## S U M M A R Y

Components of bovine ligamentum nuchae and aortic tissues were extracted with 5M guanidine to remove most of the soluble collagen, proteoglycan and glycoproteins. Further extractions with dithiothreitol in guanidine selectively removed the microfibrillins. These extracts were further purified by gelfiltration on Sepharose CL4B in 6M urea - 0.02M EDTA - 0.2 percent SDS at pH 7.0. Gel electrophoresis of each extract, in the presence of sodium dodecylsulphate revealed single protein bands. Equilibrium sedimentation analysis carried out on the carboxymethylated materials indicated a molecular weight of about 30000 daltons. The isolated microfibrillin preparations were rich in dicarboxylic amino acids, contains galactose, glucose, mannose, N-acetylgalactosamine, N-acetylglucosamine and sialic acid. It was observed by electron microscopy that non carboxymethylated microfbillin tend to aggregate after the removal of guanidine by dialysis.

# CHARACTERIZATION OF MICROFIBRILLIN OF BOVINE LIGAMENTUM NUCHAE AND AORTIC TISSUES

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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Th 9119

# D E C L A R A T I O N

I hereby declare that the following thesis is based on work carried out by me with the exclusion of molecular weight estimation by Ultracentrifugation and electron microscopy investigations which have been carried out by other members of the group. A summary of these results has however been included in the thesis for completeness. The research was conducted in the Department of Biochemistry, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr. A. Serafini-Fracassini.

# C E R T I F I C A T E

I hereby certify that Raymond Akinwumi Iroko has spent seven terms engaged in research work under my direction and that he has fulfilled the conditions of Ordinance No. 51 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 thanks to Dr. A. Serafini-Fracassini for his constant supervision, help and encouragement during this research. I am indebted to Professor G.R. Tristram for his help in obtaining my financial assistance from the Nigerian Government.

My thanks also go to Dr. M. Field and the team for their practical advice; to Mr. J. Hunter for operating the amino acid analyser and Mr. W. Blyth for photographic assistance.

DEDICATION.

I dedicate this thesis with love to my wife Adekemi and the children for withstanding the headaches and hardship caused by me during this course of study in St. Andrews.

## Abbreviations.

| | |
|---|---|
| PMSF: | Phenylmethyl sulfonyl flouride |
| NEM: | N-ethylmaleimide |
| EDTA: | Ethylenediaminetetra acetic acid disodium salt. |
| D.T.T.: | Dithiothreitol |
| SDS: | Sodium dodecyl sulphate |
| TEMED: | N N N' N' tetramethylethylenediamine |
| TRIS: | Tris hydroxymethylaminomethane |
| GuHCl: | Guanidine Hydrochloride |
| G.L.C.: | Gas-liquid chromatography. |

# C O N T E N T S

# SECTION ONE

# I N T R O D U C T I O N

Elastic fibres in vertebrates have long been recognised by Histologists to be one of the principal constituents of the connective tissue especially in those areas that exhibit elastomeric properties such as the elastic ligaments, arterial walls and skin. The elastic tissues are made up of three fibrous proteins namely, elastin, collagen and the microfibrillar component. The elastic fibres are filamentous, highly refractive and fluorescent. They stain selectively with phenolic dyes to give a red colour when viewed under light microscope. However, upon examination by electron microscopy in thin tissue sections, the elastic fibre have been shown to consist of two morphologically distinct components: a central amorphous core of elastin surrounded by an envelope of microfibrils which are approximately 10-12 nm in diameter (Usuku, 1958; Fahrenbach et al., 1966; Greenlee et al., 1966; Ross, 1973). The microfibrils have an affinity for cationic stains such as lead, osmium and uranyl acetate, whereas the amorphous component has an affinity for anionic stains like phosphostungstic acid.

During the embryogenesis of elastic tissues the first observable structure that can be identified under electron microscopy are aggregates of microfibrils in parallel array (Karrer, 1961; Low, 1962; Greenlee et al., 1966; Fahrenbach et al., 1966). Bundles of these fibrils which appear tubular in profile are first found in direct contact with, or in close proximity to the outer surface of both fibroblasts and smooth muscle cells. At a later stage, elastin appears, in the form of an amorphous material, in several discrete areas within each bundle. As more elastin is deposited in the central position, the microfibrils are displaced to the periphery, so that at maturity the elastic fibre which is several microns in diameter consist largely of the amorphous elastin core with a relatively thin envelope of the microfibrils (Haust, Mare, Bahs and Beniosine, 1965;

Fig. 1.

Longitudinal section of Ligamentum Nuchae showing elastin and collagen in close association with microfibrillin.

A. Elastin

B. Microfibrillar component

C. Collagen.

Staining: Uranyl Acetate and lead Citrate. x 38000.


A
B
C

Greenlee et al., 1967; Ross and Bornstein, 1969). In addition, collagen fibrils are found intimately associated with the surface of each elastic fibre (Fig. 1). This very close structural and morphological relationship poses serious problems in the isolation and characterisation of any of these three fibrous proteins.

Most chemical studies of elastic tissues have been made on elastin preparations isolated by alkaline treatment (Lansing et al., 1952) or by thermal treatment (Partridge et al., 1955) or by a combination of these two techniques with very few chemical studies reported on the microfibrils. This is due to the inherent difficulties in the isolation and purification of this protein. Among the problems encountered is the treatment of the tissue with extremes of temperature or pH, which are bound to catalyse the hydrolysis of labile peptide bonds and alter the secondary structure of the protein. This in effect renders any chemical and physio-chemical data derived from such preparations dubious.

In 1969 Ross and Bornstein demonstrated that microfibrils can be selectively cleaved by enzymes such as trypsin or chymotrypsin, or released in soluble macromolecular form by reduction and alkylation in the presence of denaturing agents. Serafini-Fracassini et al., 1975 isolated microfibril using a similar procedure as Ross and Bornstein but utilising collagenase purified by affinity chromatography to remove contaminating collagen. The tissue was first exhaustively extracted with guanidine solution, which removes collagen and glycoproteins, the microfibril then being solubilised by treatment with dithiothreitol under nitrogen.

In the quest for information on the structure and chemical composition of microfibrils, Muir et al., 1976 turned to tissue culture. These workers isolated and characterised newly synthesized microfibrils by studying the protein secreted by cultured arterial smooth-muscle cells

Table 1.

Amino Acid Composition of Elastin Microfibrillins (Residues/1000)

| AMINO ACIDS: | LIGAMENT (a) ELASTIN | NUCHAE (b) MICRO-FIBRIL | (c) MICROFIBRIL FIBRIL | Monkey Cell Culture MICRO-FIBRIL (d) | Pig Aort MICROFIBRIL FIBRIL (e) |
|---|---|---|---|---|---|
| HYDROXYPROLINE | 10.7 | - | - | | |
| ASPARTIC ACID | 6.4 | 114 | 105.8 | 87.6 | 94.9 |
| THREONINE | 8.9 | 55.9 | 59.1 | 75.3 | 54.1 |
| SERINE | 9.9 | 62.2 | 77.6 | 101.0 | 66.0 |
| GLUTAMIC ACID | 15.0 | 114.0 | 101.8 | 130.0 | 136.4 |
| PROLINE | 120.0 | 63.5 | 43.9 | 69.9 | 62.3 |
| GLYCINE | 324.0 | 110.0 | 82.4 | 131.0 | 104.2 |
| ALANINE | 223.0 | 65.1 | 66.8 | 65.5 | 79.1 |
| CYSTINE/2 | 4.1 | 48.0 | 24.9 | 10.2 | - |
| VALINE | 135.0 | 56.3 | 59.1 | 61.9 | 50.9 |
| METHIONINE | - | 15.3 | 8.6 | 10.7 | 3.6 |
| ISOLEUCINE | 25.5 | 47.7 | 49.2 | 35.6 | 54.6 |
| LEUCINE | 61.1 | 68.6 | 100.5 | 57.9 | 91.0 |
| TYROSINE | 7.1 | 36.0 | 32.8 | 33.0 | 23.2 |
| PHENYLALANINE | 30.1 | 37.7 | 51.1 | 27.0 | 38.1 |
| LYSINE | 7.4 | 45.0 | 67.0 | 37.0 | 57.4 |
| HISTIDINE | 0.6 | 15.4 | 17.2 | 22.4 | 15.0 |
| TRYPTOPHAN | n.c. | n.c. | 9.7 | n.c. | 8.2 |
| ARGININE | 5.4 | 45.2 | 42.5 | 44.1 | 47.7 |
| DESMOSINE/4 | 7.9 | - | - | - | - |

Columns (a) & (b) from Ross & Bornstein (1969)

(c) from Serafini-Fracassini *et al.*, (1975)

(d) Muir *et al.*, 1976

(e) Moczar *et al.*, (1977)

from monkeys. The microfibrils were selectively removed by extraction under reducing conditions. Muir et al., (1976), isolated a protein fraction which produces a single band when subjected to gel electrophoresis in the presence of sodium dodecylsulphate. This protein had an apparent molecular weight of 270000 which they claim presumably represents a subunit of connective tissue microfibrils.

The amino acid composition of this preparation from monkey's arterial smooth-muscle cells is reported in Table 1 together with those of microfibrillin preparations from ligamentum nuchae isolated by Ross and Bornstein (1969), Serafini-Fracassini et al. (1975), and Pig Aortic extract by Moczar et al. (1970). The lack of hydroxyproline, hydroxylysine or desmosines shows that microfibrils are not related in any way to elastin or collagen, as it had been propounded by early investigators in this field. The preparations are rich in polar amino acids especially cystine (20-50 residues/1000 residues). From the data in Table 1 it is clear that the amino acid analysis of the microfibrils from various sources are similar but the detailed compositions are not identical. However, some of these differences could reflect the presence of tissue and/or species specificity.

Studies by Ross, Moczar et al., and Serafini-Fracassini, have also shown that microfibrils contain a number of carbohydrate residues including hexoses and hexosamines. The microfibrillar protein also cross-reacts immunologically with purified structural glycoprotein preparations (Robert et al., 1971). It is not yet clear, whether these represent a single species of glycoprotein or several different species. However, the investigations carried out by these workers took no account of any proteolytic degradation which could occur during extraction. Muir et al., (1976), however, carried out their extraction in the presence of inhibitors ethylene diaminoetetra acetic acid, phenylmethyl sulfonyl fluoride and

N-ethylmaleimide.

In view of the discrepancy in composition of microfibrillin preparations obtained in different laboratories the aim of this work, has been the isolation of the glycoprotein, and its characterisation under conditions which minimises proteolytic degradation.

# SECTION TWO

## ISOLATION AND PURIFICATION

## 2. ISOLATION AND PURIFICATION OF MICROFIBRILLIN

Enzymes have been used to isolate microfibrillin from elastic tissue. In this study a non-enzymic method has been used to solubilise microfibrillin by reduction and alkylation in the presence of dithiothreitol.

### 2.1. MATERIALS AND METHODS

The materials used in this study were taken from ligamentum nuchae and aortic tissue of adult cattle.

#### 2.1.1. CHEMICALS

All reagents used were Aristar or Analar grade when available and were obtained from BDH Chemicals Ltd. unless otherwise stated. Guanidine HCl from Sigma, and Dialysis tubing $^{18}/_{32}$ and $^{23}/_{32}$ obtained from Union Carbide. Phenylmethylsulfonyl fluoride was prepared as a stock solution, (50m Molar) in isopropanol and stored at 4°C. N-ethylmaleimide solution was prepared fresh just before use as this compound is unstable. Distilled water was used in all experiments.

#### 2.1.2. PURIFICATION OF GUANIDINE HYDROCHLORIDE (Nozaki 1972)

For the extraction of tissue material the practical grade Sigma guanidine was used after filteration. But for gel filteration, and ultraviolet spectrophotometer studies, it was found necessary to purify the guanidine, as biguanidine, guanylurea and related compounds were probable impurities.

Guanidine Hydrochloride (250 g.) was dissolved in 1 litre of hot absolute ethanol. It was decolourised with freshly activated charcoal and filtered through a heated large Buchner-funnel. Gradually 500 ml of benzene was added to the still hot ethanol solution, and the mixture kept in the cold (4°C) overnight before the crystalline needles were

collected and rinsed with a small amount of chilled ethanol-benzene mixture. Recrystallisation from water, was found to be unfavourable because of low yields, and the small increase in purity associated with this step.

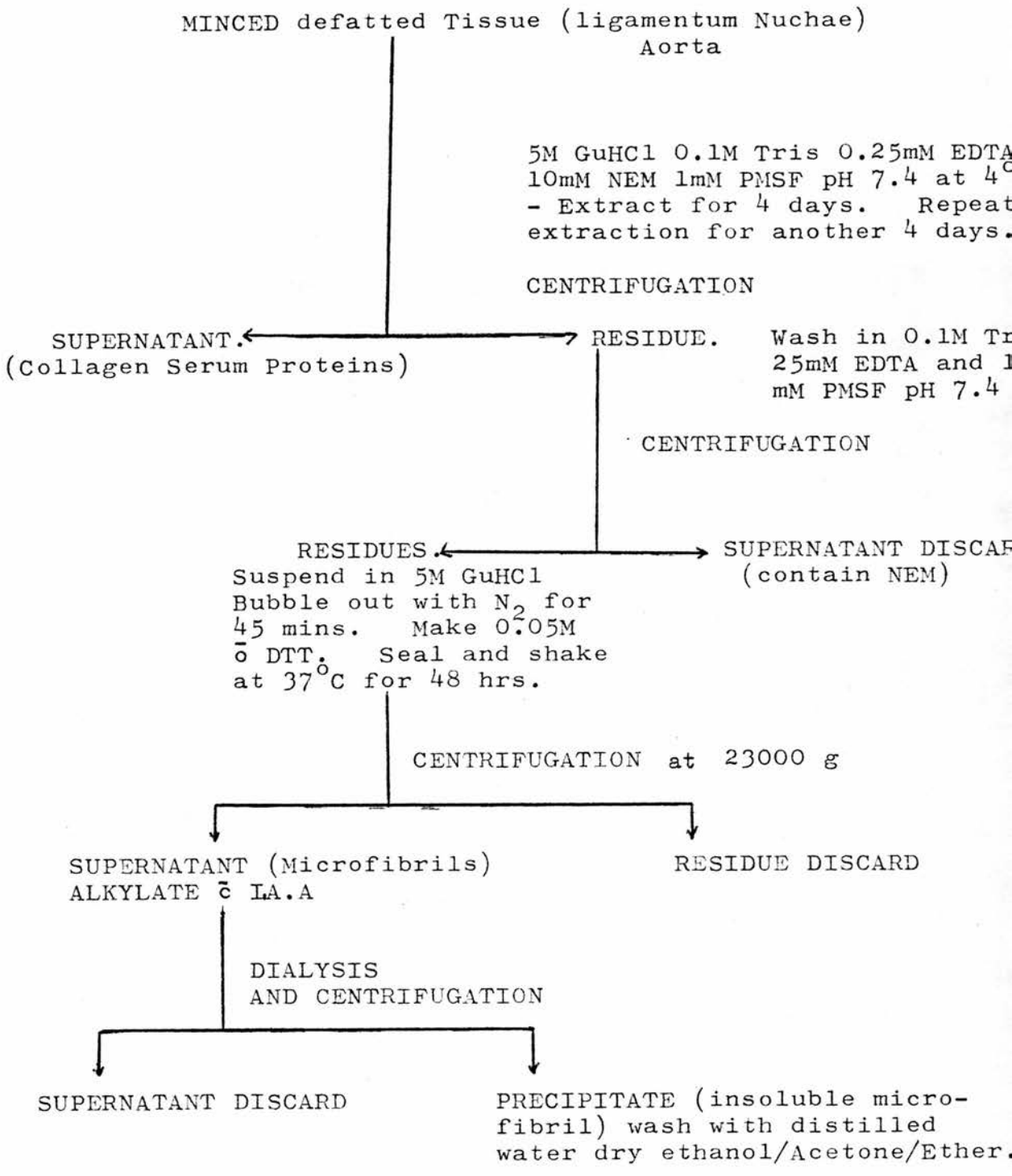
Recrystallisation from methanol was preferred; the guanidine hydrochloride recrystallised from the ethanol-benzene was dissolved in 320 ml of near boiling methanol, cooled in a dry-ice acetone mixture for several hours, and collected on a cooled Buchner funnel. The crystals were moistened with chilled methanol and drained.

## 2.2. ISOLATION OF MICROFIBRILLIN

Ligamentum nuchae and Aorta of adult cattle were removed by careful dissection at the abbatoir. The tissues were wrapped with metal foil and kept in ice en-route to the laboratory. At the laboratory the tissues were cleaned of adhering tissues, finely minced with scissors, and homogenised in a Kenwood homogeniser. (150 g wet weight of each tissue was taken and treated separately). The material was then suspended in 500 ml of 5M Guanidine, 0.1 M tris (pH 7.4) solution containing 10mM NEM, 25mM EDTA, 1mM PMSF, and extracted with continuous stirring for 4 days at 4°C. The EDTA chelates metal ions while the other two inhibitors block sulphydryl and hydroxyl groups respectively. The guanidine extracts collagen, serum proteins and proteoglycan from the tissue. The residue was collected by centrifugation, and the extraction procedure repeated in fresh guanidine solution as above.

The residue was washed several times in 5 M guanidine solution containing no NEM. The NEM needs to be removed because it would react with DTT in the next step of isolation. The suspension was degassed and placed under $N_2$ barrier. Dithiothreitol was added and (final

Fig. 2.2.1.




PROCEDURE USED FOR ISOLATION OF MACROMOLECULAR COMPONENTS OF ELASTIC FIBRE

concentration 0.05M) the container sealed with wax, and autoclave tape to ensure complete anaerobic condition. The suspension was shaken at 37°C for 48 hours after which, it was centrifuged at 23000 g for 30 mins. still under Nitrogen barrier.

2.2.2. BLOCKING THE SULPHYDRYL GROUPS

Iodoacetic acid was added in a four-fold molar excess over dithiothreitol to the supernatant, the pH was adjusted and maintained at 8.6, and the reaction mixture was stirred for 45 minutes in a light-proof container. This precaution was taken since light could cause formation of iodine which in turn could iodinate histidine, and tyrosine residues in the sample.

2.2.3. DIALYSIS OF ALKYLATED SUPERNATANT.

After the addition of a five-fold molar excess of β-mercapto-ethanol over iodoacetic acid, the solution was dialysed exhaustively against water, until a white precipitate formed within the dialysis sac. Three volumes of ethanol were added to the retentate, and the precipitate collected. The residue was dehydrated through ethanol, acetone, ether, and the weights were recorded.

2.3. CHROMATOGRAPHY ON SEPHAROSE CL4B

Pre-swollen sepharose CL4B was deaerated, and poured carefully into the column down a glass rod. The flow was started after filling the column in order to obtain an even sedimentation, then three volumes of eluant (6M urea, 0.02M EDTA 0.2% SDS pH 7.0) were passed through to stabilise, and equilibrate the gel bed. The flow rate was set at 10 ml/hour.

2.3.1. PURIFICATION OF MICROFIBRILLIN

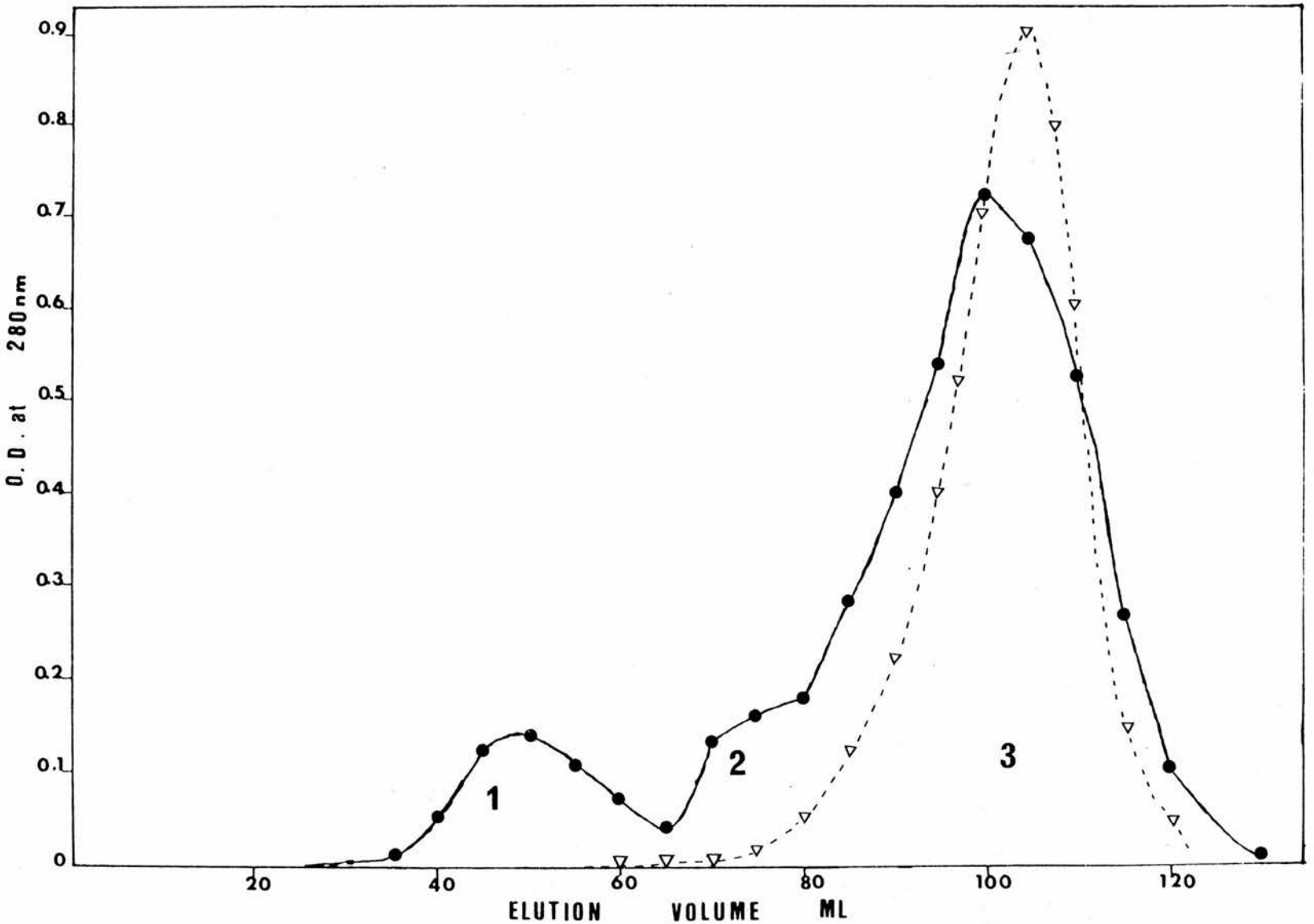
61.4 mg of each S-carboxymethylated proteins (crude sample iso-

Fig. 2.3.1.

Gelfiltration on Sepharose CL4B column of S-carboxymethylated protein extract. Elution was with 6M Urea 0.2% SDS. 0.02M EDTA pH 7.0.

●---● first chromatographic step.

▽--▽--▽ Peak. $C_3$ of the first run rechromatographed.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O. D. at 280nm
20
40
60
80
100
120
ELUTION VOLUME ML
1
2
3

lated 2.2.3 above) from Aortic tissue and ligamentum nuchae were dissolved separately in 3 ml of eluant. The solutions were allowed to stand overnight, then centrifuged to remove insoluble materials. The supernatant of ligamentum nuchae was loaded on the sepharose column and the fractions collected read at 280 nm on a spectrophotometer. The test tubes containing the trailing edge (Fig. 2.3.1), were pooled and exhaustively dialysed, against water until a white precipitate formed within the dialysis sac. The precipitate collected by centrifugation, was dissolved in 2 ml of eluant and reloaded onto the sepharose column. The fractions collected were treated as above to give the purified microfibrillin preparation. A solution of the Aortic preparation was similarly chromatographed on sepharose.

The precipitate was dehydrated, and treated with three changes in acetone to remove residual SDS that could be present in the sample. This was then hydrated again through acetone ethanol, 50% ethanol/water. The residues were freeze dried and weighed.

## 2.4. RESULTS AND DISCUSSION

The preparations from ligamentum nuchae and Aorta were isolated as S-carboxymethylated derivatives. The weights obtained from each preparation were in ligamentum nuchae 0.74 g; Aortic tissue 0.79 g. This gave 1.5% yield relative to dry weight of ligamentum nuchae, and 2.1% of the Aorta. Aliquots of these crude preparations purified on sepharose CL4B gave three fractions. The major peaks taken to dryness were 25%, 22%, of the dry crude preparations of ligamentum nuchae and aorta respectively. These results indicate that 70-80% of the preparations are mainly collagen impurities.

SECTION THREE

# DETERMINATION OF MOLECULAR WEIGHT

## 3. DETERMINATION OF MOLECULAR WEIGHTS.

Determination of the molecular weights of polypeptide chains in oligomeric proteins is an important problem. The most frequently used physicochemical method is equilibrium centrifugation in guanidine -HC1 solution. In this investigation the molecular weight determination by Dodecyl sulphate-Polyacrylamide gel electrophoresis technique has been used in addition to equilibrium centrifugation in guanidine HC1 solution.

### 3.1. BY DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

#### 3.1.1. MATERIALS AND METHODS:

Analytical grade $NaH_2PO_4.H_2O$ $Na_2HPO_4.7H_2O$, urea, sodium dodecyl sulphate, acrylamide, methylene bisacrylamide and NNN'N' tetramethyl-ethylene-diamine were obtained from BDH Chemicals Ltd. Glacial acetic acid and methanol were reagent grade chemicals. $\beta$-mercaptoethanol, ammonium persulphate were Eastman products, Bromophenol blue and Coomassie brilliant blue (R250), molecular weight markers, for SDS gel calibration (Human albumin, ovalbumin myoglobin , cytochrome C) were obtained from Mann, Orangberg N.Y.

#### 3.1.2. ALKYLATION OF STANDARD PROTEINS

1 mg protein was dissolved in 1 ml hot buffer, (0.1M tris 6M GuHCl pH 8.5) with mercaptoethanol (30 µl) and the mixture incubated at 37°C for 2 hours. This was alkylated with iodoacetic acid and the pH was adjusted to 9.0 with 2M NaOH, then raised to 10.5 with additional drops of iodoacetic acid solution. The mixture was incubated for 10 mins. and mercaptoethanol (50 µl) added to excess. The pH was adjusted to 7.0 and the solution was dialysed against 9M Urea 0.1M tris HCl; then against 0.01M sodium phosphate solutions containing 0.1% SDS.

All the standard proteins used were treated similarly.

The rechromatographed extracts from ligamentum nuchae and Aorta were dissolved in 0.01M sodium phosphate buffer and $\beta$-mercaptoethanol (30 ul) was added to each preparation. These were then incubated at 37°C together with standard protein solutions for 2 hours before application to the gels.

### 3.1.3. PREPARATION OF GELS

The gel buffer contained 7.8 g $NaH_2PO_4.H_2O$; 38.6 g $Na_2HPO_4.7H_2O$, 2 g of SDS per litre. For the 10% acrylamide solution, 22.2 g of acrylamide, and 0.6 g methylene bisacrylamide were dissolved in water to give 100 ml of solution. Insoluble material was removed by filtration through Whatman No. 1 filter paper. The solution was kept in a dark bottle at 4°C.

The glass tubes were 10 cm long with an internal diameter of 6 mm. Before use they were soaked overnight in chromic acid, rinsed and oven dried. For a typical run of 12 gels, 15 ml of gel buffer were degassed, and mixed with 13.5 ml of acrylamide solution. After further deaeration, 1.5 ml of freshly made ammonium persulphate solution (15 mg/ml) and 0.05 ml of TEMED were added. After mixing, each tube was filled with 2 ml of solution. Before the gel hardened, a few drops of water were layered on top of the gel solution. After 15 mins. an interface could be seen indicating, that the gel had solidified. Just before use, the water layer was sucked off and the tubes were placed in the electrophoresis apparatus (SHANDON).

### 3.1.4. PREPARATION OF SAMPLES

For each gel 3 ul of tracking dye (0.05% bromophenol blue in

water) 1 drop of glycerol, 5 ul of mercaptoethanol and 50 ul gel buffer were mixed in a small test tube. 30 ul of the protein solutions was added and the mixture applied to the gel. Gel buffer diluted 1:1 with water was carefully layered on top of each sample to fill the tubes. The two compartments of the electrophoresis apparatus were filled with diluted gel buffer and the run performed at a constant current of 8 ma per gel with the positive electrode in the lower chamber. Under these conditions the marker dye moved three-quarters through the gel in 4 hours. After such a time the gels were removed, from the tubes by squirting water from a syringe between the gel, and glass wall. The length of the gel, and distances moved by the dye were measured.

### 3.1.5. STAINING AND DESTAINING

The gels were stained with Coomassie brilliant blue solution for 2 hours at room temperature. (1.25 g coomassie brilliant blue R 250 dissolved in 4.54 ml 50% methanol, 4.6 ml of glacial acetic acid). The gels were rinsed with water and destained (destaining solution 7.5% Acetic acid, 5% methanol solution). Several changes of the destaining solution were made until the gel became clear again. The length of the gels after staining and the position of the blue protein bands were recorded. The mobilities were plotted against known molecular weights expressed on a semilogarithmic scale.

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length of gel after staining}} \times \frac{\text{length of gel before staining}}{\text{distance of dye migration}}$$

## 3.2. ULTRACENTRIFUGATION

The molecular weights of the S-carboxymethylated protein extracts from ligamentum nuchae and Aorta were estimated by menicus depletion

Fig. 3.1.1.4.

Determination of the molecular weight of microfibrillin from a set of 3 individual standard gels. The three marker proteins used were serum albumin ovalbumin and myoglobin.

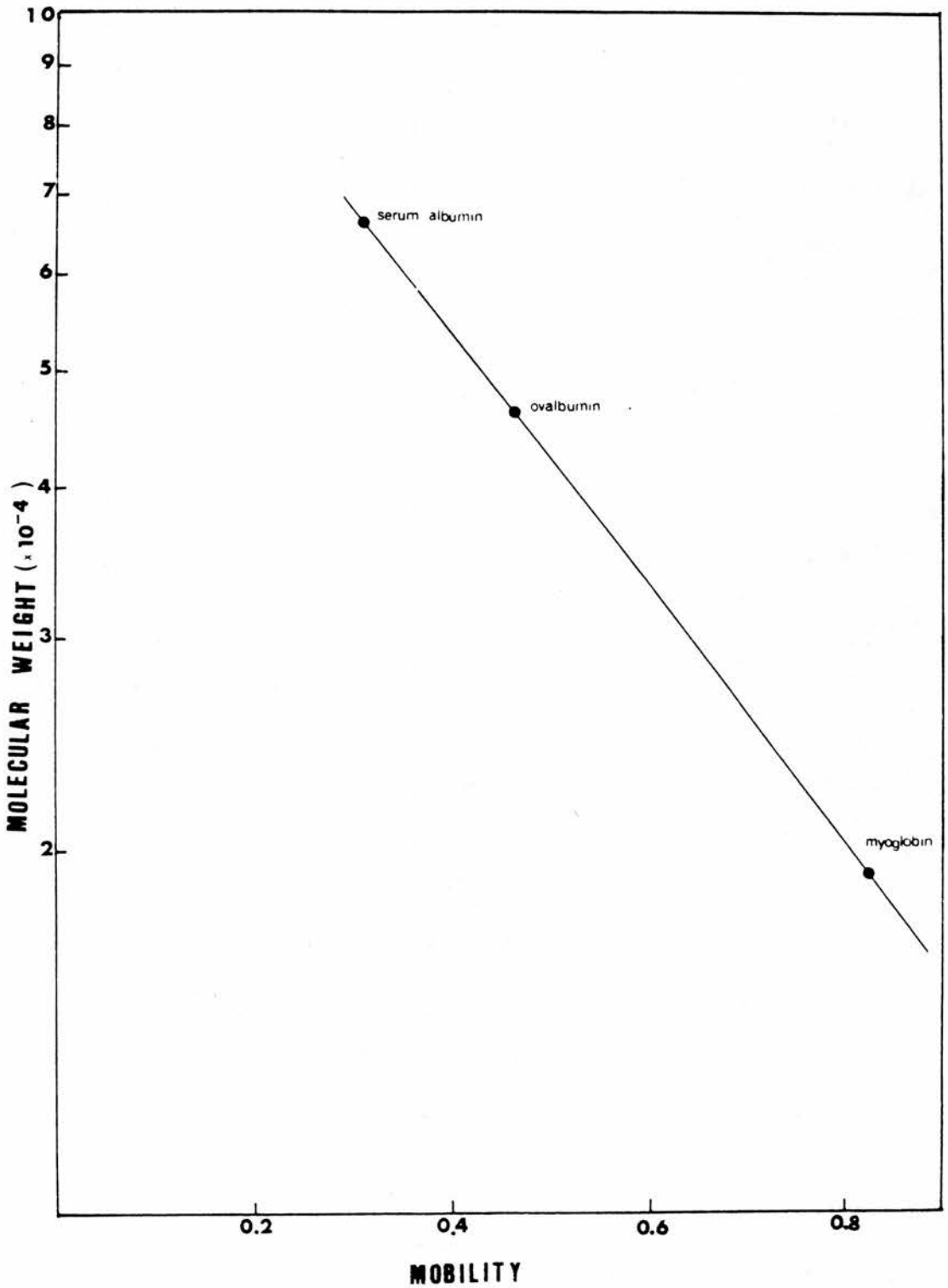
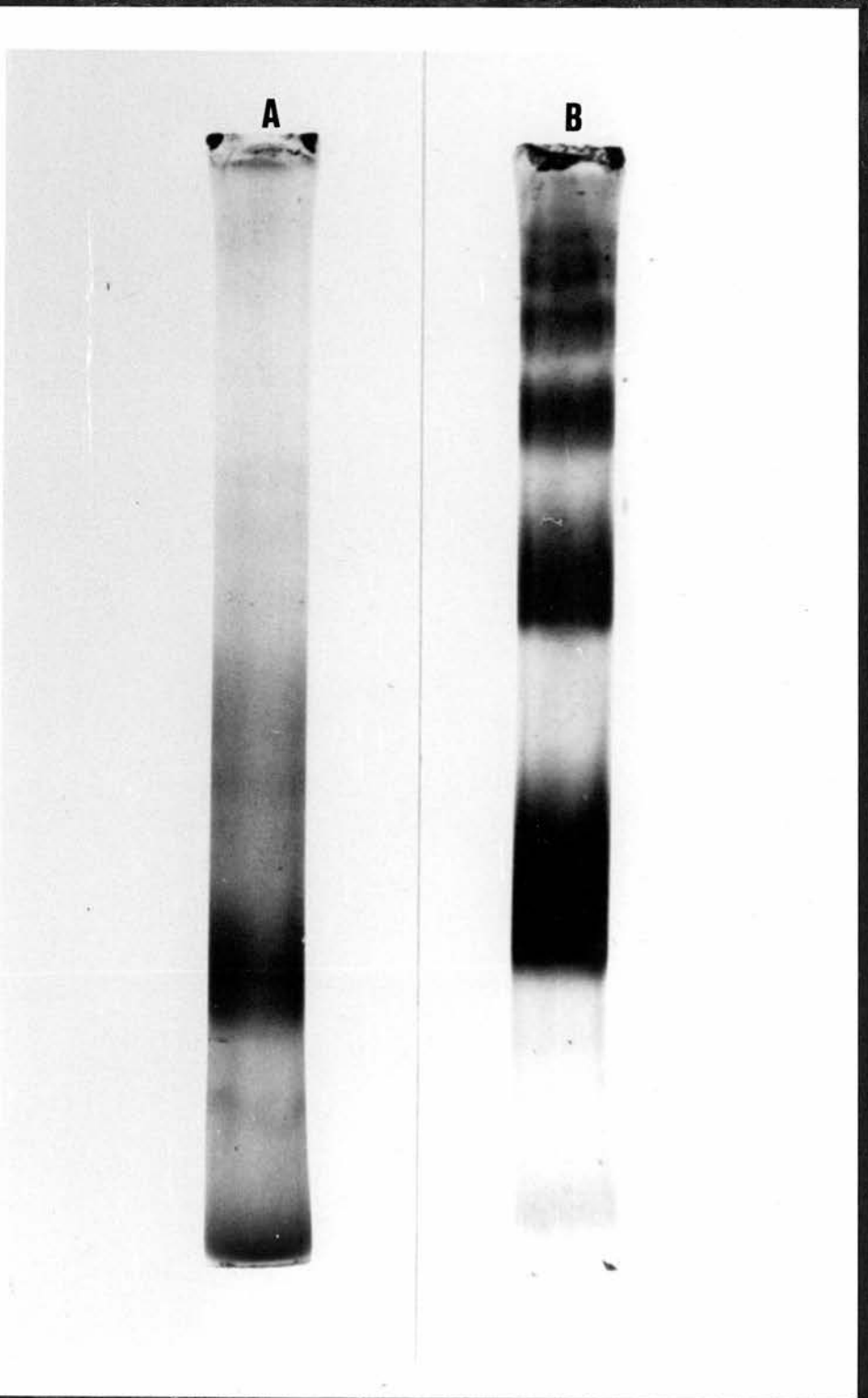

10
9
8
7
6
5
4
3
2
MOLECULAR WEIGHT ($\times 10^{-4}$)
serum albumin
ovalbumin
myoglobin
0.2
0.4
0.6
0.8
MOBILITY

Fig. 3.1.1.5.

Disc gel electrophoresis pattern of ligamentum nuchae microfibrillin. The gels were run under the same conditions, stained with coomassie brilliant blue and destained in 7.5% Acetic acid.

Gel A = Pure microfibrillin preparation from Ligamentum nuchae.

Gel B = Same preparation as A but only 50% carboxymethylated.


A
B

sedimentation equilibrium analysis (Yphantis 1964, Chervenk, 1970).

Aliquots of these preparations were dissolved in 5M guanidine 0.1M tris (pH 7.4), and dialysed exhaustively against several changes of the same buffer. The molecular weight determination was carried out, on a Spinco Model E Ultracentrifuge using Rayleigh Interference Optics. Fringe displacements were analysed, according to the procedure of Roark and Yphantis (1969). The partial specific volumes were calculated, from the amino acid compositionaldata (Zamyatnin, A.A. 1972).

## 3.3. RESULTS AND DISCUSSION

The major peak, fraction $C_3$ from ligamentum nuchae and Aorta gave single bands on electrophoresis in the presence of Urea and Sodium dodecyl sulphate (Fig. 3.1a). They gave apparent molecular weights of 38,000, and 37,000 for ligamentum nuchae and Aorta respectively. The figure 3.1. /b/ is an electrophoretic run of a ligamentum preparation that was not fully S-carboxymethylated. It shows several discrete bands of protein with increasing molecular weights. These in fact are aggregates of the single bands in figure 3.1. /a/.

The apparent molecular weights of fractions $C_3$ from ligamentum nuchae and Aorta determined by equilibrium ultracentrifugation, at an initial sample concentration of 0.3 mg $ml^{-1}$, and calculated, using partial specific volumes 0.688 ml $g^{-1}$, 0.692 $mlg^{-1}$ respectively are reported in Tables 3.2.I and 3.2. II. At vanishing concentrations the four moments selected for analysis converge in both instances to a value of about 30000 daltons. The accuracy of these extrapolations are supported by the coincidence of their intercepts with those of the ideal moment. Figures 3.2.(a) and 3.2.(b) shows graphs of concentration

against molecular weights from ultracentrifugation experiments.

It is interesting to note the differences in the results of the molecular weight determination by these two methods described above. The one explanation here is that the high molecular weight obtained in gel electrophoresis is due to the carbohydrates present in the glycoprotein preparations.

TABLE 3.2. I.

APPARENT MOLECULAR WEIGHTS - AORTA

MOMENTS AT MIDPOINT.

| | |
|---|---|
| N | 31386 ± 1046 |
| W | 33493 ± 413 |
| Z | 40463 ± 753 |
| $Y_2$ | 30675 ± 775 |

MOMENTS AT VANISHING CONCENTRATION:

| | |
|---|---|
| N | 29904 ± 1936 |
| W | 30619 ± 1936 |
| Z | 32038 ± 704 |
| $Y_2$ | 29317 ± 2205 |

TABLE 3.3. II.

APPARENT MOLECULAR WEIGHTS IN NUCHAE.

MOMENTS AT MIDPOINT.

| | |
|---|---|
| N | $34427 \pm 1244$ |
| W | $40657 \pm 509$ |
| Z | $51387 \pm 965$ |
| $Y_2$ | $31916 \pm 885$ |

MOMENTS AT VANISHING CONCENTRATION.

| | |
|---|---|
| N | $30199 \pm 1964$ |
| W | $32343 \pm 1232$ |
| Z | $36845 \pm 913$ |
| $Y_2$ | $28317 \pm 2471$ |

FIG. 3.2.(a) Aorta Preparation

Standard and Ideal moments. Curve for Aorta preparation showing Molecular weight Versus concentration.

● N = Number Average

■ W = Weight Average

**0** Z = Z Average

▲ $Y_2$ = Weight Average which has been corrected for 2nd varial coefficient.

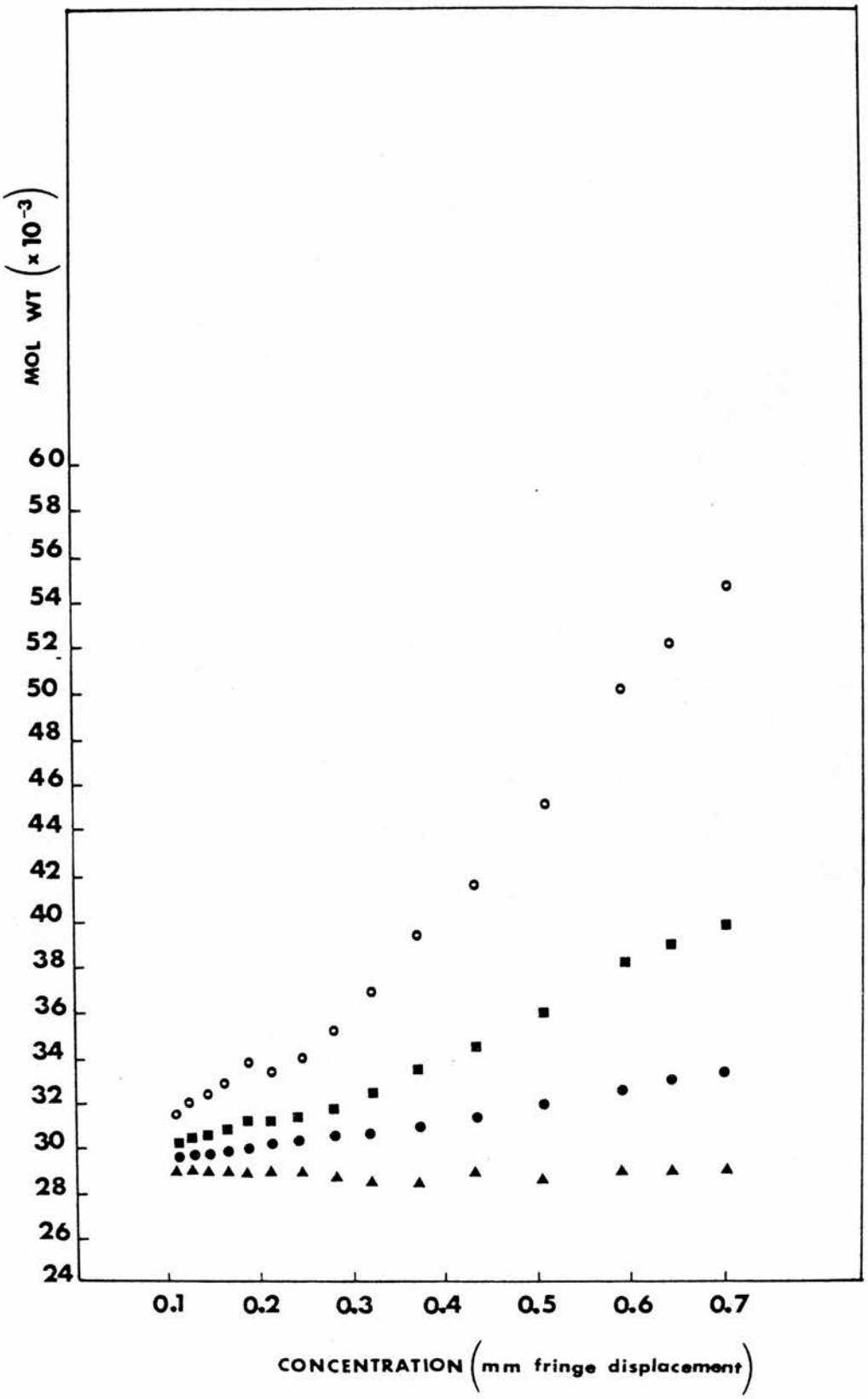

MOL WT (x 10⁻³)
60
58
56
54
52
50
48
46
44
42
40
38
36
34
32
30
28
26
24
0.1
0.2
0.3
0.4
0.5
0.6
0.7
CONCENTRATION (mm fringe displacement)

FIG. 3.2.(b).

Standard and Ideal moments curve for ligamentum nuchae microfibrillin.

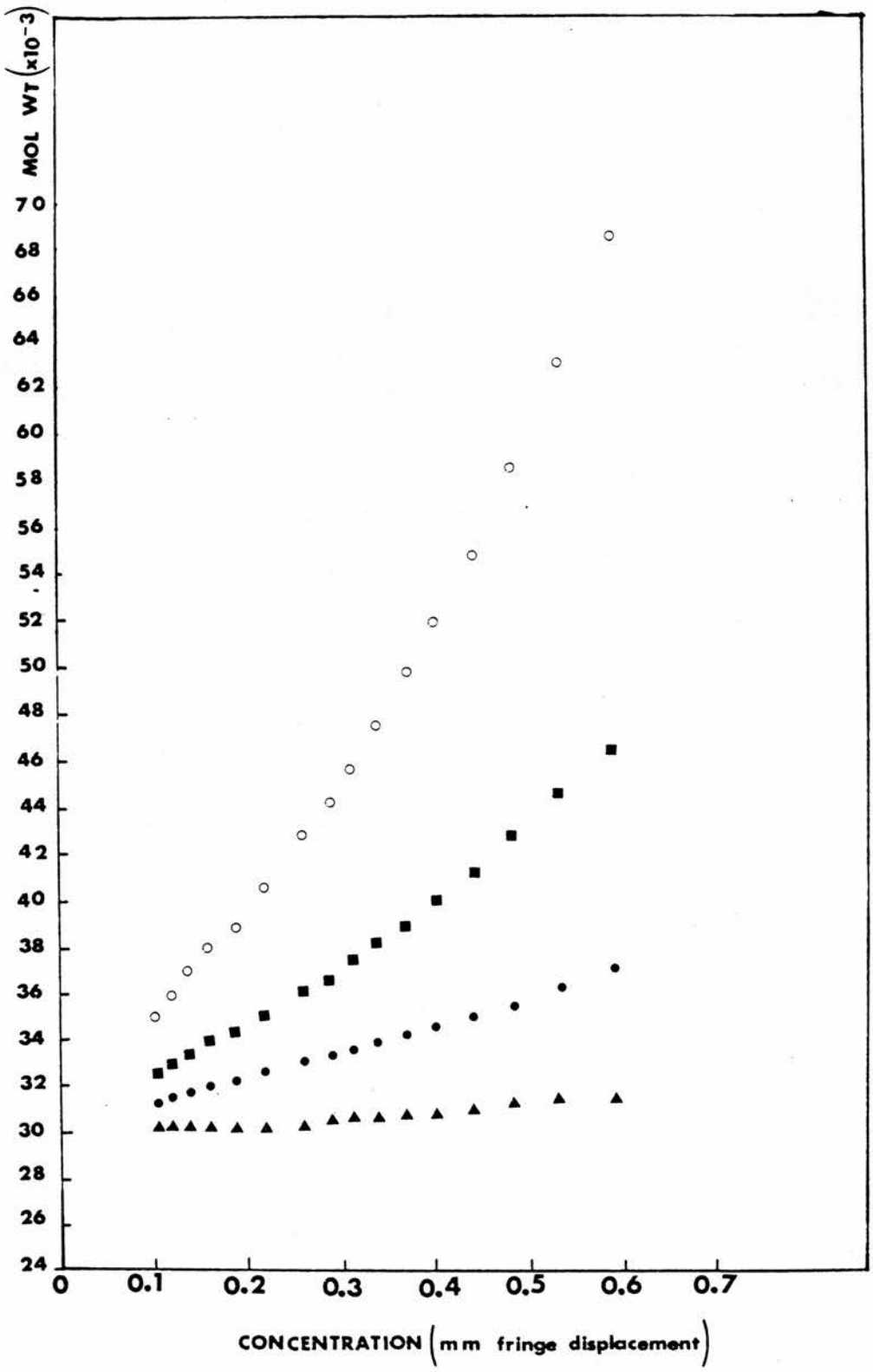
Showing Molecular weights Vs Concentration.

● N = Number Average

■ W = Weight Average

O Z = Z Average

▲ $Y_2$ = Weight Average which has been corrected for 2nd varial coefficient


MOL WT (×10⁻³)
70
68
66
64
62
60
58
56
54
52
50
48
46
44
42
40
38
36
34
32
30
28
26
24
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
CONCENTRATION (mm fringe displacement)

# SECTION FOUR

# IDENTIFICATION AND QUANTITATION OF CARBOHYDRATES

# 4. IDENTIFICATION AND QUANTITATION OF CARBOHYDRATES BY G.L.C.

## MATERIAL & METHODS.

### 4.1. MATERIALS:

All solids were dried to a constant weight in a vacuum dessicator over phosphoric oxide. All reagents were Aristar or Analar grade where available and were obtained from BDH chemicals Ltd. unless otherwise stated. N-Acetyl-glucosamine, N acetyl neuraminic acid and perseitol were obtained from Koch-light laboratories Ltd.

### 4.2. GAS CHROMATOGRAPHY

The glass columns of internal diameter 0.32 cm were used for carbohydrate analysis on the GLC (250 cm long). The support material was 3% SE30 on Diatomite CO (Pye Unicam) while the carrier gas used was Argon at a flow rate of 46 ml per min.

### 4.3. PREPARATION OF METHANOLIC HCL.

Dry methanol was prepared by adding magnesium turnings (2.5 g) and iodine (0.1 g) to 500 ml methanol. The mixture was heated for 1 hour under reflux and the dry methanol distilled into a clean container. Dry HCl gas was then slowly bubbled into the solution until saturated (1.5M).

### 4.4. TRIMETHYLSILYLATING AGENT

A mixture consisting of pyridine, trimethylchlorosilane, and hexamethyldisilazane (Ratio 5:1:1 by volume) was prepared fresh and centrifuged before use.

### 4.5. PREPARATION OF SAMPLES

0.1 ml of standard perseitol solution (0.7 μmol/ml) was put into

FIG. 4.1

Separation of microfibrillin monosaccharides subjected to gas chromatography as methyl glycosides. Peaks were numbered in their order of emergence.

| | |
|---|---|
| Mannose | 1, 3 |
| Galactose | 2,4,5 |
| Glucose | 6,7 |
| N Acetyl-glucosamine | 8,10,12,13 |
| N-acetyl Galactosamine | 9,11 |
| Perseitol (internal standard) | 14,15 |
| N-acetylneuraminic acid | 16. |

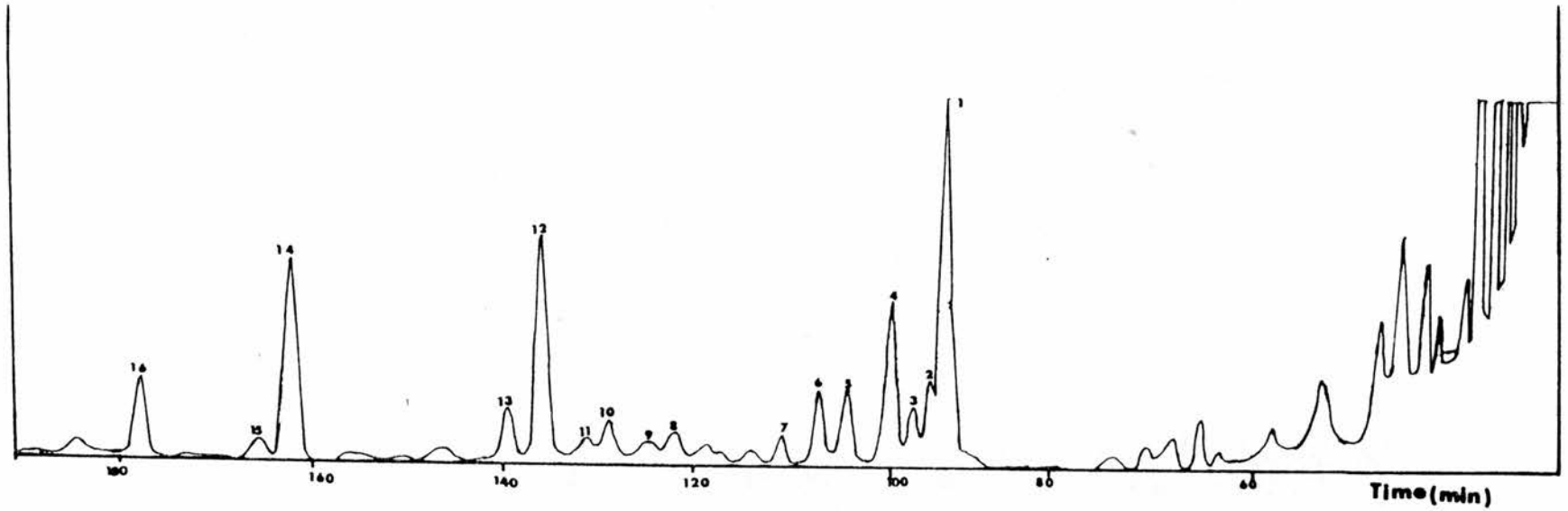
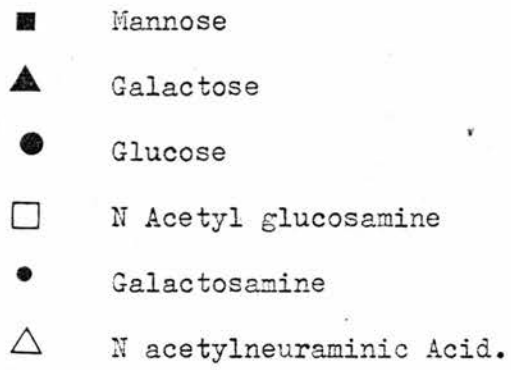
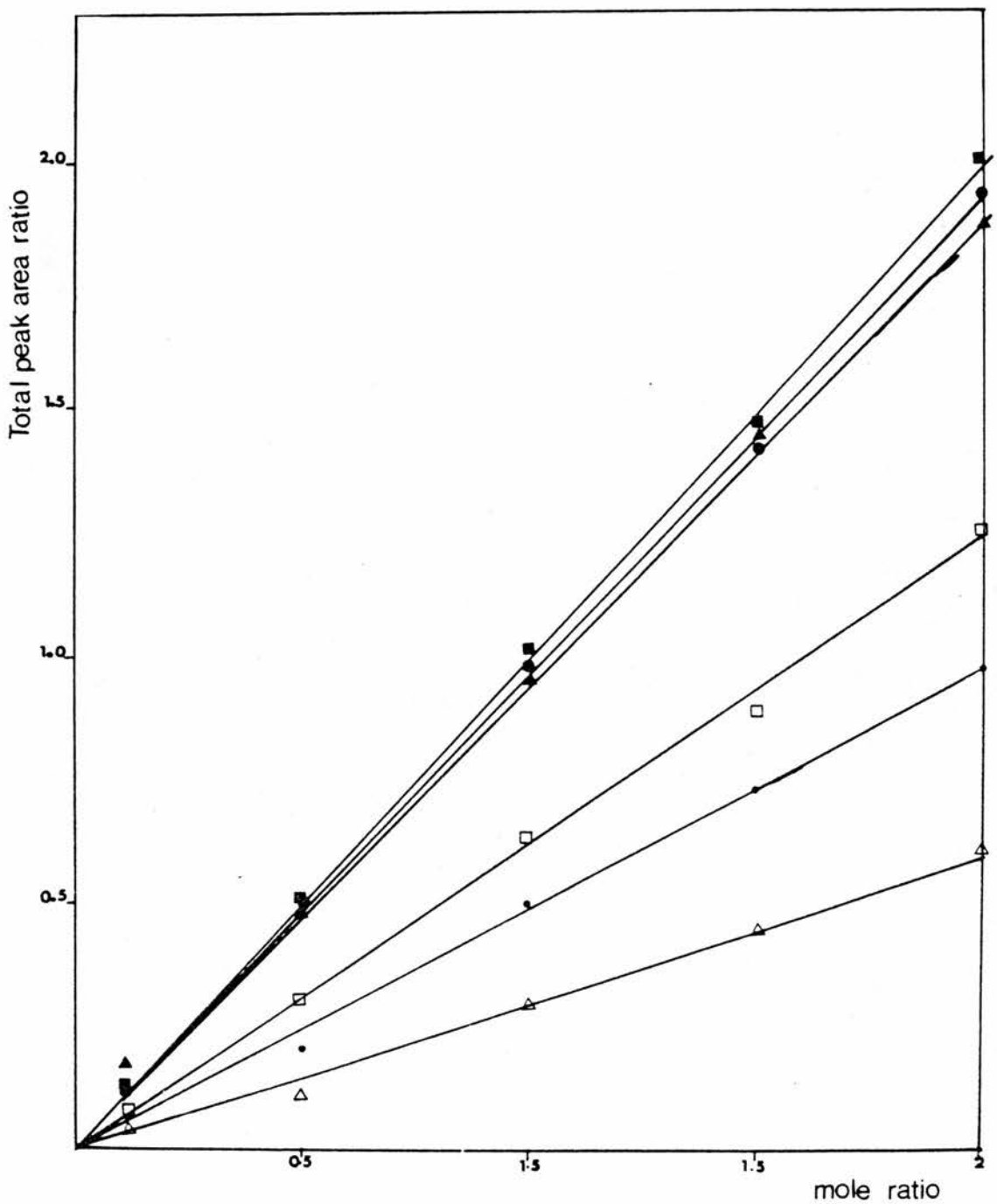

16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
160
140
120
100
80
60
Time(min)

FIG. 4.2.

Graph showing relationship of total peak area ratio to mole ratio of monosaccharides to internal standard (perseitol)




Total peak area ratio
2.0
1.5
1.0
0.5
0.5
1.5
1.5
2
mole ratio

small test tubes (chromic acid washed) and evaporated to dryness. 1.5 mg of microfibrillins from ligamentum nuchae and Aorta were accurately weighed out and added to the test tubes containing dried perseitol. The sample and standard mixtures were dried overnight in vacuum dessicator over phosphoric oxide. 0.5 ml of methanolic HCl was added to each tube and bubbled out with nitrogen. The tubes were sealed and placed in an oil bath at 90°C for 24 hours after which the acid was neutralised by the addition of solid silver carbonate. Acetic anhydride (0.05 ml) was added to each sample and the mixture kept at room temperature for 6 hours. After thorough trituration each tube was centrifuged and the supernatant transferred to a 5 ml pear-shaped flask. This procedure was repeated three times with methanol (0.5 ml). The pooled supernatants from each preparation were evaporated under reduced pressure at 35°C and placed in a dessicator over phosphoric oxide for 12 hours. 0.05 ml of trimethylsilylating agent was added to the dried material and allowed to stand for 30 mins.

After centrifugation, 5 ul of the supernatant was injected into the gas chromatographic apparatus which had been temperature programmed from 110°C to 200°C at 0.5°/min the upper limit being held until the end of the run. Quantitative results were obtained by internal standardisation technique (Bhatti, T. et al., 1970).

## 4.6. RESULTS AND DISCUSSION

Figure 4.1 shows the gas chromatograph separation of the carbohydrates present in the ligamentum nuchae and Aortic preparations. They contain three hexoses; and hexosamines; glucosamine, N acetylglucosamine; sialic acid. Fig. 4.2. also shows the calibration curve for the carbohydrates present. From the values obtained Table

5.1.(b) there are more carbohydrate residues in the ligamentum nuchae than the Aorta. This may be due to the fact that the Aortic tissue contain more cells than the ligament and with more lysosomal enzymes present, degradation of carbohydrate moieties may have occurred.

# SECTION FIVE

# AMINO ACID ANALYSIS

## 5. AMINO ACID ANALYSIS.

### 5.1. AMINO ACID ANALYZER:

2 mg aliquots of ligamentum nuchae and Aortic preparations were dissolved separately in 4 ml 6M HCl. Thioglycolic acid (4 ul) was added to each sample and these were then bubbled out with nitrogen for 10 mins. The tubes were sealed and placed in an oil bath at 110°C for 24 hours. The hydrolysates were taken to dryness by rotary evaporation at 40°C and left overnight under reduced pressure in the presence of NaOH pellets to remove HCl.

The amino acid analyses were performed on a Locarte (single column operation) amino acid analyzer. Standard colour values were obtained from the chromatography of suitable volumes of a standard solution of amino acids. Both tryptophan and hydroxyproline were assayed independently.

### 5.2. TRYPTOPHAN ESTIMATION

#### 5.2.1. SPECTROPHOTOMETRIC METHOD:

The tryptophan content of a protein can be determined from its absorption at 288 nm if the tyrosine and cystine contents are known from amino acid analysis and their absorption accounted for. So absorption measurements were taken at 288 and 280 nm.

The two preparations (ligamentum nuchae and aorta) were dissolved in 6M purified guanidine hydrochloride (pH 6.5) 0.02M phosphate buffer. With the cystine concentrations taken from the amino acid analysis, the tyrosine and tryptophan concentrations were calculated from absorbance measurements taken at 288 nm and 280 nm by using the following equations (Edelhoch, 1967).

E288 = NTrp 4815 + MTyr 385

E280 = NTrp 5690 + MTyr 1280

Where N and M are the numbers of moles of tryptophan and tyrosine per mole of protein; consequently

$$NTrp = \left(\frac{E_{288}}{3103}\right) - \left(\frac{E_{280}}{10318}\right)$$

5.2.2. AMINO ACID ANALYZER METHOD (HYDROLYSIS)

Hydrolysis was carried out in heavy-walled test tubes which had been washed with $H_2SO_4$:$HNO_3$ (3:1) rinsed in deionised water and oven-dried. 2 mg aliquot of each protein preparation (Aorta and ligamentum nuchae) were hydrolysed under vacuum at 110°C for 24 hours with 1 ml of 3N p-toluenesulfonic acid containing 0.2% tryptamine. At the end of hydrolysis, 2 ml of 1N NaOH was added to each tube and the solutions were quantitatively transferred to 5 ml volumetric flasks. The solutions were made up to mark with deionised distilled water and 1 ml of each was loaded on the amino acid analyser. Quantitations were made as before.

5.3. HYDROXYPROLINE:

The hydroxyproline content was determined by the method of Cessi and Serafini-Cessi (1964). The amino acid was oxidised to an unsaturated heterocyclic compound which was coupled with p-dimethylaminobenzaldehyde to give a coloured product. The coloured (pink) solution was read at 550 nm after 15 mins. The concentrations were calculated from standard solutions which were similarly treated. The hydroxyproline determination was done to check the purity of the preparations for the presence of collagen or elastin.

## TABLE 5.1

## COMPOSITION OF MICROFIBRILLIN

| SOURCE | LIGAMENTUM NUCHAE. | | AORTA | |
|---|---|---|---|---|
| | A. | B. | A. | B. |
| Hydroxyproline | 0.0 | | 0.0 | |
| Aspartic Acid | 106.4 | | 103.4 | |
| Threonine | 62.3 | | 63.1 | |
| Serine | 72.7 | | 67.5 | |
| Glutamic Acid | 109.7 | | 112.5 | |
| Proline | 56.8 | | 54.6 | |
| Glycine | 97.0 | | 102.0 | |
| Alanine | 64.9 | | 70.8 | |
| Valine | 52.4 | | 54.6 | |
| ½ Cystine | 24.9 | | 24.8 | |
| Methionine | 7.8 | | 9.7 | |
| Isoleucine | 43.8 | | 50.7 | |
| Leucine | 88.9 | | 81.2 | |
| Tyrosine | 41.4 | | 34.8 | |
| Phenylalanine | 39.7 | | 41.3 | |
| Tryptophan | 16.1 | | 14.9 | |
| Lysine | 44.1 | | 49.6 | |
| Histidine | 17.9 | | 15.8 | |
| Arginine | 53.2 | | 48.7 | |
| Total | 1000 | 944.2 | 1000 | 967.7 |
| Mannose | | 14.2 | | 8.2 |
| Galactose | | 9.9 | | 5.8 |
| Glucose | | 2.7 | | 3.4 |
| N-acetyl glucosamine | | 15.0 | | 6.9 |
| N-acetylgalactosamine | | 5.2 | | 5.6 |
| Sialic Acid | | 8.8 | | 2.4 |

A. Residues/1000 amino acid residues. Values are corrected f hydrolytic losses.

B. Moles/1000 moles. Values are based on the sum of the mole of the components presented in the table normalised to 1000.

## 5.4. Results and Discussion

The amino acid composition of the two preparations is reported in Table 5.1. There was no hydroxyproline or hydroxylysine indicating the purity of the preparations. The dicarboxylic amino acids make up about 1/5 of the total amino acid residues. The amino acid compositions are very similar.

# SECTION SIX

# DISCUSSION.

# D I S C U S S I O N.

It has been demonstrated that microfibrillin can be solubilised with dithiothretol in guanidine, and purified using collagenase (Ross, R. and Bornstein, P. 1969). But this preparation has much less microfibrillar elements than expected. This could be due to proteolytic degradation of the glycoproteins caused by the use of collagenase in the extraction procedure. In the present study the use of proteolytic enzyme had been avoided for the removal of contaminating collagen and in addition protease inhibitors have been used during the extraction to prevent proteolytic degradation caused by tissue enzymes.

The amino acid composition of the two preparations from ligamentum nuchae and Aorta have a broad similarity with previously published data for connective tissue structural glycoproteins. They show a high content of polar amino acids and clearly differ from collagen or elastin with the absence of hydroxyproline, hydroxylysine or Desmosenes (Tables 5A & B). However, when compared to the preparations of Ross and Bornstein reported in Table 1(b), the two preparations from ligamentum nuchae and Aorta have more threonine, leucine and tyrosine residues. The methionine content is about half the amount reported in Table 1b. The results of Serafini-Fracassini et al., (1975) are rather more similar to those obtained in this present study but there are still some substantial differences with respect to the lysine and glycine residues. The amino acid composition of the two preparations (ligamentum nuchae and Aorta) are very much in agreement with a more recent report by Moczar et al., (1977). The glycine, aspartic acid and isoleucine are identical to the Aortic preparation. The differences in other amino acids composition may be due to species specificity. This phenomenon is known to occur in many connective tissue systems. It is

worth noting that the protein moiety of microfibrillin might be considered to be genetically related to those of other connective tissue structural glycoproteins as it exhibits a similar total content of amino acids coded by the triplet X-Cytosine-X (review ref. Mathew, M.B. 1975). This fact is supported by the chemical similarities (Tables 1, 5) between the amino acid compositions of the ligamentum nuchae and Aortic preparations.

The carbo hydrate determinations done on the two preparations demonstrated the presence of N Acetyl neuraminic acid, hexoses and hexosamines (GLC profile Fig. 4.1). The values differ from those published by Ross and Bornstein. These workers found 4-7% hexoses and 0.7% for hexosamine. Values in this present investigation were 2.3% hexose, 3.6% hexosamine in the Aorta and 3.7% hexose, 6.0% hexosamine in ligamentum nuchae. It is very difficult to compare these results, with those of Ross and Bornstein because their findings were based on foetal preparation. However, the carbohydrate composition of the preparations isolated above, are in general agreement with these reported for the porcine structural glycoprotein by Moczar et al., (1977) which, was extracted by a similar procedure, to that adopted in the present study. From Table 5, it is interesting to note, that the content of the neutral sugars in the ligamentum nuchae preparation, is twice that of the aortic preparation. One possible explanation for this is the fact that the Aorta contain more cells than the ligamentum; the lysosomal enzymes may have degraded more of the carbohydrate moieties of the Aortic preparation. Dingle et al., 1966, 1969 proposed, that this does happen in the extracellular matrix, where lysosomal enzymes may induce degradation, of non-collagenous proteins and some collagen as well. Also the possibility does exist, that the normal turnover of protein polysaccharide in connective

tissue, may be controlled by hormonal action, which involves, the release, in controlled amounts from lysomes, enzymes capable of degrading extracellular glycoproteins. On the other hand, the carbohydrate moiety of microfibrillin may exhibit tissue specificity, this type of specificity having been detected in collagen. For review ref. Serafini-Fracassini and Smith (1974).

Glucose, is a sugar residue usually not present in glycoproteins. It could therefore, be argued that the glucose present in the two microfibrillin preparations, could arise from contamination from sepharose. However it should be noted, that a high glucose content was found in the crude preparations, before their application to sepharose. These values are in agreement with those reported for the carbohydrate contents, of structural glycoprotein (Moczar et al., 1977, Serafini-Fracassini et al., 1975). From the above result it follows therefore, that glucose is present in an appreciable quantity in microfibrillins.

On gel electrophoresis in the presence of Urea and SDS, the purified preparations gave single bands. The position of these bands corresponds to a molecular weight of about 38000 daltons, this value being in agreement with that reported by Moczar et al., 1977. The presence of high molecular weight material on the surface of the gel indicates, the possibility of aggregation taking place. In this respect it is therefore interesting to consider that an incomplete blocking of the sulphdryl groups may result in aggregation. This view is, in fact, supported by the observation of several discrete bands (Fig. 3.1.5(b)) of increasing molecular weights detected in the samples, in which the S-carboxylmethylation was only about 50% of the theoretical value.

A separate molecular weight assessment was carried out by

equilibrium sedimentation. Standard and ideal moments converged at vanishing concentration, to a value of 30200 and, 29800 in the case of ligamentum nuchae and Aortic microfibrillin respectively. The discrepancy between molecular weight assessments carried out by acrylamide gel electrophoresis, and equilibrium sedimentation analysis may be due to anomalous behaviour of the preparations, in acrylamide due to the presence of their carbohydrate moieties. These carbohydrates increased the size of the protein on electrophoresis hence the higher molecular weight obtained. Although acrylamide gel electrophoresis is a useful analytical tool, it is not a good method for quantitating molecular weights of macromolecular components especially glycoproteins.

With regard to the tendency of non-carboxymelthylated microfibrillin to aggregate, it is worth noting that electron microscopic examination of one such preparation, after the removal of guanidine by dialysis revealed the presence of short filaments, similar in diameter to those present in the tissue. These findings further indicate that additional research needs to be carried out on this protein as it may play an important part, in the biochemistry of connective tissue.

# APPENDIX

## 1. AMINO ACID ANALYSIS

### ION-EXCHANGE CHROMATOGRAPHY.

In this method solute molecules are sorted out by the differences in their acid-base behaviour. For this process a column (25 cm) is filled with cation exchanger which are solid granules of a sulfonated polystyrene previously equilibrated with NaOH solution in order to charge its sulfonic acid groups with $Na^+$.

To the washed $Na^+$ form of the resin an acid solution (pH 2.2) of the amino acid mixture is added. At pH2 amino acids are largely cations with net positive charge. The most basic amino acids (lysine, Arginine and Histidine) are bound tightly to the resin by electrostatic forces and the most acidic amino acids (glutamic and aspartic acids) are bound the least.

As the pH and NaCl concentration of the eluting aqueous medium are gradually increased, the amino acids move down the column at different rates and are continuously analysed quantitatively by the ninhydrin reaction. Areas of standard and sample peaks are measured and from this the concentration of sample is calculated from known standard values.

## BUFFERS USED IN LOCARTE AMINO ACID ANALYSER.

| pH | MOLARITY | VOL/LIT | CITRIC ACID | NaOH | CONC HCl | SODIUM OCTOATE | THIODI-GLYCOL | BRIJ 35 |
|---|---|---|---|---|---|---|---|---|
| 2.2 | 0.2M | 1 | 21 g | 8.4 g | 16 ml | 0.1 g | 5 ml | |
| 3.25 | 0.2M | 9 | 189.7g | 74.2 g | 99 ml | 0.9 g | 4.5 ml | 6.3 g |
| 4.25 | 0.2M | 9 | 189.7g | 74.2 g | 45 ml | 0.9 g | - | 6.3 g |
| 5.28 | 0.35M | 9 | 221 g | 129.6 | 61.2 ml | 0.9 g | - | 6.3 g |
| 6.65 | 1.0M | 9 | 882 g | SODIUM CITRATE | 13 ml | 0.9 g | - | |

## NINHYDRIN REAGENT

| | |
|---|---|
| Sodium Acetate buffer pH 5.5 | 250 ml |
| Ninhydrin | 60 g. |
| Distilled water | 125 ml |
| Stannous Chloride | 0.334 g. |

## THE NINHYDRIN REACTION

The amino acids react with ninhydrin to form carbon dioxide, an aldehyde derivative of the amino acid, and a blue pigment, (Fig. 7-1). The colour developed is read at two wavelengths 440 nm and 570 nm. The reaction is not entirely specific since ammonia also yields a blue colour. Proline and hydroxyproline give a yellow product.

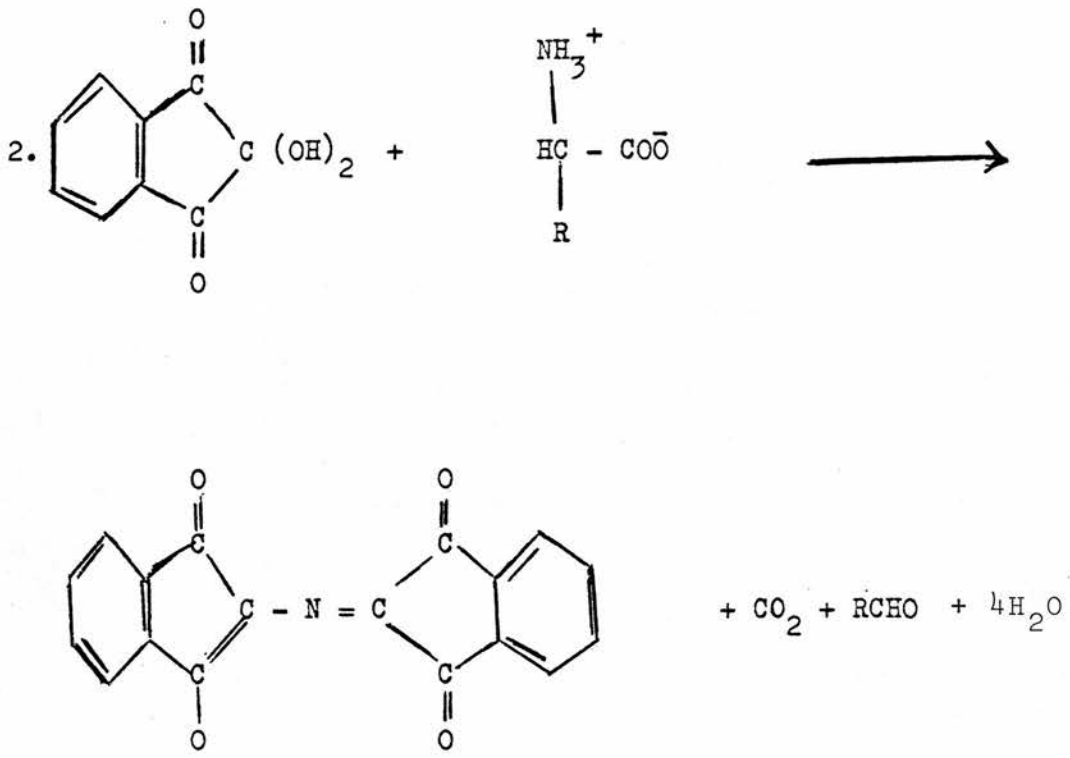
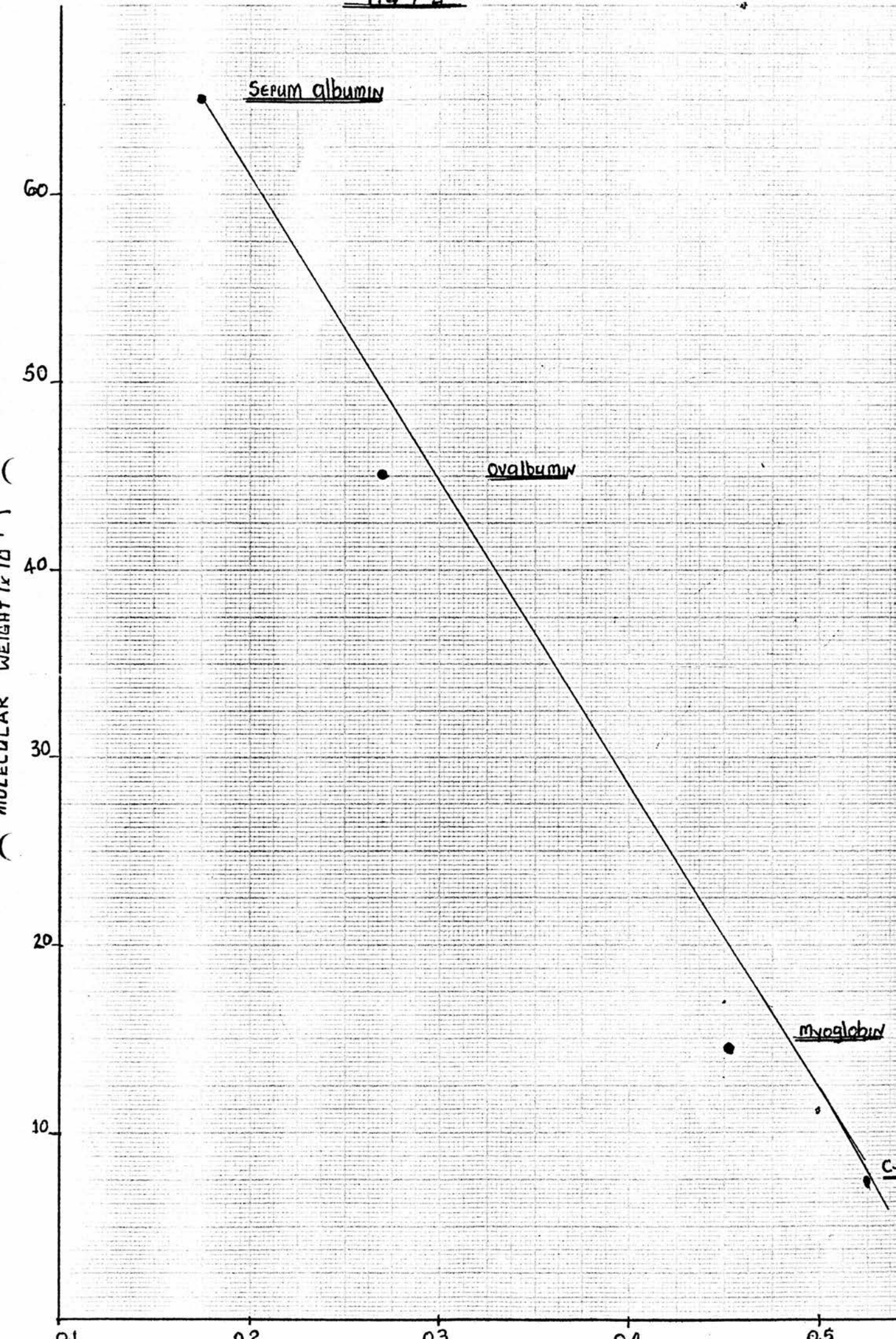


Fig. 7-1. The reaction of ninhydrin with the $\alpha$-amino group of an amino acid to form a blue pigment.


Serum albumin
ovalbumin
myoglobin
60
50
40
30
20
10
MOLECULAR WEIGHT (x 10⁻³)

2. DENSITOMETER:

Attempts were made to calculate the molecular weights by Vitatron TLD100 Densitometer. Samples of gels stained from section 3.1 were scanned on the instrument. The Vitatron TLD100 was set at wavelength 570 nm, and slit width 2.5 x 0.5mm.

The three main peaks of standard proteins and the interpreted values were plotted against the molecular weight values. As figure 7.2 illustrates there is no linearity between these standards and peak values. One possible reason for this is the gel, on the account of its composition, was difficult to destain. Similar results were obtained from the ligamentum and Aorta preparations. The calculation of molecular weight by Vitatron TLD100 densitometer was therefore abandoned.

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