bromosuccinimide. NBS (6mg) was added and the suspension stirred for 24hr at room temperature. The residue was recovered by centrifugation, washed with water, acetone, ether and dried. The supernatant was lyophilized and the residue checked for the presence of protein, with fluorescence labelling, ANS. Amino acid analysis was performed on the residue.

2.6.2 p-Toluenesulphonylfluoride treatment.

The serine residue can be selectively modified by substitution of its OH group with a strongly electron attracting group. Spande et al (1970) showed that the derivative can easily be split by alkali induced β-elimination. In the case in question, water is removed and a double bond is formed between α- and β-carbon atom, forming a dehydroalanine residue (Fig. 6). The groups that have been found effective are tosyl (Patchornik et al 1963) and O-diphenylphosphoryl groups (Photaki 1963, Riley et al 1957). Weiner et al (1966) and Strumeyer et al (1963) have used tosylfluoride to modify serine in chymotrypsin with subsequent formation of the dehydroalanine derivative of the enzyme.

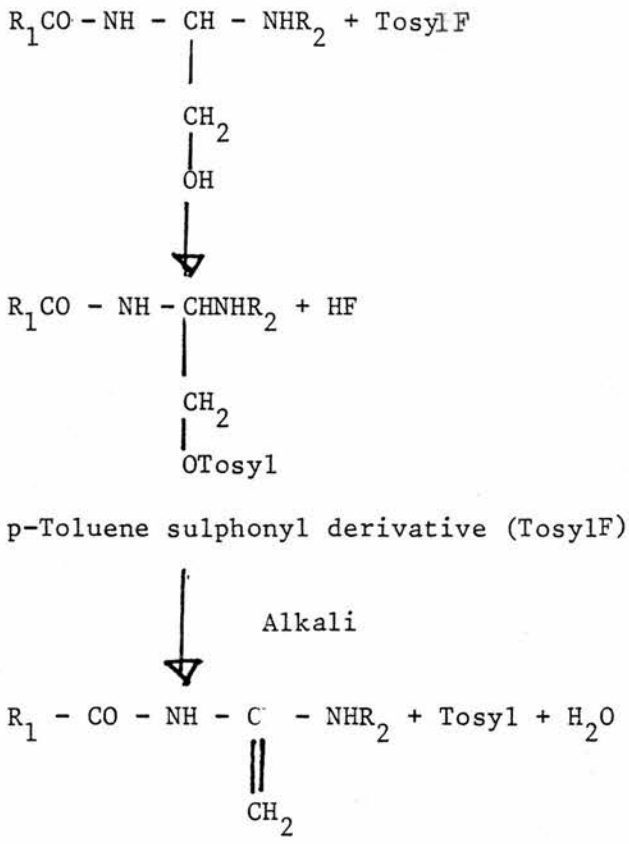


FIG 6

The procedure adopted in this work was that of Weiner et al (1966) with slight modification.

Purified elastin (35mg) was suspended in 10ml of distilled water and left at 4°C for 24hr to soak. 1ml of dioxane containing 20mg of p-toluenesulphonylfluoride (TosylF) was added to the suspension. The mixture was stirred at room temperature for 24hr. The residue was recovered by centrifugation, washed 3 times with dioxane to remove unreacted TosylF and twice with distilled water. This was followed by two washes with acetone and the precipitate left in fresh acetone overnight covered with aluminium foil. The acetone treated residue was washed with ether and air dried in the fume cupboard.

2.6.2 β-Elimination

(a) Diethylamine: The procedure described is essentially that of Photaki et al (1963).

Tosyl elastin (35mg) was suspended in 10ml of ether for 24hr at 4°C. Diethylamine (1ml) was added and the mixture stirred at room temperature for 2-4hr, left at 4°C for 1hr for the precipitate to settle. The addition of diethylamine in the organic solvent causes precipitation of toluene-sulphonum salt of diethylamine which is insoluble. The residual mixture was recovered by centrifugation and washed with more ether, which removed excess diethylamine, 2ml of absolute ethanol or methanol were added and the suspension stirred for 30min. This treatment dissolved the p-toluenesulphonum salt which was recovered by rotary evaporation at 37°C. The insoluble elastin was washed with more ethanol, and the washings added to the previous one. The amount of diethylammonium salt of p-toluene sulphonate was determined and the amount of modified serine residue calculated.

(b) β-Elimination with 0.1M NaOH: Tosyl elastin (30mg) was soaked in 10ml of water for 24hr at 4°C, 10ml of NaOH was added, mixed by stirring and left for several hours at 4°C with continuous mixing. The residue was recovered by centrifugation, washed well with distilled water, twice with acetone, then ether and dried as before.

2.6.3 Diphenylphosphorylchloridate treatment

The procedure is that of Riley et al (1957) with minor modifications.

Purified elastin (40mg) was suspended in 5ml of dry pyridine and left overnight to soak at 4°C. 0.8ml of diphenylphosphorylchloridate (DPPCl) was added and stirred at 4°C for 36hr. The brownish yellow coloured mixture was washed successively with one molar HCl, 0.02M HCl and washed thoroughly with water until a test of the washings with silver nitrate solution gave no white precipitate. This indicated complete removal of chloride ions which would

interfere in subsequent treatment with performic acid. The residue was washed with ethanol until no more colour was observed in the supernatant and finally with ether. Samples were treated at $0^{o}C$ and room temperature, while a control was carried through the procedure without DPPCl.

2.6.3 (a) β-Elimination with diethylamine

To the residue from above 10ml of diethylether were added followed by 0.8ml of diethylamine, mixed and left at room temperature for 2hr with constant stirring. Diethylammonium diphenylphosphate precipitated. Subsequent treatment was as in 2.6.2(a).

2.7.1 Oxidative cleavage with performic acid

Proteins and peptides modified by tosylation or diphenylphosphorylation and converted to dehydroalanine derivative have been shown to be easily oxidised by performic acid to form a labile intermediate which is easily cleaved by raising the pH to 11. The modified peptides have been shown to cleave in distilled water at $100^{o}C$ and in HCl pH 2 (Patchornik et al 1964). In each case the scission occurs at the bond between the α-carbon atom and the amide nitrogen of the serine residue giving rise to an amide peptide and hydroxy-pyruvyl peptide (Fig. 7).

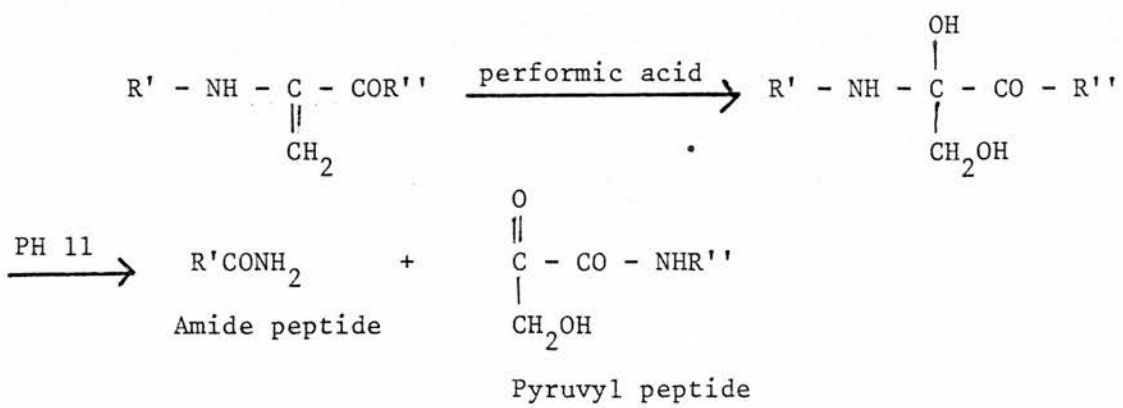
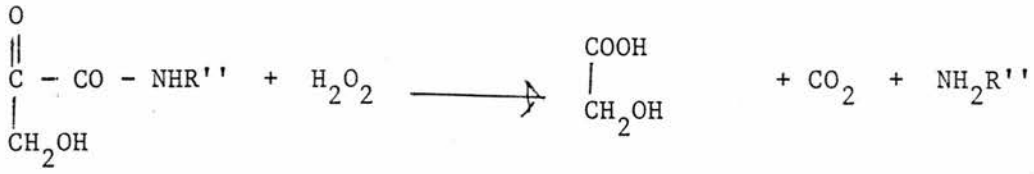


FIG 7

Addition of H_2O_2 oxidises pyruvyl peptide further producing a new amino acid terminal, glycolic acid, and CO_2.



The oxidative procedure adopted is that of Patchornik et al (1964) slightly modified. Performic acid was prepared by adding 10.6ml of 88% formic acid to 1ml of 30% $^{v}/_{v}$ H_2O_2 in a test tube, mixed, stoppered and left for 2hr at room temperature. Modified elastin (35mg) was suspended in 5ml of distilled water and left at $4^{o}C$ for 24hr. The mixture was spun down at 2,000 rpm for 40min and the water carefully removed. 1ml of cold performic acid was added to the residue and left at $4^{o}C$ with constant stirring overnight. The solution was diluted 10-fold with distilled water, frozen immediately and lyophilized. The freeze drying was repeated to remove every trace of formic acid.

Distilled water (10ml) was added to the lyophilised sample which was left to soak at $4^{o}C$ for 24hr. The pH of the solution was adjusted to 11 with diethylamine. Immediate cleavage was observed and the residue disappeared. This was stirred at room temperature for 40min. The supernatant was recovered by centrifugation and amino acid analysis was performed on an aliquot. The remainder was lyophilised. The residue was washed four times with water, acetone and finally ether and dried, and an amino acid analysis performed. The experiment was repeated as above with 0.1M NaOH and the cleavage was the same. However, diethyamine was preferred because it could easily be removed by lyophilisation. The control, and the samples treated at different temperatures were oxidised and cleaved as above. The purpose of the control was to monitor the effect of alkaline treatment. There was no change in the residue, and no cleavage or solubilization.

2.7.2 Oxidation by alkali hydrogen peroxide

The modified sample (15mg) was added to 5ml of distilled water left at 4°C for 24hr. Five ml of 0.2M NaOH was added followed by 1ml of 30% hydrogen peroxide. The mixture was incubated at 37°C for 5hr with constant mixing. The recovery of the supernatant and residue was as in 2.7.1 and an amino acid analysis was also performed.

2.8 Identification of Peptides

2.8.1 SDS Gel Electrophoresis

Polyacrylamide gel electrophoresis is perhaps the most useful and versatile electrophoretic system for the separation and analysis of macromolecules. In combination with sodium dodecyl sulphate (SDS) it has become invaluable in the identification, assessment of purity of protein preparations and determination of molecular weight of polypeptide chains. SDS an anionic detergent combines with proteins forming a complex of rod shaped random coil conformation and dissociating it into its constituent polypeptide chains (Shapiro et al 1967; Weber et al 1969; Weber et al 1972).

The protein treated in this way behaves as having a uniform shape and identical charge-to-mass ratio because of the binding of a constant amount of SDS (1.4g/g of protein) per unit weight of protein. The negative charge acquired by proteins is that of SDS rather than the intrinsic charge of the constituent amino acids (Freifelder 1976). Hence the effective mobility, of reduced proteins only, is proportional to the molecular weight as a result of the sieving property of the gel. In this work the separation and identification involved the following steps:

2.8.1.1 Reagent preparation:

This was according to Weber et al (1972) with slight modification.

(a) Acrylamide solution A. 20g of acrylamide and 0.54g of N N' methylenebisacrylamide were made up to 100ml with distilled water.

(b) Acrylamide solution B. Acrylamide (30g) and N N' methylenebisacrylamide

(0.8g) were dissolved and made up to 100ml with distilled water. Both solutions were filtered through Whatman No. 1 paper, and stored at $4^{o}C$.

(c) Gel buffer. 0.1M Tris/HCl buffer pH 7.4 containing 1% SDS and 8M urea.

(d) Reservoir buffer stock. Tris (121.1g) and SDS (20g) were dissolved and made up to 1 litre with distilled water after adjustment of the pH to 8 with HCl and diluted one in ten prior to use.

(e) Ammonium pensulphate 1%. Ammonium persulphate (1g) was dissolved in a small quantity of distilled water and made up to 100ml. This solution was prepared immediately before use.

(f) N'N'N'N' tetramethylethylenediamine (TEMED). This was supplied in solution and 0.03ml of this was used for all gel concentrations. It was kept at $4^{o}C$.

(g) Staining solution. Coomassie blue (CBBR 250) (2.5g) was dissolved in 450ml of methanol, 72ml of glacial acetic acid was added and the solution made up to 1 litre with distilled water.

(h) Destaining solution. Methanol (250ml) was mixed with 75ml of glacial acetic acid and made up to 1 litre with distilled water.

TABLE 3/

TABLE 3

COMPOSITION OF ACRYLAMIDE GEL OF VARIOUS POROSITY

Final concentration of acrylamide %	5	7.5	8	10	15
Solutions (ml)					
Acrylamide Sol. A	7.5	11.3	12.0	-	-
Acrylamide Sol. B	-	-	-	10	15
Gel buffer	15.0	15.0	15.0	15.0	15.0
Distilled water	6.5	2.7	2.0	4.0	-
1% Ammonium persulphate	1.0	1.0	1.0	1.0	10mg
TEMED	0.03	0.03	0.03	0.03	0.03

2.8.1.2 Gel preparation

Glass tubes, 7.5cm long and 5mm in diameter, were soaked overnight in chromic acid, washed thoroughly with tap water and rinsed well with distilled water and oven dried. One end of the gel tube was closed with parafilm, and placed in a vertical position. The appropriate solutions to make up required gel concentrations were selected from Table 3 and mixed in that order and deaerated for 5min. Ammonium persulphate and TEMED were added and mixed. Using a pasteur pipeete, each tube was filled with acrylamide solution to a 6.5cm mark or any other selected mark provided that all the gels for the same experiment were of the same height, and tapped to remove air bubbles trapped at the bottom. A small volume of distilled water was carefully layered on the top of the gel solution with a pasteur pipette

whose tip was bent at 90°. This prevents air-meniscus formation and maintains the even surface of the gels. The gels were left at room temperature to set. The water on the top of each gel was replaced with the reservoir buffer, covered with parafilm and stored at 4°C overnight before use. The buffer was replenished regularly. Gels with SDS at pH 11.5 were also prepared.

2.8.1.3 Preparation of protein standards

The protein standards used were bovin serum albumin, ovalbumin, myoglobin, and cytochrome C. These were dissolved in 0.1M Tris buffer pH 7.4 containing 1% SDS, 8M urea and 3% mercaptoethanol. The solution was left at room temperature with constant stirring for 24hr under nitrogen. The final concentration of protein was 1mg/ml. Samples were heated for 5min at 100°C, cooled under tap water and centrifuged to remove insoluble materials.

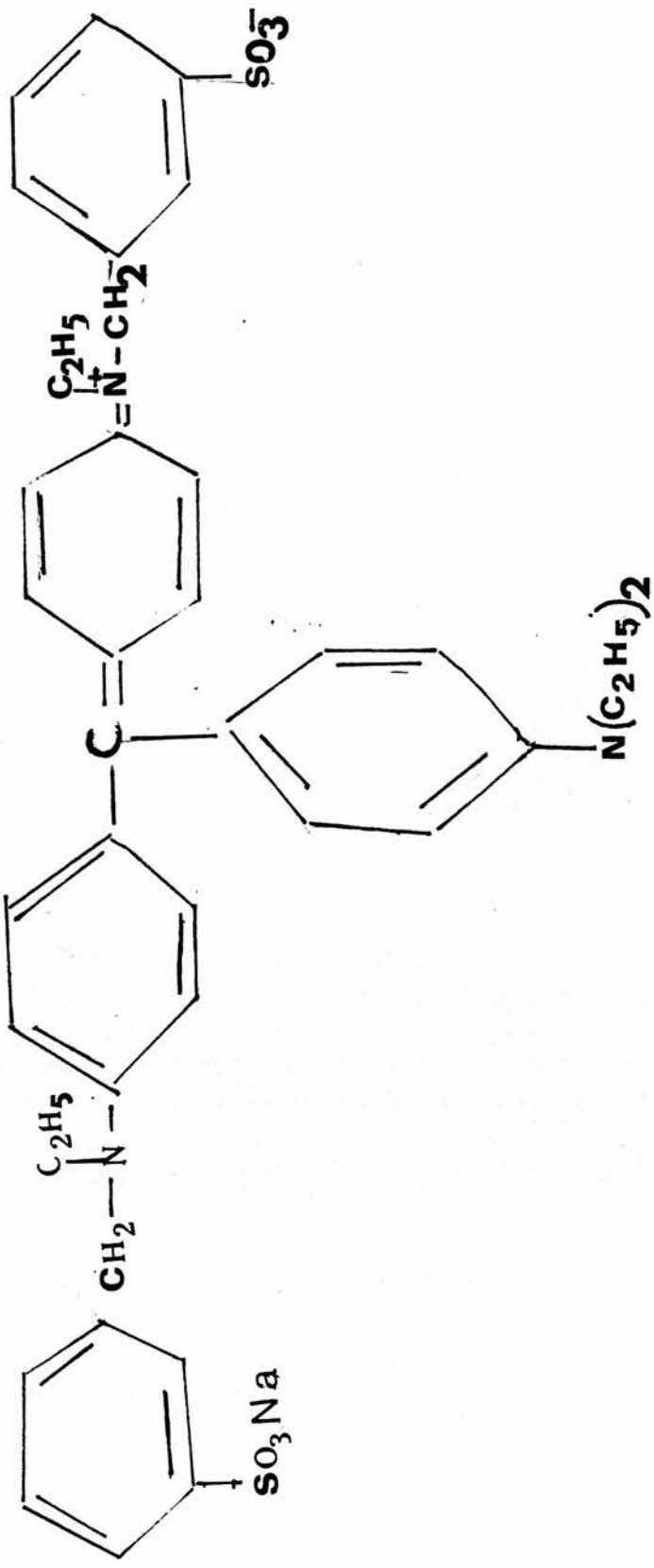
2.8.1.4 Sample preparation

An aliquot (3mg) of the material obtained from the supernatant of performic acid oxidation was dissolved in 1ml of 0.1M Tris pH 8.0. An identical aliquot dissolved at pH 11.5 and the preparation procedure was the same as in 2.8.1.3 with the exception that β-mercaptoethanol and bubbling with nitrogen were omitted.

2.8.1.5 Gel loading

The parafilm covering the bottom of the gel tubes was removed, the buffer on the top shaken off and the gel surface rinsed with fresh reservoir buffer. The tubes were inserted vertically in the upper compartment of the electrophoretic tank. For each tube 10µl of 0.05% bromophenol blue in 0.1M Tris buffer pH 11.5, 1 drop of glycerol and 10µl each of sample or standard

FIG 8



STRUCTURE OF COOMASSIE BLUE

and reservoir buffer were mixed on a square piece of parafilm. Reservoir buffer (250ml) was added to the lower tank chamber. The sample was loaded on the top of the gel with a micropipette, and the reservoir buffer carefully layered on the solution with a fine pipette so that the two solutions did not mix. The upper chamber was filled with the buffer pH 11.5 and covered with the lid which served as a cathode.

The power supply was switched on and the current adjusted to 5mAmps per gel or less as appropriate. The electrophoresis was carried out at room temperature. As soon as the dye reached a suitable position, the electrophoresis was stopped and the tubes removed. The electrophoresis was repeated at pH 8.0.

2.8.2 Acrylamide Gel Electrophoresis without SDS

This was carried out as in 2.8.1 except that the SDS was omitted and the pH of the buffers was adjusted to 11.5. This was to avoid the aggregation of the elastin peptides at a pH lower than 10.

2.8.3 Staining

2.8.3.1 Coomassie Blue

This is a triphenylmethane dye that stains proteins very strongly. It has been extensively used in the location of proteins separated in SDS acrylamide gel electrophroesis (Weber & Osborne 1969; Weber et al 1972; Bennett et al 1971). Because of its high sensitivity, Fazaka et al (1963) and Bennett et al (1971) used it in quantitative estimation of proteins separated in cellulose acetate and acrylamide gels. Its structure is as shown in Fig 8.

Procedure: Each gel was removed from the tube by squirting in distilled water through the sides of the gel by means of a long needle attached to a syringe. This was carried out carefully to avoid fragmentation of the gel. The latter came out smoothly under the pressure exerted. The gels were stained in test tubes containing the commassie blue solution for 24hr at room temperature, destained in the destaining solution until the bands were clear. A little of DEAE ion exchange resin was added to absorb excess dye.

2.8.3.2 Orcein stain

Orcein is a complex organic compound with empirical formula $C_{23}H_{24}N_2O_7$ (Weiss 1954; Engle et al 1954). It is a mixture of related amphoteric dyes (Engle & Dempsey 1954; Hans 1956). It is insoluble in water but soluble in alcohol with optimum staining pH of 3-8.5. The specificity is due to the alcohol soluble cationic F1 and F11 of Engle and Dempsey (1954).

Preparation: Orcein (1g) was dissolved in 75ml of absolute ethanol and 1ml of con. HCl acid was added. The solution was made up to 100ml with deionised water. It was then filtered and left at room temperature for one week before use.

Staining procedure: The gels were removed from the tubes as in 2.8.3.1 and stained in test tubes containing orcein solution for at least 24hr at room temperature. After staining, the gels were destained by leaving them in distilled water until the excess dye was removed, and the bands became visible. This required several changes of distilled water.

2.8.3.3 1.Anilino-8-Naphthalene sulphonic Acid (ANS)

This is a compound that does not fluoresce in water but does so when bound to protein in aqueous solution or when dissolved in organic solvent (Weber et al 1954). This unique property is attributed to its binding to hydrophobic sites on the protein surface (Stryer 1965; McClure & Eldman 1966).

(a) Preparation: The method of preparation was that of Nerenberg et al (1971) modified. Anhydrous megnesium salt of ANS (100mg) was dissolved in 90ml of deionised water with constant stirring. When dissolved, it was made up to 100ml with deionised water and stored in a brown bottle, as a stock solution, at $4^{o}C$.

(b) Staining procedure: An aliquot of the stock solution (3ml) was diluted to 100ml with 1% acetic acid ($^{v}/_{v}$). The gels once removed from the tubes were stained in test tubes containing the ANS solution at $20^{o}C$ for 10min. At low protein concentration the time of staining may take longer. The bright yellow fluorescence was observed under an UV lamp using excitation wavelength of 366nm.

(c) ANS-Elastin activation/fluorescence emission spectra:

Procedure: Elastin (0.5mg) was suspended in 4ml of 0.1M Tris buffer pH 11.5, added to 1cm silica cuvette, and the fluorescence recorded using an Aminco-Bowman spectrofluorimeter. The maximum fluorescence emission of ANS-elastin complex was determined by setting the activation wavelength between 200nm and 800nm at interval of 2nm. The maximum emission wavelength found was used to mirror the maximum excitation wavelength of the complex scanning the activation wavelength between two hundred and eight hundred namoeters. Similar operation was used to check the activation/fluorescence emission maximum of ANS in buffer solution without added protein.

2.9 Isolation of Elastin Peptides

2.9.1 Sephadex Gel Filtration

(a) Preparation of G25, G50 and G100 gels

Sephadex fine grade gels were suspended in Tris buffer pH 11.5 in a Buchner flask and heated in a boiling water bath for 1hr and cooled.

G100 was heated for 5hr . Each slurry was degassed to remove any remaining air bubbles by connecting the arm of the flask to the vacuum water pump.

(b) Column packing

A glass column, diameter 1.4cm, was mounted vertically and the dead space under the bottom filter and the outlet tubing filled with eluent buffer. This was accomplished by pouring about 10ml of eluent into the column and sucking away air bubbles under the net with a syringe attached to the outlet tubing. The outlet was closed and the gels prepared as previously explained was carefully poured down the wall of the column. When all the slurry had been poured into the column, the flow was started and the column allowed to pack under gravity at a rate of 12ml per hour. After the gel had sedimented, three column volumes of buffer were passed through to equilibrate the gel bed, by means of a pump which maintained the flow rate at 0.2ml per minute. The void volume was determined using 2mg of dextran blue per ml.

(c) Sample application

The top of the column bed was protected with filter paper cut to size and laid on the top of the gel bed. The eluent above the gel surface was removed by draining through the outlet tubing. 2ml of the sample containing 12mg of protein was carefully layered on the top of the gel and allowed to percolate through the gel by opening the outlet tube. When the sample entered the gel the flow was stopped and 1ml buffer was used to rinse remnants of the sample adhering to the sides of the column. Then the column was filled with eluent. The top of the column was closed and connected to the reservoir inlet. The pressure pump was turned on and 3.7ml fractions collected. The flow rate was 11.1ml per hr.

(d) Monitoring of effluent

An aliquot (0.05ml) of the stock solution of ANS was added to each tube fraction and the blank, allowed to stand for 10min at room temperature and read in the Aminco-Bowman Spectrofluorimeter with the activation wavelength set at 380nm and the effective emission wavelength at 455nm. The relative fluorescence intensity readings were plotted against the fraction volumes, and the elution profile of the peptides determined. The fractions marking each peak were pooled, lyophilized and amino acid analysis performed as detailed under 2.10.

2.9.2 Dialysis

2.9.2.1 Acetylation

Although it was stated by Craig et al (1957), that insulin with a molecular weight of 5,733 daltons does not pass through $^{18}/_{32}$ visking tubing (diameter 0.017mm), while it does so very slowly through $^{8}/_{32}$ visking tubing (0.064mm). It was discovered in this laboratory that $^{8}/_{32}$ visking tubes do not completely retain solute of molecular weight of 20,000 daltons. Hence the recommendation of Pitt-River and Impiombato (1968) to acetylate this grade of membrane when used with solutes of molecular weight lower than 25,000. It is therefore safer to acetylate $^{18}/_{32}$ when one is separating or dialysing peptides with unknown molecular weight.

Procedure: This is according to that of Craig and Konigsberg (1961).

A suitable length of $^{18}/_{32}$ visking tubing was soaked in distilled water and the lower end tied firmly. The open end was slipped over a suitable glass tube. After removal of the water, the tube was washed with dry pyridine. Both the glass tube and the membrane tubing were filled with 10% acetic anhydride in pyridine (v/v) and left for 15hr at 25°C. The membrane was rinsed with distilled water and washed thoroughly with 0.01M acetic acid

until all the pyridine had been removed as shown by the levelling of the absorbance at 260nm of subsequent washings. The membranes were stored at $4^{o}C$ in distilled water.

2.9.2.2 Dialysis in distilled water

Because of the large amount of salt present in each lyophilized sample, membrane dialysis with acetylated $^{18}/_{32}$ tubing was undertaken to remove the excess salt in the high molecular weight fractions obtained from gel filtration. The pooled residues were dissolved in 3ml of Tris pH 11.4 and dialysed against 4 changes, 250ml each of distilled water for 48hr at $4^{o}C$. The solution was lyophilized and forzen for subsequent use.

2.9.3 Assessment of purity of fractions

The purity of the two fractions separated by column chromatography was assessed by SDS acrylamide and acrylamide gel electrophoresis. The procedure is as detailed in 2.8.1 using orcein and ANS as stains.

2.10 Chemical Analysis

2.10.1 Amino acid analysis

Preparations were hydrolysed in 5ml of 6N, constant boiling, hydrochloric acid. A drop of thioglycolic acid was added, the tubes were flushed with nitrogen for 10min, sealed and placed in an oil bath set at $110^{o}C$ for 24hr. The hydrolysates were taken to dryness with a vacuum rotary evaporator, and left under reduced pressure in the presence of sodium hydroxide pellets.

Procedure: Amino acid analyses were performed using a Locarte single column amino acid analyser. Norleucine was used as internal standard while the solution of amino acid standards was obtained from Sigma Chemical Co Ltd. The quantitation was done manually from the chromatograms.

2.10.2 Hydroxyproline analysis

This was carried out independently according to the method of Blumenkrantz et al (1973). In this procedure, the hydroxyproline is oxidised in acid medium by periodic acid to form Δ^1 pyrroline 4 hydroxy 2 carboxylic acid, which in an excess of periodic acid is further oxidised to pyrrole-2 carboxylic acid, a heterocyclic unsaturated compound. The latter is coupled to p-dimethylaminobenzylaldehyde to form a coloured complex, the intensity which is proportional to the concentration of hydroxyproline. The maximum absorbance is at 565nm. (Fig 9)

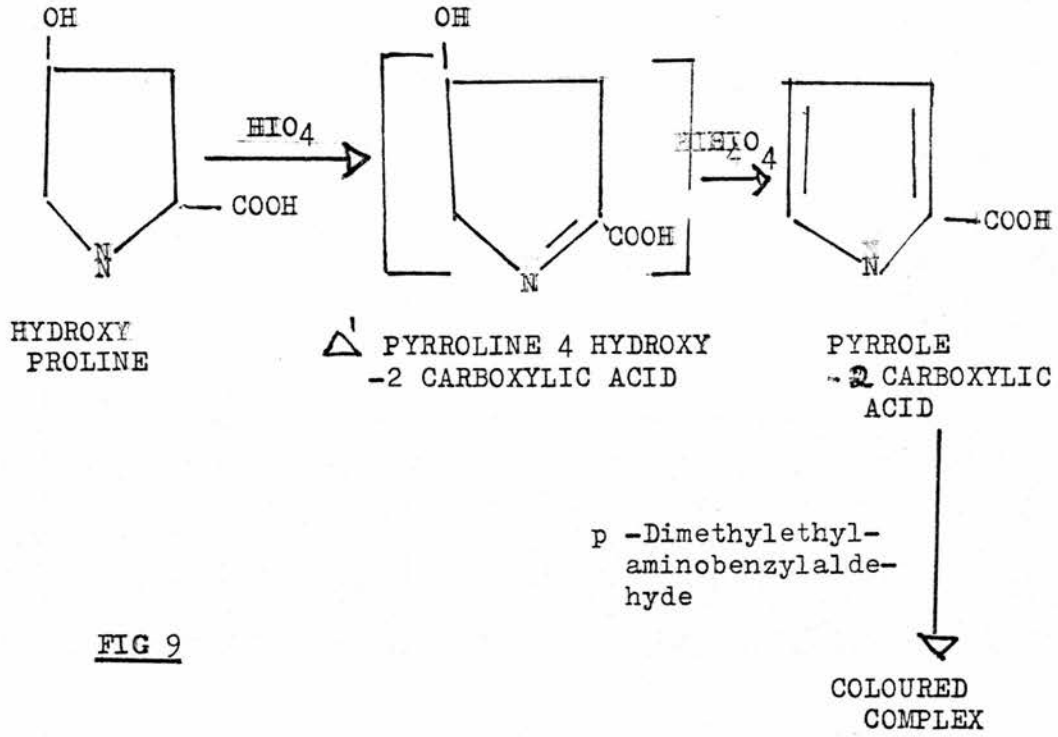


FIG 9

Reagents

1. Periodic acid solution

1-M periodic acid in 9M H_2PO_4. 14.3ml of periodic acid was made up to 50ml with 9M H_3PO_4 and stored in a dark brown bottle in the dark.

2. Citrate-Phosphate buffer

154ml of 0.15M Citric acid was mixed with 34.6ml of 0.6M Na_2HPO_4 giving a pH of 7.

3. Extraction solution

125ml of toluene, 125ml of iso-butanol and 50ml of normal propanol were mixed.

4. Standard hydroxyproline solution

L-hydroxyproline was prepared at 50mg/ml.

5. p-dimethylaminobenzaldehyde

4g of p-dimethylaminobenzaldehyde was dissolved in 15ml of isobutanol and 4.5ml of perchloric acid was added. The solution was mixed and stored in dark bottle at $4^{o}C$ for several weeks.

Procedure:

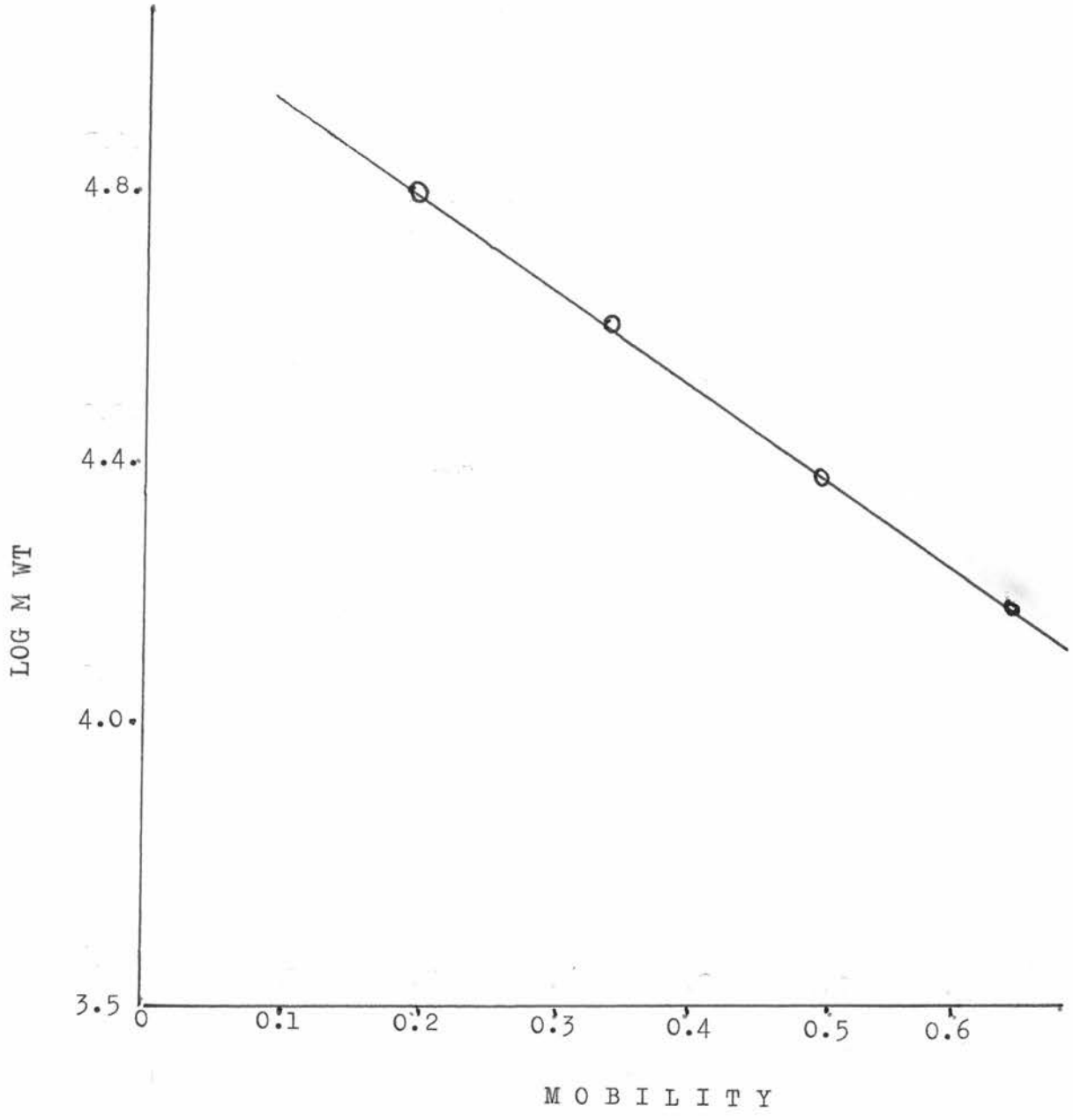
Samples from purified elastin, supernatant of the peptides, Fractions F_1 and F_2 isolated from chromatographic procedure were hydrolysed in 6M constant boiling hydrochloric acid in a sealed tube at $110^{o}C$ for 24hr. The hydrolysates were brought to dryness using a rotary evaporator. The residues were dissolved in the appropriate amounts of distilled water.

Assay procedure:

0.5ml of samples and standards were added to 3ml of buffer followed by 0.5ml of 1M periodic acid solution, and carefully mixed. 1.75ml of extraction mixture was added and the solution mixed in a Vortex mixer. The tubes were then placed in a test tube rack, covered with aluminium foil and shaken in a horizontal shaker for 30min. The tubes were centrifuged at 2,000rpm for 10min to separate the two phases.

To 0.6ml of organic phase, 0.15ml of Ehrlich's reagent was added. The tubes were stirred vigorously. The colour was allowed to develop for 15min at room temperature and the absorbance was read at 565nm in a Beckman DB-GT grating spectrophotometer.

FIG M



2.11 Molecular Weight Determination

2.11.1 SDS acrylamide gel electrophoresis

The electrophoresis of F_1, F_2 and the supernatant were carried out in SDS acrylamide as described in 2.8.1 except that the pH 11.5 was employed throughout the experiment, and the stain used was orcein. Cytochrome C was omitted in the standared.

The length of the gels and the distances migrated by the dye and the proteins were measured before and after staining. From the data obtained the mobilities of the standards and the unknowns were calculated as shown below:

$$\text{Mobility} = \frac{\text{Length of gel before staining}}{\text{Length of gel after staining}} \times \frac{\text{Distance migrated by protein}}{\text{Distance migrated by dye}}$$

A standard curve of the mobility versus Lag of Molecular Weight was constructed from which the molecular weights of the unknowns were determined by extrapolation.

2.11.2 Meniscus deplection

Equilibrium Dialysis:

F_1 (3mg) obtained from chromatographic separation was dissolved in 1ml of 0.1M Tris buffer pH 11.5 and dialysed against 0.1M Tris pH 11.5 for 3 days at $4^{\circ}C$ with 3 changes of buffer using acetylated visking tubing. A suitable aliquot of this was Loaded in Spinco Model E analytical ultracentrifuge.

Condition for ultracentrifugation for molecular weight determination:

Temperature	=	$20^{o}C$
Speed	=	44,000rpm
Partial Specific vol. $\bar{v}$	=	0.700
Specific gravity of solvent ρ	=	1.004
Cell used	=	12mm double sector centre piece
Optical system	=	Rayleigh interference optics
Instrument used	=	Spinco Model E analytical Ultracentrifuge.

Fringe displacements were measured by means of a travelling microscope from Projection Cope PQ Ltd. Recordings were taken at 200 microns interval commencing at the meniscus along the X-scale until a deflection of more than 10 microns occurred on the Y-scale between consecutive readings. The readings were then made at 100 microns intervals along the X-scale. The partial specific volume of elastin was calculated from compositional data (Zamyetnin 1972). The density of the solution was measured with pycnometer. The program of Yphantis and Roark (1972) was utilized in the computation of the molecular weight values from experimental data.

S E C T I O N 3

RESULTS

3. RESULTS

3.1 Isolation of Elastin from Bovine Aorta

The scheme followed in the isolation and purifiction of elastin from bovine aorta is shown in 2.4. Table 4A shows the amino acid composition of the purified protein. There is a slight increase in the polyfunctional amino acid, desmosine and isodesmosine.

3.2 N-Bromosuccinimide (NBS) Treatment

The modification of tyrosine residues in purified elastin with NBS was carried out as in 2.6.1. The amino acid analysis of the modified elastin is shown in Table 4B. There is evidence of loss of tyrosine residues and hence cleavage without solubilization. There is no peptide released in the supernatant as indicated by fluorimetric measurement using 1-anilino-8-naphthalene sulphonic acid.

3.3 p-toluenesulphonylfluoride Modification

Several attempts to modify serine residues in elastin with p-toluene-sulphonylfluoride (TosylF) were unsuccessful. The amino acid composition of treated elastin is shown in Table 4C. This shows that the serine residues were not modified and there was no evidence of solubilization. No TosylF was recovered after β-elimination.

TABLE 4

AMINO ACID COMPOSITION OF ELASTIN FROM BOVINE AORTA

Values are expressed as residues/1000 total amino acid residues and corrected for hydrolytic losses.

TREATMENT:	PURIFIED ELASTIN	NBS TREATED ELASTIN	TOSYLFLUORIDE TREATED ELASTIN
	A	B	C
AMINO ACID			
1. Hydroxyproline	10.3	9.6	10.0
2. Aspartic acid	7.8	7.5	7.4
3. Threonine	9.2	9.6	9.3
4. Serine	8.5	8.3	8.4
5. Glutamic acid	17.0	16.5	16.0
6. Proline	113.0	118.0	114.0
7. Glycine	320.0	322.0	321.0
8. Alanine	232.0	233.0	321.0
9. Valine	150.0	150.0	150.0
11. Isoleucine	24.0	23.6	24.0
12. Leucine	58.0	58.0	58.1
13. Tyrosine	7.8	-	7.5
14. Phenylalanine	26.0	26.0	25.5
15. Lysine	3.8	4.6	4.2
16. Arginine	5.0	5.4	5.1
17. Isodesmosine	3.1	3.0	2.9
18. Desmosine	5.0	5.1	4.9

3.4 Diphenylphosphorylchloride (DPPCl) Modification

Diphenylphosphorylchloride modification of serines was carried out as detailed in 2.6.3. β -Elimination of the diphenylphosphonyl group by alkali produced dehydroelastin which was subsequently cleaved by formic acid oxidation following the adjustment of the pH to 11. Only accessible serine residues were modified. The amino acid analysis of the supernatant showed that only half of the serine residues were modified. Very little residue was left. The amino acid composition of the residue (Table 5R) showed glycine predominating, followed by alanine, proline and valine. There was no indication of the presence of desmosine or isodesmosine or arginine. Table 5 shows the amino acid profile of the solubilized elastin. The supernatant was also subjected to gel filtration (see Fig. 13).

3.5 Identification of the Peptides

Identification of the peptides was carried out by acrylamide gel electrophoresis with and without SDS followed by coomassie blue, ANS and orcein staining. The gel electrophoresis was carried out in triplicates for each type of acrylamide gel.

The coomassie blue stained two bands. However, the stained bands disappeared before the destaining was completed. ANS identified two bands as bright yellow fluorescence in the gel without SDS. These bands failed to appear in SDS acrylamide gel. Staining with orcein showed two bands but the gel containing SDS stained very faintly (see Fig. 16) and the bands were not permanent.

3.6 Activation and Emission Spectra of the ANS-elastin Complex

The maximum activation and fluorescence emission spectra of ANS and ANS-elastin Complex are shown in Figures 10-12. The maximum excitation and the fluorescence emission wavelengths found for the ANS-elastin complex are 380nm and 455nm respectively. These were measured in Aminco-Bowman spectrofluorimeter as detailed in 2.8.3.3c. The maximum activation and emission wavelengths found for ANS without added protein are 365nm and 505nm respectively.

3.7 Isolation of the Peptides

Figure 13 shows the elution profile of the supernatant fraction from Sephadex G50. This shows two defined peaks F_1 and F_2 when eluted with 0.1M Tris buffer pH 11.5. The F_1 peak exhibited a slight trail and had an elution volume of 55.5ml. This corresponds to the void volume. F_2 is a symmetrical peak with maximum elution volume of 148.0ml. The rechrometography of Fractions 51.8-111ml on G100 produced a single peak as shown in Figure 14. The assessment of purity with acrylamide gel electrophoresis indicated one single band. The F_1 fraction eluted in the void volume, in G100 (42.0ml).

F_2 was rechrometrographed on Sephadex G25 and the result showed a single peak (Fig 15) which eluted in the void volume (61.0ml). ANS was used in the monitoring of the effluent fractions in all the chromatography done.

FIGURE 10

Activation spectrum of ANS elastin complex in 0.1M Tris pH 11.5 showing maximum at 380nm.

FIG 10

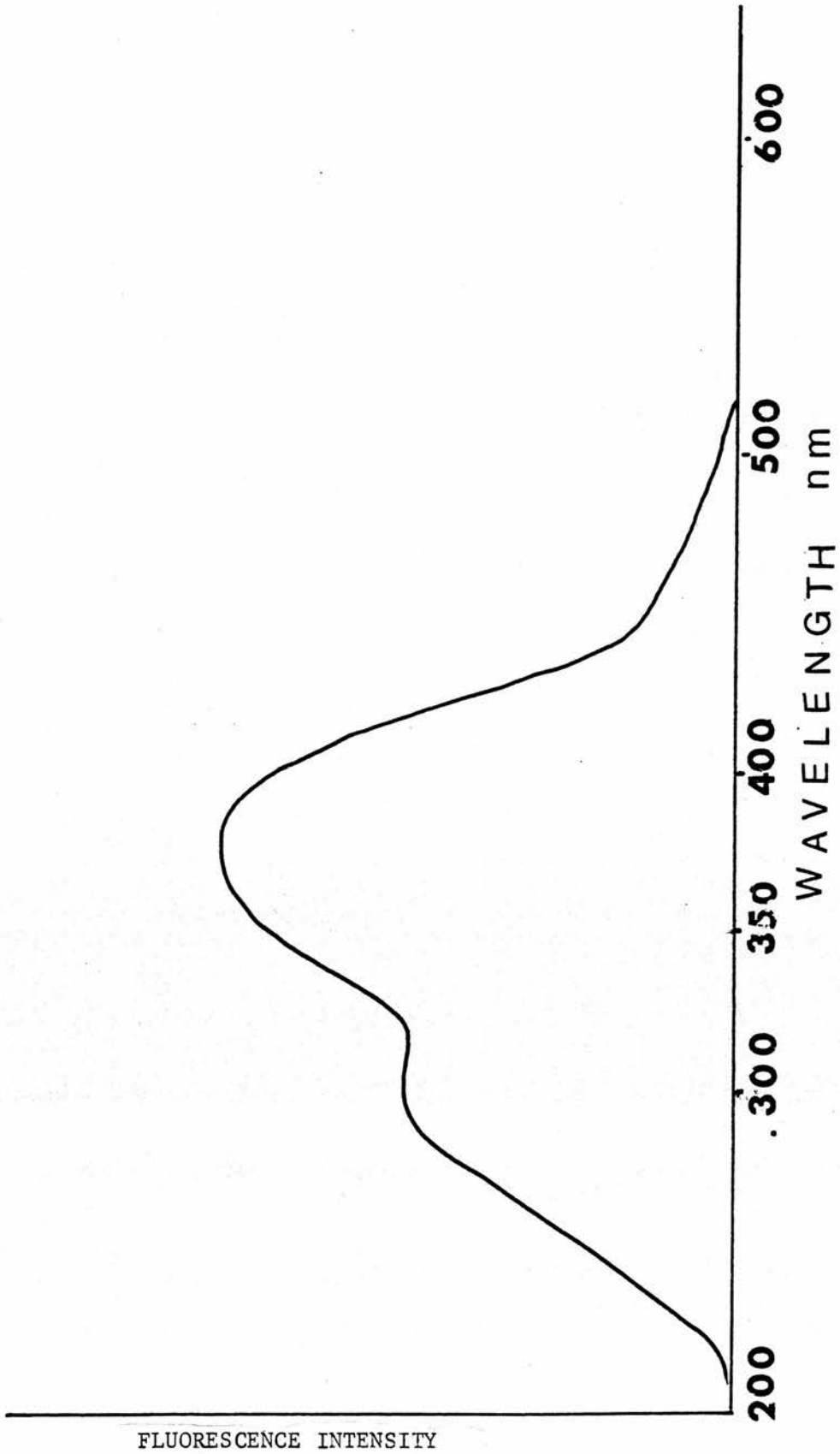

200
300
350
400
500
600
WAVELENGTH nm
FLUORESCENCE INTENSITY

FIGURE 11

Emission spectrum of ANS-elastin complex in 0.1M Tris pH 11.5, showing two peaks at 455nm and 725nm.

FIG 11

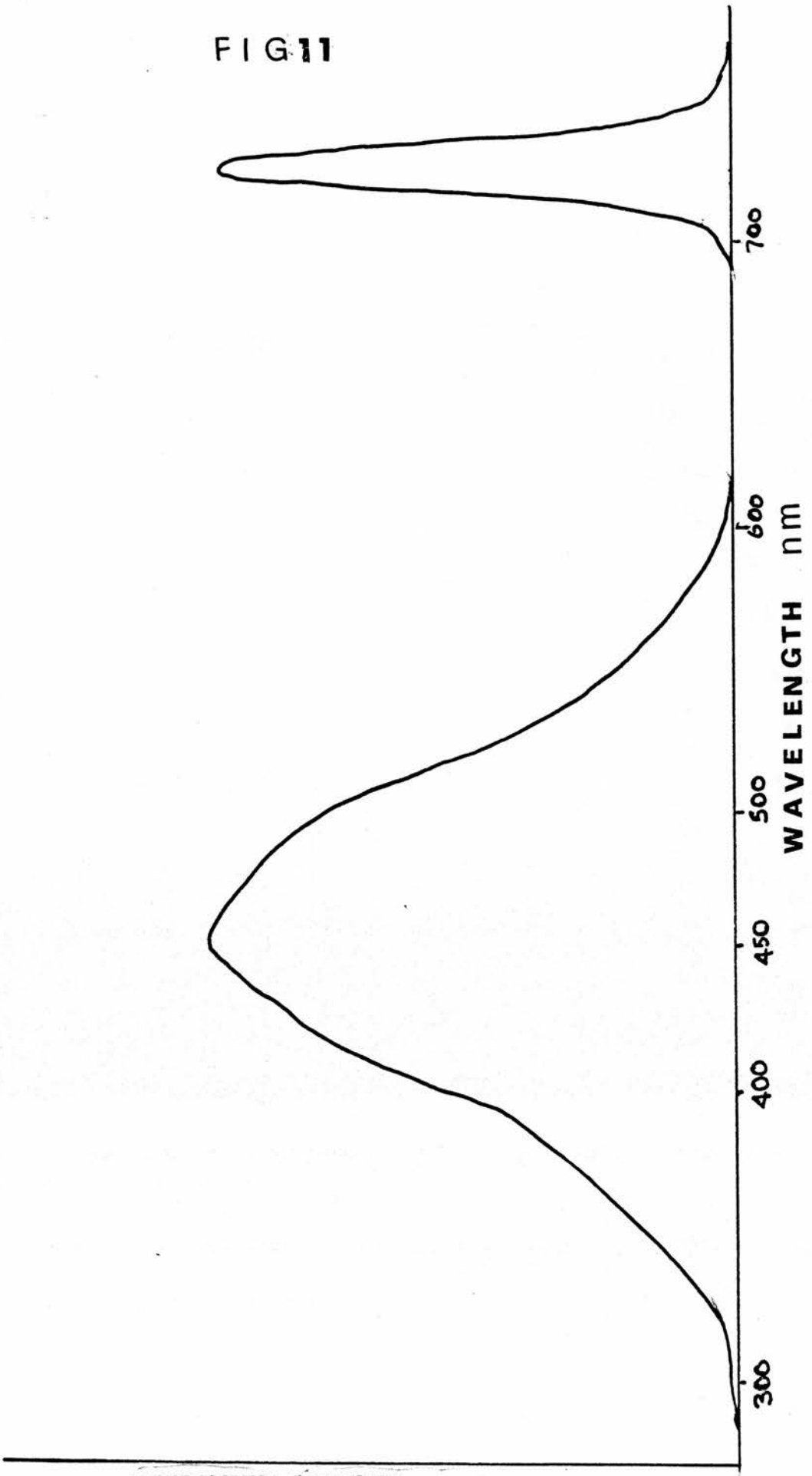

300
400
450
500
600
700
WAVELENGTH nm
FLUORESCENCE INTENSITY

FIGURE 12

Activation and flueorescence emission spectra of ANS in 0.1M Tris buffer pH 11.5 without addition of protein.

———————— Activation

---------- Fluorescence Emission

FIG12

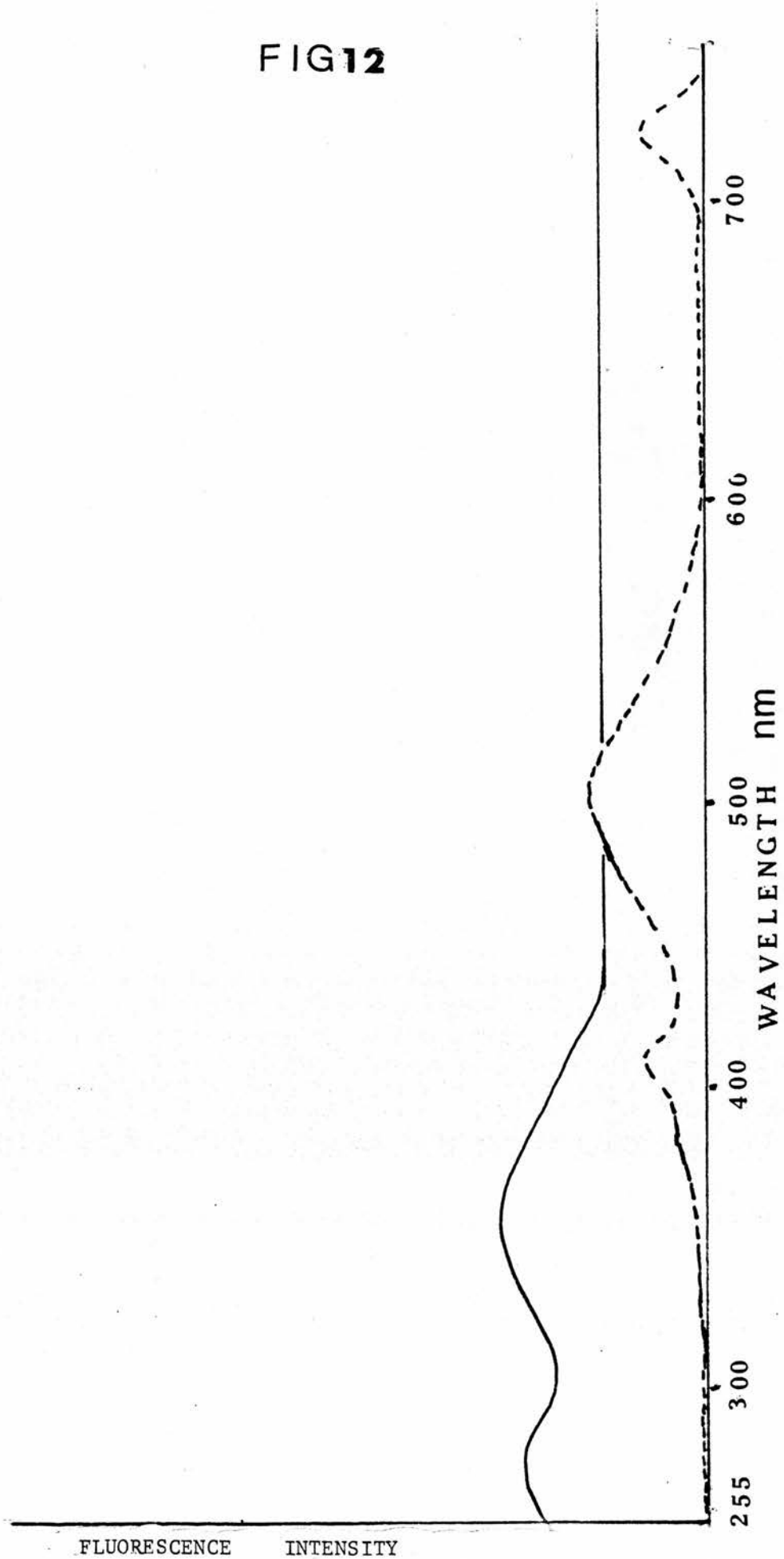

255
300
400
500
600
700
WAVELENGTH nm
FLUORESCENCE INTENSITY

FIGURE 13

Elution profile of supernatant fraction separated on Sephadex G50

F_1 shows slight trail.

Bed dimensions:	1.4 x 104 cm
Flow rate:	11.2ml/h
Fraction volume:	3.7ml
Eluent:	0.1M Tris buffer pH 11.5

FIG13

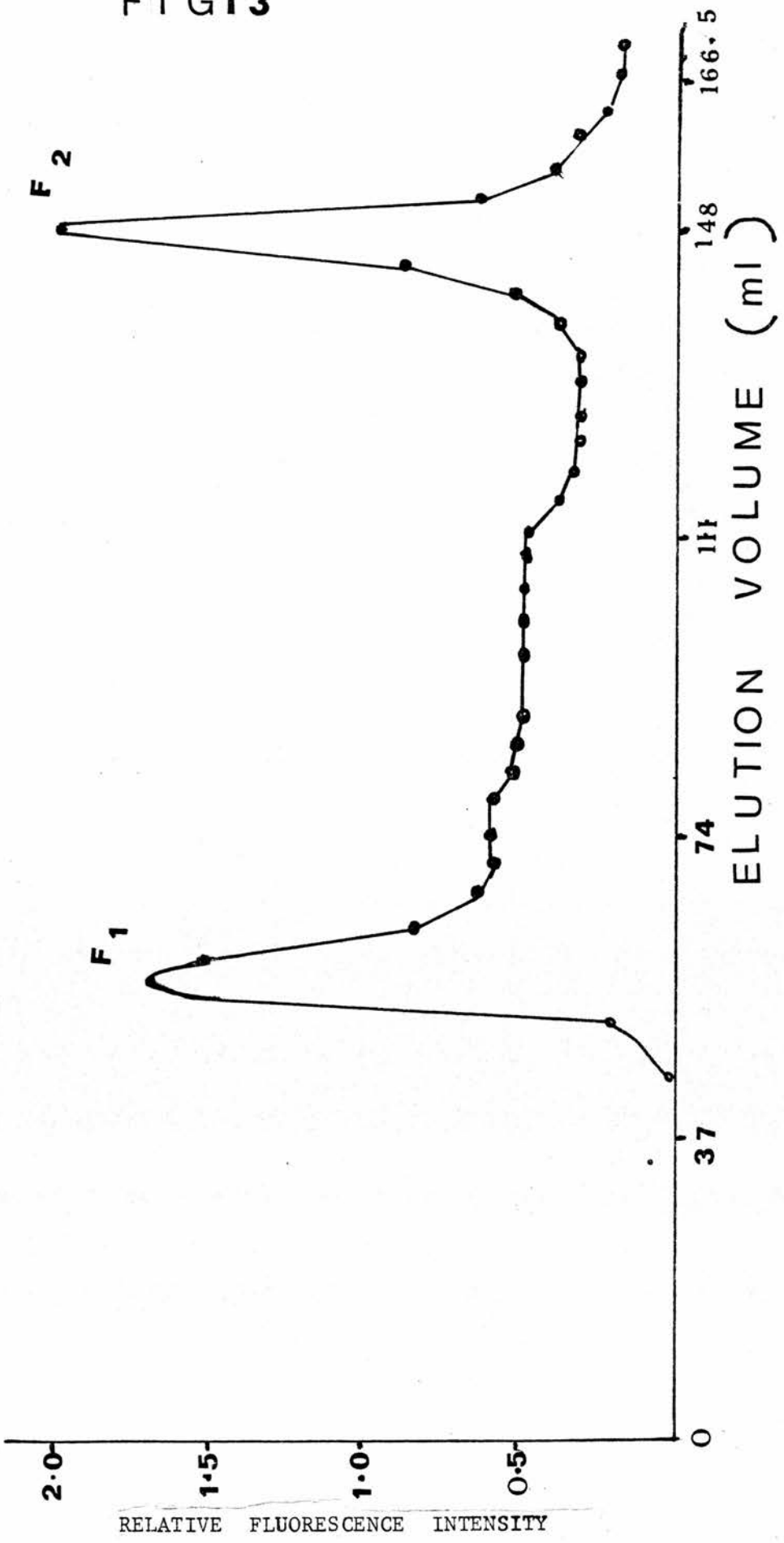

F2
F1
2·0
1·5
1·0
0·5
0
37
74
111
148
166·5
ELUTION VOLUME (ml)
RELATIVE FLUORESCENCE INTENSITY

FIGURE 14

Rechromatography of F_1 on Sephadex G100 showing single peak.

Eluent:	0.1M Tris buffer pH 11.5
Fraction volume:	3.0ml
Flow rate:	9ml/h
Bed dimensions:	1.5 x 92cm

FIG 14

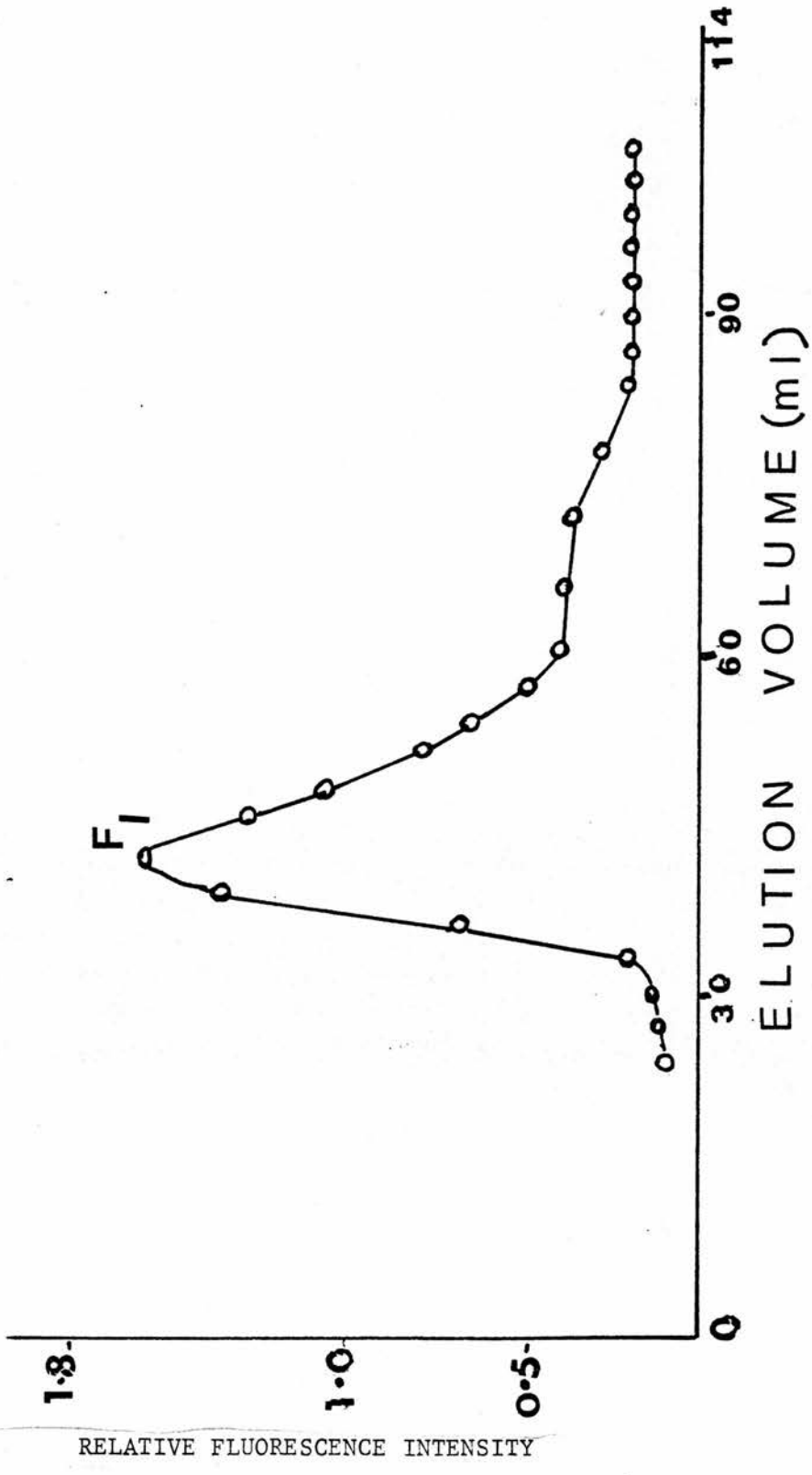
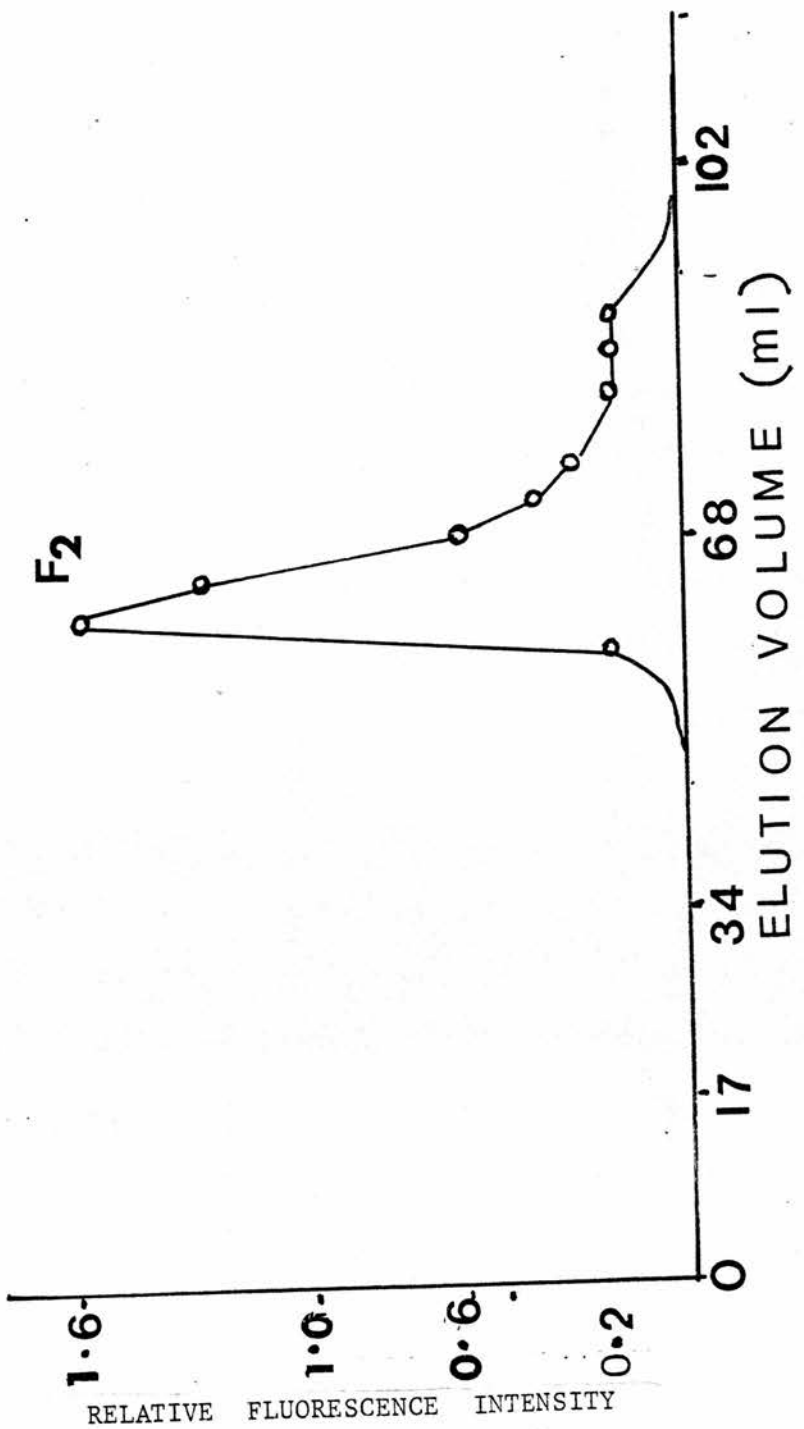

F I
0
30
60
90
114
ELUTION VOLUME (ml)
1·8
1·0
0·5
RELATIVE FLUORESCENCE INTENSITY

FIGURE 15

F_2 rechromatographed on Sephadex G25 using 0.1M Tris pH 11.5

Bed dimensions:	1.4 x 104cm
Flow rate:	10.2ml/h
Fraction volume:	3.4ml

FIG 15


F2
0
17
34
68
102
ELUTION VOLUME (ml)
0·2
0·6
1·0
1·6
RELATIVE FLUORESCENCE INTENSITY

3.8 Amino Acid Analysis

Amino acid profiles of supernatant, F_1 and F_2 are as shown in Table 5. The amount of sample solubilized is 30mg out of the 35mg used. This represents 86% of the starting material. The remaining 14% is accounted for in the residue. Supernatant fraction showed that the values of glycine and alanine are reversed, with low glycine and high alanine content. It is also rich in glutamic acid. All others agreed closely with the amino acid composition of the purified elastin.

F_1 shows that this fraction is enriched in alanine and low in glycine. F_2 shows very high content of glycine, valine, proline and isoleucine. The value of alanine in this fraction is half the value of glycine. The crosslinks and arginine are absent, while lysine is very low. All the desmosine and isodesmosine together with arginine are in F_1. The ratio of F_1 to F_2 is 6 : 1, F_2 accounting for 17% of the solubilized elastin and 14% of the starting material.

3.9 Molecular Weight Determination

3.9.1 SDS Gel electrophoresis. The determination of molecular weight of F_1 by SDS acrylamide gel electrophoresis was carried out as in 2.8.1 at pH 11.5 and stained with orcein. The apparent molecular weight by this method is 42,000,while gel filtration in Sephadex indicates over 68,000 daltons for F_1 and about 3,000 daltons for F_2.

3.9.2 Analytical Ultracentrifugation.

The molecular weight data derived from ultracentrifugation analysis are reported in Figure 17. The analysis was performed in 0.1M Tris buffer pH 11.5. At vanishing concentration the molecular weight obtained using a partial specific volume of 0.70ml g^{-1} is about 10,000. This is supported by the plots of the number-, weight-, and z-average molecular weights versus concentrations in the cell,which converged to the same value at infinite dilution. There is no significance difference between curves Mn and Mw average molecular weights.

TABLE 5

AMINO ACID COMPOSITION OF DPPCl TREATED ELASTIN

Values expressed as residues/1000 total amino acid residues and corrected for hydrolytic losses

Amino Acid	Supernatant	Fraction (F_1)	Fraction (F_2)	Residue (R)
Hydroxyproline	9.8	10.0	ND	ND
Aspartic Acid	12.9	14.1	6.1	44.7
Threonine	4.1	5.6	8.0	17.0
Serine	4,4	7.7	19.8	20.8
Glutamic Acid	36.2	35.0	31.7	87.7
Proline	127.6	87.7	150.5	130.0
Glycine	203.0	220.0	274.0	330.0
Alanine	311.3	400.9	125.7	140.0
Valine	156.0	120.5	217.9	112.0
Isoleucine	25.8	17.5	41.3	25.0
Leucine	58.0	40.0	77.3	50.0
Tyrosine	7.8	9.3	8.0	10.3
Phenylalanine	33.2	27.5	37.3	22.5
Lysine	3.7	7.4	1.1	10.0
Arginine	6.3	5.1	-	-
Isodesmosine	4.0	3.0	-	-
Desmosine	6.0	7.2	-	-

ND = not determined

FIGURE 16

Acrylamide gel electrophoretic patterns of isolated fragments of elastin (from Sephadex gel filtration) stained with orcein.

(a) F_1 (from G100) using 8% acrylamide gel

(b) F_2 (from G25) using 10% acrylamide gel

FIG 16

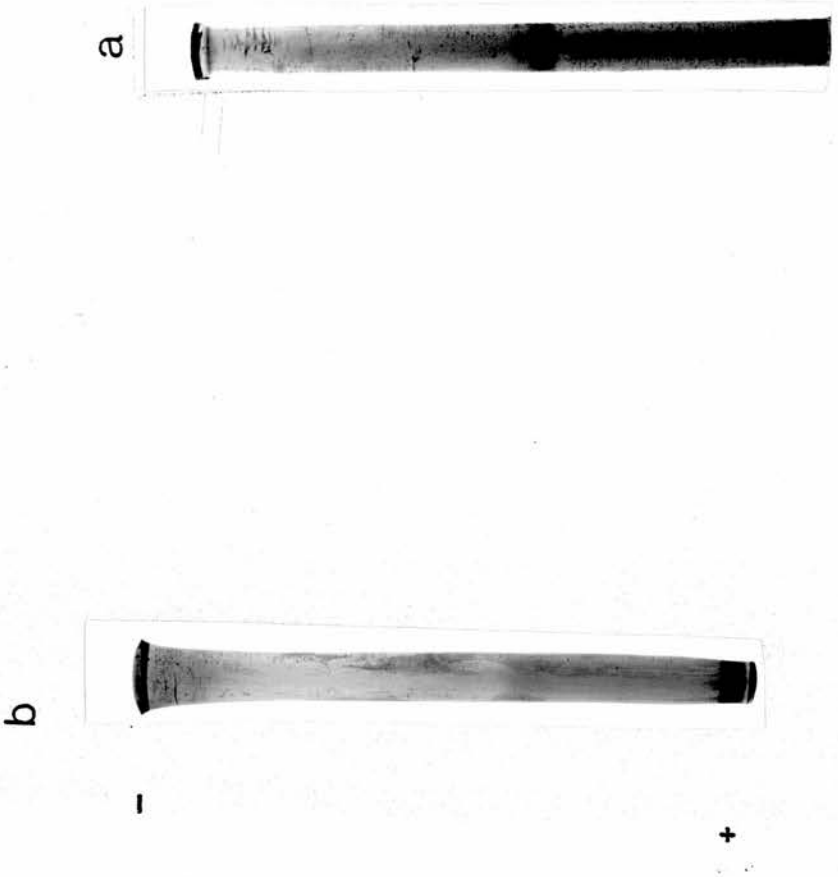


FIGURE 17

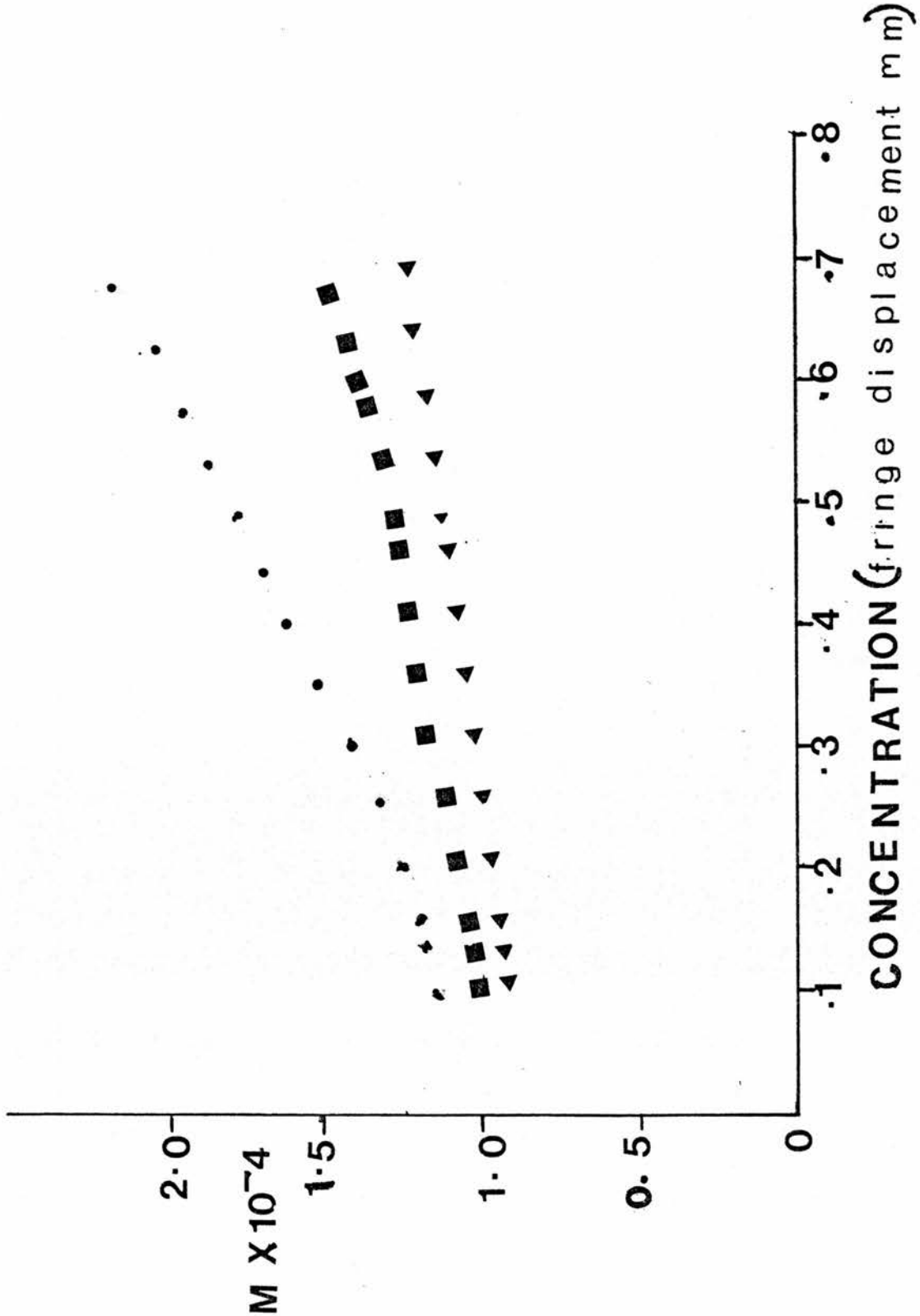
Plot of molecular weight averages versus concentration (fringe displacement) in the cell.

(◀) : Mn

(■) : Mw

(●) : Mz

FIG 17


M X10⁻⁴
2·0
1·5
1·0
0·5
0
·1
·2
·3
·4
·5
·6
·7
·8
CONCENTRATION (fringe displacement mm)

S E C T I O N 4

DISCUSSION

4. DISCUSSION

Many attempts have been made in recent years to isolate elastin in an undegraded form (Miller & Fullmer 1966; Ross & Bornstein 1969; Richmond 1974; Rasmussen et al 1975; Serafini-Fracassini et al 1975). In the work under consideration bovine aortic elastin was isolated employing the method of Serafini-Fracassini et al (1975). The amino acid composition of the elastin obtained here is shown in Table 4A and is similar to that previously described for elastin from bovine aorta (Table 1). Tryptophan, histidine and the sulphur containing amino acids are absent. However, there is a slight increase in the desmosine and isodesmosine crosslinks. This is probably due to the presence of tetrahydrodesmosine and dihydrodesmosine (Paz et al 1971,1974), since it has been pointed out (Sandberg 1976) that the desmosine peak seen in a chromatogram is a mixture of desmosine and some of these reduced analogues.

In this work tyrosine and serine have been chosen for chemical modification because of their position in the primary sequence of elastin. Tyrosine follows the lysine residues which provide the nitrogen atom of the pyridine ring(sandberg 1976, Sandberg et al 1972, Foster et al 1974),while serine does not exhibit any special relationship withthe crosslinking sites. From the data in table 6, it is however apparent in all but two of the tryptic peptides of the tropoelastin, which represent the intercrosslink segments of mature protein, that only one of these amino acids is present. Consequently, modification of tyrosine and serine should afford maximum cleavage.

Attempts to solubilize elastin using N-bromosuccinimide (NBS) alone were not successful although the cleavage of tyrosine was indicated by the absence of these residues in amino acid analysis(Table 4B). NBS has been shown to be effective in cleaving at tyrosine residues (Schmir et al 1961) according to the mechanism detailed in 2.6.1. Under the condition of the experiment, it does not react with other

Table 6

AMINO ACID COMPOSITIONS OF PURIFIED TRYPTIC PEPTIDES FROM TROPOELASTIN.[a]

AMINO ACIDS	PEPTIDES																
	T1	T2	T4	T6	T7a	T7b	T9a	T9b	T9c	T10	T12	T13a	T13b	T14a	T14b	T14c	T15
LYS	1.0	1.0	2.8	1.0	1.0	1.0	1.3	1.3	1.0	1.2		1.0	1.7	1.0	1.0	0.9	2.8
ARG											1.1						
HYP	2.4				0.7						1.0				0.7		
ASX									1.2	2.8							1.0
THR				1.0	1.0	2.6				0.9	1.3		0.9			1.9	
SER	0.6			0.9	0.8			0.8		1.7			0.9				
GLX		1.0	0.8	1.0	1.1	1.1		1.0		1.0			1.2		2.0		
PRO	10.7	9.3	12.1	1.7	2.6	1.0	5.1	2.8	1.9	3.3	4.1		2.4		6.0	3.1	9.8
GLY	21.6	20.2	34.7	15.4	23.9	4.4	14.9	13.1	10.8	7.1	8.3		15.2		12.2	4.7	33.0
ALA	9.1	12.8	33.1	8.8	14.8	8.4	12.9	9.2	7.6	6.8	0.8	2.7	8.0	2.0	6.0		20.0
VAL	16.2	10.0	14.1	5.6	7.6	1.3	6.1	3.5	2.9	3.0	5.0		6.1		3.4	4.4	8.0
ILE		3.8	5.2		1.0		3.4		0.9	0.6			(0.3)		1.8		
LEU	1.0	1.0	3.9	3.1	1.4		1.2	2.0	2.0	3.9	1.8		2.8		1.6	0.9	7.5
TYR	0.5		1.5			0.5	0.7		1.1	(0.2)	1.1				1.0		0.8
PHE	0.8	0.9	1.2		3.1			1.8		0.6			(0.3)		2.0	1.2	3.8

[a]VALUES ARE EXPRESSED AS RESIDUES PER PEPTIDE. A SPACE INDICATES LESS THAN 0.2 RESIDUE. RESIDUES IN PARENTHESES ARE FRACTIONAL RESIDUES THOUGHT TO BE IMPURITIES.

(Foster et al 1973b)

amino acids except tryptophan. However, the absence of this amino acid in elastin meant that NBS could be used to specifically modify tyrosine residues. Since its introduction(Schmir et al 1959), NBS has been extensively used in the fragmentation of proteins and peptides through the modification of tyrosine residues(Corey & Haefele 1959; Eylar &Hashim 1969; Schmir et al al 1959). However, the inability of this reagent to solubilise elastin can be attributed to the position of the tyrosine residue in relation to the desmosines, and the splitting at this point would not cause the release of any peptides from the elastin network(see Fig X below. A & C are tyr near the crosslinks).

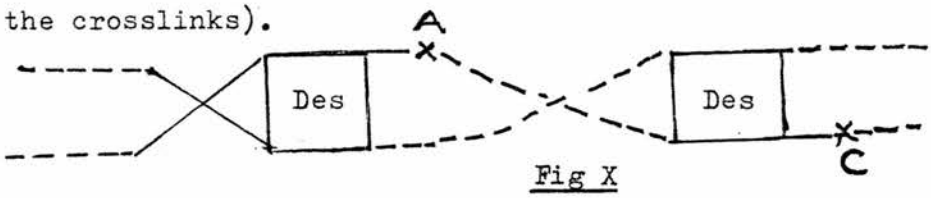


Fig X

As the modification of tyrosine residues did not result in the solubilization of elastin, another attempt was made to fragment the protein using p-toluenesulphonylfluoride(TosylF)to modify serine residues and convert them to dehydroalanine by β-elimination(2.6.2) These modified serine residues are labile and treatment with performic acid should cause cleavage of the peptide backbone at this point. However, this method did not meet with any success(see Table4C). This is rather surprising when one considers that extensive use of this compound has been made to carry out specific modification of serine to give dehydroalanine in other proteins. This has been utilised in the synthesis of cystein peptide from serine by addition of thiol to the double bond(Photaki et al 1963,1965,Zioudrou et al 1964,1965). The active site of chymotrypsin has been shown (Weiner et al 1966, Strumeyer et al 1963) to possess a serine residue. These dehydroalanine protein have been cleaved by dilute acid hydrolysis(Photaki et al 1963; Patchornik et al 1964), by performic acid oxidation followed by adjustment of pH to values greater than 10(Patchornik et al 1964) or even by heating in water at 100^{o}C. The lack of success could be attributed to the lack of penetration of tosylfluoride into the elastin network caused by steric effects since Tosylfluoride is a large molecule. However, this

explanation seems unlikely since diphenylphosphorylchloride (DPPCl), an even larger molecule, was successfully used to modify the protein. The inertness of elastin to tosylfluoride might be explained by the fact that this reagent is relatively hydrophilic and therefore could not penetrate the elastin molecule, while diphenylphosphorylchloride, which is highly hydrophobic, could penetrate. The high affinity of elastin for hydrophobic molecules has been demonstrated by its affinity for ANS and hydrophobic dye orcein, while hydrophilic substances such as coomassie blue or SDS do not bind to any great extent.

The peptides solubilized using diphenylphosphorylchloride were identified using acrylamide gel electrophoresis both with and without SDS. Staining of gels with coomassie blue was not very successful since the binding of this dye to elastin was very weak and transient. This reagent is a very sensitive stain which has been extensively used in the identification of protein separated on acrylamide gel (Weber & Osborne 1969; Weber et al 1972; Bennett et al 1971) (see also 2.8.3.1). However, its low affinity for elastin is not surprising when one considers its structure (2.8.3.1) and binding mechanism. In slightly acid medium the SO_3^- groups dissociate and combine with the NH_3^+ groups of proteins to form a complex which is stabilized by Van der Waals forces (Fazaka de St Groth et al 1963). Since elastin contains very few of NH_3^+ groups (in fact, there are only about 9 to 10 NH_3^+ groups/1000 amino acid residues) it is not surprising that it will not bind this dye. The lack of affinity between elastin and coomassie blue prompted attempts to use orcein which is a specific stain for elastin (Unna 1890). It has in fact been shown to be a mixture of closely related amphoteric dyes (Engle & Dempsey 1954; Hans Husso 1956). It has been demonstrated that the mechanism of its reaction with elastin involved hydrogen bonding (Engle & Dempsey 1954; Weiss 1954). However, it is still not clear which groups on elastin take part in the hydrogen bonding with the phenolic group of the dye. Since its discovery, orcein has been used as a specific

stain for identification of elastin (Fullmer & Lillie 1956; Lillie 1948; Mallory 1938; Kraus 1926-27).

Weiss (1954) has advanced the following reason for the specificity of this dye for elastin. He suggested that Fractions I and II (as defined by Engle and Dempsey 1954) are cationic and therefore carry positive charge in the acidic medium in which they are employed. These would be repelled by the positivly charged side chains present in most proteins at such a pH. This repulsion does not however occur in elastin as it contains very few of such charged groups. It should be emphasised here that the use of alcohol as solvent in the staining of elastin serves two purposes: it solubilises fraction I and fraction II of orcein which are specific for elastin and it excludes the binding of the dye to other proteins.

The use of orcein to locate the peptides in SDS acrylamide gel met with little success. The bands stained very faintly making it difficult to measure accurately the mobilities of the peptides. However, two bands were identified representing F_1 and F_2. The latter was located at the bottom of the gel just ahead of the tracking dye. The inability of orcein to stain elastin in SDS is not surprising if one remembers that orcein binds by hydrogen bonds. It therefore appears that the presence of SDS, a negatively charged detergent prevented the formation of such a bond. This suggestion is supported by the fact that the same fractions separated in acrylamide gel without SDS stained excellently with orcein (Fig 16).

It should be pointed out here that a pH 11.5 was employed in the electrophoretic separations to avoid aggregation of the peptides which was observed at pH below 10. This, however, created some problems because of high electrophoretic mobility at this pH both in SDS and without SDS. All separations at this pH were completed in 10 to 15 minutes. The reason for this phenomenon is not quite clear.

The lack of success in staining elastin with coomassie blue has led to the use of ANS. This stain was also used to monitor the column effluents.

As pointed out previously, (2.8.3.3), this dye fluoresces only when bound to proteins (Stryer 1965; McClure & Eldman 1966). The fluorescence is greatly enhanced as the hydrophobicity of the protein increases but is suppressed in a polar environment. ANS has proved useful in elucidation of protein structure (Stryer 1968; Winker 1962) and has been employed in the detection of conformational changes in elastin (Gosline et al 1975). It has also been used to demonstrate the existence of hydrophobic regions in elastin (Gosline 1976). The discovery that chemical or thermal denaturation, (Galley & Eldman 1967; Hartman & Undenfriend 1969; Alexander & Eldman 1965) and precipitation of protein (Nerenberg et al 1971) enhanced binding and fluorescence was utilised (Hartman & Undenfriend 1969; Nerenberg et al 1971) in the staining and identification of proteins separated on acrylamide electrophoresis. Separated elastin peptides were stained in ANS as detailed in 2.8.3.3(b) and the results showed two yellow fluorescence bands under UV using an activation wavelength of 365nm. Many attempts to stain the peptides when separated in SDS gels failed. This is understandable since SDS detergent is negatively charged and therefore increases the polarity of the environment and this suppresses fluorescence.

The low absorbance of elastin at 280nm (due to absence of tryptophan, low content of tyrosine and weak absorbance of phenylalanine) rendered UV monitoring of column effluents useless. However, the use of ANS in the monitoring of column effluent proved successful. The maximum activation emission wavelengths of ANS-elastin complexes were determined as described in 2.8.3.3(a). The fluorescence maximum of ANS-elastin complexes is at 455nm and that of ANS in aqueous solution (although very weak) is observed at 505 to 510nm. The activation wavelength of ANS is at 365nm. These figures agree with those given by Chen et al (1969). However, the authors did not state the activation wavelength for the elastin-ANS complexes which was found here to be 380nm. It has to be added here that although ANS

fluoresces in aqueous or polar environment the emission wavelength is displaced far into the red, hence the emission peak found at 725nm. These spectra are shown in Figures 10 - 12.

The discrepancy between themolecular weight values obtained by SDS acrylamide gel electrophoresis and ultracentrifugation could be attributed to low SDS binding. It has been shown (Kagan et al 1972; Gosline 1976) that elastin binds 0.5g of SDS per gram of elastin. This low affinity of elastin to SDS could be due to the extensive crosslinking of the protein, which would restrict the binding of the detergent. It is known that proteins with disulphide crosslinks such as ribonuchease and bovine serum albumin (Pitt-River & Impiombato 1968; Reynold & Tomford 1970) do not bind appreciable amount of SDS in their native state and therefore do not migrate according to molecular weight. However, they behave ideally when reduced and denatured. Similarly introduction of artificial chemical crosslinks to proteins restricts SDS binding (Davies & Stark 1970). In each case lowered electrophoretic mobilities occur and mobility versus molecular weight relationship are no longer valid and high molecular weights are to be found. The swelling of elastin in aqueous solution which increases its size and volume may also add to the reduced mobility in SDS gel electrophoresis.

The molecular weight obtained from gel filtration is very high. Although gel filtration separates molecules according to their size, separation is also governed by the shape of the molecules. In this case the shape factor would appear to be very important. The high molecular weight values could be explained if it is assumed that F_1 is made up of several polypeptide chains each arranged in random coil fashion which extend in all directions. Another possible explanation is that F_1 consists of several peptide chains arranged in parallel to form a rod. In each case the molecule would tend to be excluded from the gel used and would behave like a protein of higher molecular weight.

The amino acid composition of the supernatant from which F_1 and F_2 were obtained was almost identical to that of purified elastin (Tables 4A and 5). The only difference being the lower content of glycine and higher content of alanine. The residue left after cleavage on the other hand was very high in glycine. Indeed the level of glycine is twice that of alanine.

The values of serine and threonine were reduced to half their original values. This is not surprising (diphenylphosphorylchloride also modifies threonine) and is in agreement with the amount of diphenylphosphoryl-ammonium salt recovered by β-elimination. This was equal to twice the amount used to modify serine. Since serine is preferentially modified, the modification of threonine seems to indicate that some of the serine residues are inaccessible to the reagent. Usually all the serine residues are modified before threonine is affected. This suggestion is supported by preliminary experiment which showed that irrespective of the duration of treatment, the same amount of serine is modified.

F_1 is very rich in alanine, lysine and contains all desmosines and arginine (Table 5). The value for alanine is twice that of glycine, while glutamic acid, tyrosine and phenylalanine are present on a molar basis in the ratio of 1,1,and 2. This result shows that F_1 is a crosslinked peptide and its amino acid content is consistent with the results of sequencing carried out on crosslinked peptides, isolated from mature elastin (Foster et al 1974; Gerber & Anwar 1974; Foster et al 1973a). The main feature of crosslinked peptides previously noted is that each peptide contains one mole of serine, one mole of tyrosine, one of glutamic acid, two or three of phenylalanine and several of alanine. These amino acids tend to cluster near the area of crosslinks (Foster et al 1973a, 1974; Sandberg 1976). Composition of F_1 seems to be consistent with this.

In conclusion, this experiment has succeeded in solubilizing elastin by specific chemical modification of serine residues with diphenylphosphoryl-

chloride producing two fragments. The serine residues which were not modified seem to be hidden in the elastin network and inaccessible to the reagent.

However, the number of polypeptide chains in each fragment was not determined and no attempt was made to sequence any part of the peptides. These will be left for future research. It will also be useful to investigate further the reason for the inactivity of the p-toluenesulphonylfluoride towards elastin, in spite of its extensive documentation and the reason for high electrophoretic mobility of protein in SDS acrylamide and acrylamide gel without SDS electrophoresis at pH above 10.

SECTION 5

REFERENCES

5. REFERENCES

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