CHARACTERISATION OF MICROFIBRILLIN A STRUCTURAL CONNECTIVE TISSUE GLYCOPROTEIN

A Thesis presented by

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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 been presented previously for a higher degree.

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

CERTIFICATE

I hereby certify that Nkechi I. Onyezili has spent seven terms as a matriculated post-graduate student under my direction and that she has fulfilled the conditions of Ordinance General No.12 and Resolution of the University Court 1967, No.1 and that she is qualified to submit the accompanying thesis for the Degree of Master of Science.

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ABBREVIATIONS

The following abbreviations are used.

CMC	Critical micelle concentration
DTE	Dithioexythritol
EDTA	Ethylenediaminetetraacetic acid.
GLC	Gas-liquid chromatography
GuHCl	Guanidinium hydrochloride
HMDS	Hexamethyldisilasane
NEM	N-ethylmaleimide
PAS	Periodic acid - Schiff
PMSF	Phenylmethyl sulphonylfluoride
SDS	Sodium dodecyl sulfate
TEMED	Tetramethylethylenediamine
TMCS	Trimethylchlorosilane
TMS	Trimethylsilylation
TRIS	Tris-(hydroxy-methyl) - amino ethane

Symbols:

δ	g. of detergent/g of protein
$\bar{\nu}$	true partial specific volume of the protein
ϕ	effective partial specific volume which includes the true partial specific volume and effects of interaction with detergent.
$\bar{\nu}_d$	partial specific volume of detergent when bound to the protein
ρ	density of solution
σ	Sigma moment.

CONTENTS

ABSTRACT

Minced bovine ligamentum nuchae was freed of adhering tissue and extracted in 1% w/v sodium chloride for 24hr. The tissue was then dehydrated in acetone and defatted by treatment with methanol-chloroform (3:1 by volume x 2) and (2:1, x 2) for two 24 hr. periods. Microfibrillin was extracted with guanidinium hydrochloride in the presence of proteinase inhibitors and β-mercaptoethanol. Carboxymethylation of reduced sulphydryl groups was carried out using iodoacetic acid. Sodium dodecyl sulfate acrylamide gel electrophoresis of the crude preparation showed several Coomassie blue stained bands, two of which were also PAS positive. Attempts were made to isolate the major of these two glycopeptides by Sepharose 4B-CL gel chromatography in sodium phosphate buffer (ionic strength = 0.004) containing 0.2% sodium dodecyl sulfate (pH 7.2). Sodium dodecyl sulfate acrylamide gel electrophoresis of the purified material revealed a single protein band, the apparent molecular weight which was determined to be about 35,000 using a range of 5% to 10% acrylamide concentration. Ultracentrifugation analysis carried out by sedimentation equilibrium revealed, at vanishing concentration, a molecular weight of 21,000. The amino acids, contained galactose, mannose, N-acetylglucosamine and sialic acid. It contained no hydroxyproline nor fucose.

SECTION 1

INTRODUCTION

It has been noted by several workers that both foetal and mature elastic fibrils consist of two morphologically distinct components: a centrally located amorphous core constituted of elastin surrounded by a layer of microfibrils of about 110Å in diameter (Haust, 1965; Low, 1962; Usuku, 1958; Gotte and Fracassini, 1963; Ross, 1968, 1973; Ross and Bornstein, 1969, 1970, 1971). It has therefore been suggested that microfibrillin plays an important part in the biogenesis of the elastic fibre, and indeed in foetal bovine ligamentum nuchae, elastic fibres comprise bundles of microfibrillar component lacking an amorphous elastin core. As the tissue ages elastin appears in the centre of the fibril surrounded by a sheath of microfibrillar component which comprises approximately 5-10% of total dry weight of the fibril (Ross and Bornstein, 1969).

This glycoprotein may therefore be important in aligning tropoelastin molecules prior to enzymic oxidation of lysine residues and subsequent cross-link formation (Greenlee et al., 1966; Fahrenbach et al., 1966; Ross and Bornstein, 1969 and 1971; Sandberg, 1976).

The high content of acidic residues such as sialic acid, aspartic and glutamic acids, as will be seen later, confers upon the microfibrillar component the property of an anionic polyelectrolyte and therefore it can be stained with both cationic lead and uranyl acetate.

Very few chemical studies have been reported on the microfibrillar component. This is due to the inherent

difficulties encountered in its isolation and purification. The microfibrillar component is, in fact, insoluble in solvents normally used for the isolation and purification of proteins because of the presence of numerous disulfide bridges. It has also been suggested that this difficulty in isolation and purification may partly be due to its intricate association with collagen and other macromolecules such as structural or acidic glycoproteins (Wolff et al., 1971; Anderson et al., 1972; Fracassini and Smith, 1974). The microfibrillar component also has a strong tendency to form aggregates by self-aggregation (Moczar et al., 1976; Kadar et al., 1973; Shipp and Bowness, 1975).

Ross and Bornstein isolated the microfibrillar component from bovine ligamentum nuchae, after removal of the collagen by collagenase digestion followed by cleavage of the disulfide bonds with dithioerythritol in 5M guanidine hydrochloride. The resultant supernatant was then, either alkylated or dialysed against water where two fractions were obtained. As a control, the collagenase-treated preparation was subjected to tryptic (EC 3.4.4.4) and chymotryptic (EC 3.4.4.5) digestion followed by the isolation of the microfibrillar peptides.

The author noted that this selective enzymic cleavage resulted in the formation of a degraded protein, the amino acid composition of which differed from that of the material isolated with dithioerythritol. Equally the two fractions obtained after dialysis differed in the content of several amino acid residues. These compositional discrepancies were attributed to possible contamination by small peptides resulting from partial digestion of residual collagen and/or

the elastin.

Fracassini et al., (1975) applied a method essentially identical to that of Ross and Bornstein with the exception that the collagenase used was purified by affinity chromatography to remove non-specific proteolytic activity.

Moczar et al., (1977) followed a procedure in which the microfibrillar component (from 6 month old procine aortae) was isolated and solubilised in 6M urea containing β-mercaptoethanol and ethylenediamine tetraacetic acid after a series of salt extractions intended to remove collagen, proteoglycans and other glycoproteins. The protein was isolated by dialysis against water followed by a further extraction in 4M GuHCl and dithioerythritol. The preparation was carboxamidomethylated. It still contained some contaminating collagen.

In pursuit of a homogeneous preparation, Muir et al., (1976) turned to cell culture; after several unsuccessful attempts using foetal bovine ligamentum nuchae, they cultured arterial smooth muscle cells from monkey. They isolated, under reducing conditions in the presence of proteolytic inhibitors - (PMSF, EDTA and NEM) an apparently homogeneous protein the molecular weight of which, on SDS-gel electrophoresis, was 270,000, that they claimed represented a subunit of connective tissue microfibrils. However the amino acid composition of this preparation differed from that previously determined using crude microfibrillar protein. Of particular interest in this respect was a lower cystine

content. This anomaly was attributed to tissue or species specificity.

From the amino acid composition reported on table 1, one sure conclusion can be drawn irrespective of variability and that is, the microfibrillar component is rich in acidic polar amino acids-glutamic and aspartic acids and cystine.

Investigations have also shown that the microfibrillar component contains a number of carbohydrate residues including hexoses and hexosamines (Ross and Bornstein, 1970; Moczar et al., 1976; Fracassini et al., 1975). Very little work has, however, been done on this aspect of the glycoprotein. Although the percentage by weight, and molar ratios of the residues have been quoted (table 1B) little or nothing is known either of the number of carbohydrate side chains, the microheterogeneity of these chains, if any; the isomerism of the sugars or of the type of carbohydrate-protein linkage.

The molecular weight of the carboxymethylated microfibrillin isolated by Fracassini et al., was between 14,000 and 15,000 daltons by equilibrium sedimentation analysis. The preparation exhibited a low degree of polydispersity. Although the possibility of any gross contamination is excluded by the low degree of polydispersity, it is possible that the collagenase had cleaved a fragment from the microfibrillar component, such a fragment having been subsequently removed during dialysis, and therefore leaving a larger microfibrillar "core". The carboxamidomethylated

microfibrillin isolated by Moczar and colleagues gave, in fact, an apparent molecular weight of 35,000 on SDS-gel electrophoresis. It is known that glycoproteins with $\geq$ 10% carbohydrate behave anomalously on SDS gel electrophoresis exhibiting drastically reduced electrophoretic mobility. They therefore appear to be larger than they really are and hence of higher molecular weight (Schubert, 1970 Pitt-Rivers and Impiobato, 1970). The very high apparent molecular weight (270,000) obtained for the microfibrillar subunit of Muir and colleagues, could either be an artefact or could be due to gross contamination by other tissue proteins which are expected to be found in significant amount in a culture medium.

Neither the yield nor the degree of purity of these preparations was mentioned by any of these workers. For example, a single band on a single gel concentration is not a sufficient proof of homogeneity since glycoproteins and proteins are known to exhibit certain heterogeneity on gels of different porosities.

It is, therefore, the aim of this work to attempt to isolate a homogeneous preparation of microfibrillin and to further partially characterise its chemical and physical parameters.

TABLE 1 AMINO ACID COMPOSITION OF THE MICROFIBRILLAR RESIDUES/1000

	Ross and Bornstein (1969)*				Fracassini et al (1975)	Muir et al (1976)	Moczar et al (1977)
	Enzymatic digest	Alkylated	Reduced dialysed precipitate	Reduced dialysed supernatant			
Hydroxyproline	1.7	Not calcula.	-	-	0.0	0.0	0.0
Aspartic Acid	92.5	114	114	120	105.8	87.6	94.9
Threonine	47.3	55.1	55.9	56.1	59.1	75.3	54.1
Serine	52.8	58.9	62.2	65.4	77.6	101.0	66.0
Glutamic Acid	98.3	111	114	117	101.8	130.0	136.4
Proline	73.5	70.4	63.5	68.9	43.9	69.9	62.3
Glycine	142.0	120	110	123	82.4	131.0	104.2
Alanine	82.6	58.9	65.1	49.2	66.8	65.5	79.1
½ Cystine	56.3	80.3 (a)	48.2	88.1	24.9	10.2 (b)	12.0
Valine	69.7	54.1	56.3	46.2	59.1	61.9	50.9
Methionine	13.0	15.8	15.6	15.1	8.6	10.7	3.6
Isoleucine	43.8	45.2	47.7	46.6	49.2	35.6	54.6
Leucine	65.5	57.2	68.6	50.3	100.5	57.9	91.0
Tyrosine	27.6	30.0	36.0	31.0	32.8	33.0	23.2
Phenylalanine	32.8	32.1	37.7	29.6	51.1	27.0	38.1
Lysine	36.7	36.9	45.0	30.7	67.0	37.0	57.4
Histidine	11.5	14.2	15.4	13.5	17.2	22.4	15.1
Tryptophan	11.9	Not calculd.	Not calculd.	Not calculd.	9.7	Not calculd.	8.2
Arginine	42.3	45.2	45.2	41.8	42.5	44.1	47.7
Hydroxylysine	0.7	-	-	-	0.0	0.0	0.0

* Uncorrected for hydrolytic loss. (a) Includes S-carboxymethylated cysteine. (b) Includes cysteic acid.

TABLE 1B.

CARBOHYDRATE COMPONENTS OF MICROFIBRILLIN

(Percentage by weight)

	Moczar et al (1977)	Ross and Bornstein (1969)	Fracassini et al (1975)	Muir et al (1976)
Galactose	(3)		3.5	Not stated
Mannose	3.8 (4)	4 - 7	3.0	"
Glucose				"
Glucose/Galactose	(0.15-0.3)			"
N-acetyl Glucosamine	2.8 (3)	0.7	<0.3	"
N-acetyl Galactosamine			-	"
Sialic Acid	1 (1)		Trace	"

Figures in brackets indicate molar ratios.

SECTION 2

MATERIALS AND METHODS

2.1 CHEMICALS

All reagents used were of analar or aristar grade and were obtained from BDH Chemicals Ltd. (London-England), unless otherwise stated. Sepharose CL-4B was obtained preswollen from Pharmacia Chemical Company. Guanidine hydrochloride was obtained from Sigma Chemical Company, (London-England) and purified according to Nozaki (1972). Proteolytic inhibitors: Phenylmethylsulphonyl fluoride was prepared as a stock solution (50mM) in isopropanol and stored at $4^{o}C$. N-ethylmaleimide solution was prepared fresh just before use. SDS was purified by extraction in absolute ethanol and purity was assessed by measuring the critical micellar concentration according to Birdi (1976).

Acrylamide and N-N^{1}-methyl bisacrylamide were obtained specially purified for electrophoresis from BDH.

Standard marker proteins: Bovine serum albumin, ovalbumin, lysozyme and trypsin were obtained from Sigma Chemical Co. (London-England). pH of all buffer solutions was measured on Radiometer type 25 pH meter.

Spectrophotometric measurements were performed on Unicam Instrument SP500 Series 527 (Cambridge).

2.2 PURIFICATION OF COMMERCIALLY OBTAINED GUANIDINIUM HYDROCHLORIDE (GuHCl)

Commercially available GuHCl is known to contain some impurities such as biguanidine, guanylurea and related compounds. For ultra-violet photometric and especially ultracentrifugation analyses it is necessary to remove

these contaminants. The method employed for this purpose involved slight modifications of the procedure described by Nozaki (1972).

250g of GuHCl was dissolved in 1 litre of cold ethanol and gradually heated with constant stirring in a 2 litre round-bottomed flask which had overhead stirrer and a condenser attached. The decolorisation step was omitted since it was found unnecessary. 500 ml of benzene was gradually added to the still hot ethanol solution and the mixture kept at 4°C overnight. Crystalline needles were collected and rinsed with a small amount of ethanol-benzene mixture. A yield of about 70% was obtained which agreed with that quoted by the author.

Recrystallisation was performed in 320 ml of near-boiling methanol cooled in dry ice-acetone mixture. Crystals were collected on a cooled Buchner funnel, moistened with chilled methanol and drained. Residual methanol was removed by rotary evaporation at 37°C. The yield was approximately 37% of the starting material.

2.3 PURIFICATION OF SODIUM DODECYL SULPHATE (SDS)

Some commercially available batches of SDS have been found to be impure. Assessment of purity by gas liquid chromatography revealed up to 31% of tetradecyl sulphate and 2% decyl sulphate (Birdi, 1976; Rowe and Steinhardt, 1976; Matheka et al., 1977). For molecular weight characterisation by techniques such as ultracentrifugation, presence of such large quantities of impurities are unacceptable. Tetradecyl sulphate has been known to have a higher binding capacity than SDS for unfolded proteins.

It also tends to dissociate protein into their monomers much more readily than does SDS. It was observed that it remains associated with proteins during electrophoresis at room temperature (as opposed to SDS, which within detection limits, is completely removed) thus leading to anomalous staining of proteins with pinacryptol yellow (an organic dye which forms a fluorescent complex with detergents) (Dohnal and Garvin, 1979).

20 g of commercial SDS were suspended in 200 ml of absolute alcohol and heated under reflux in a 1 litre round-bottomed flask till dissolved. The mixture was allowed to cool at room temperature, centrifuged at 23,000 r.p.m. (4°C). The residue was rinsed with about 200 ml of cold absolute ethanol, filtered through a Buchner funnel, and residual alcohol removed by rotary evaporation at 37°C.

2.4. Determination of the critical micelle concentration (CMC) (Birdi, 1976).

It is known that the log of CMC varies linearly with the alkyl hydrocarbon chain length (Tanford, 1973). Accurate CMC measurement should theoretically reflect the presence of high contents of tetradecyl and decyl sulphates.

Solutions containing 0.5 g/litre to 4.0 g/litre of SDS were prepared. 20mg of naphthalene was added to 5 ml of each sample solution in a test tube capped with parafilm and allowed to shake at room temperature for 48 hours. Excess naphthalene was removed by centrifugation at 2,000 r.p.m. and the amount of dissolved naphthalene

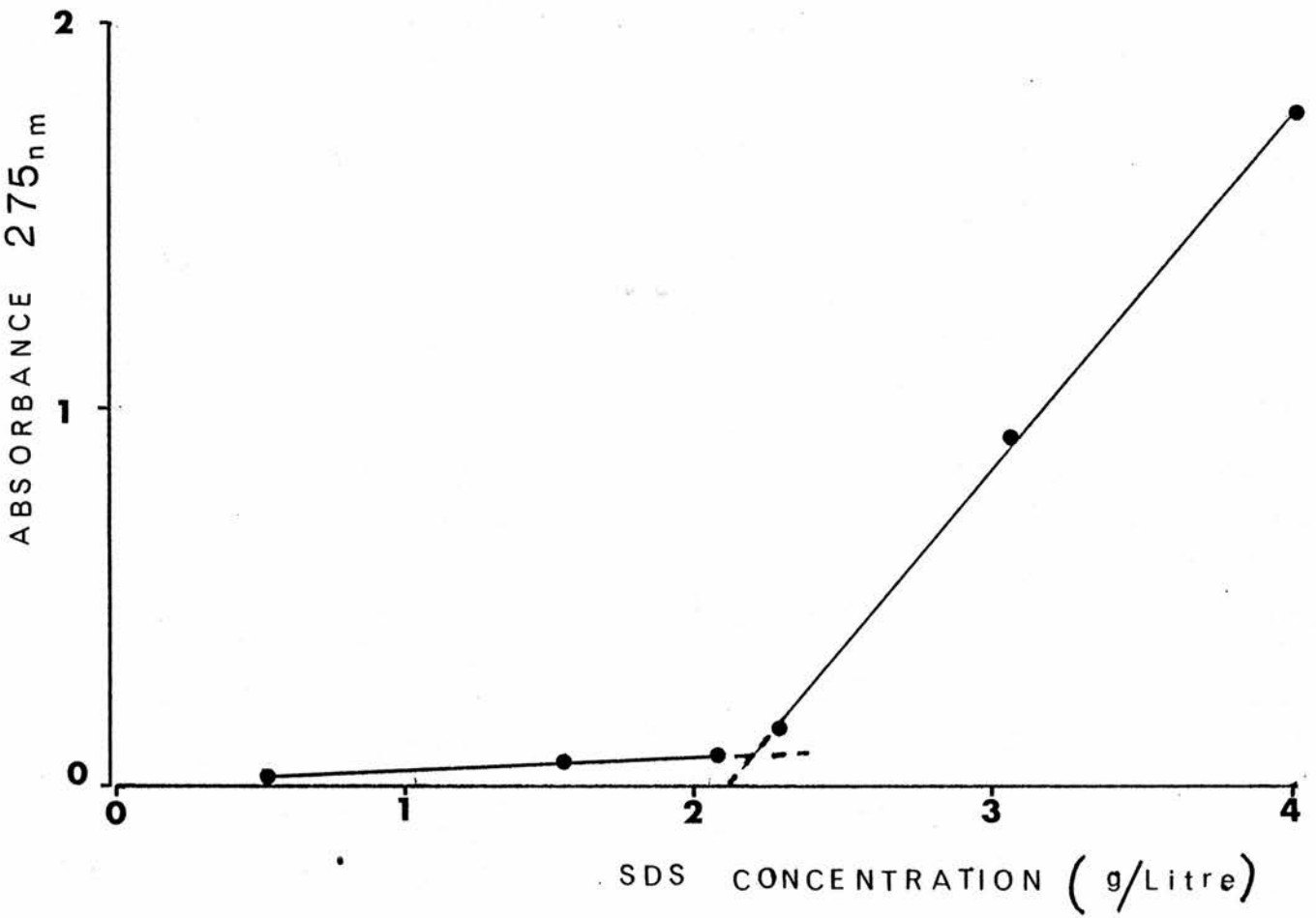

Fig. 1 Determination of the critical micelle concentration (CMC) of SDS in distilled water. CMC value = 2.13 g/litre.

determined by measuring the absorbance at 275nm. A plot of absorbance versus concentration of SDS is given in Fig. 3. The value of CMC as determined for our preparation from the break in the curve was about 2.13 g/litre which was very close to the figure of 2.3g/litre quoted by the author.

2.5 Isolation of the microfibrillar component from bovine Ligamentum Nuchae.

The ligamentum nuchae from an adult cow was freed from adhering tissue and homogenised in 1% (w/v) sodium chloride and left in same solution for 24 hours with continuous stirring to remove serum proteins and blood. It was then defatted and dehydrated in chloroform - methanol (3:1 by volume) for 2 x 24 hr. periods at 4°C followed by a second treatment in same solvent (2:1 by volume) for 2 x 24 hr. periods. The residue was allowed to dry in a fume cupboard. The dried material was suspended in 2 litres of 5M GuHCl in 0.1M Tris buffer pH 7.4 containing 25mM EDTA, 1mM Phenylmethanesulphonyl-fluoride (PMSF) and 10mM N-ethylmaleimide (NEM) (PMSF was added fresh every 12 hr. as it has a half-life of about 6 hr.). The material was extracted with continuous stirring at 4°C for 24 hr., the residue collected by centrifugation at 2,700 r.p.m. for 2 hr. at 4°C. This extraction step was repeated for another 24 hr. period. It removes proteoglycans, serum proteins and some of the

collagen. The residue was washed with 0.1M Tris buffer pH 7.4, containing 25mM EDTA, 1mM PMSF overnight at 4°C to remove NEM which might react with PME. 2 litres of 5M GuHCl in 0.1M Tris buffer pH 7.4 containing EDTA and 40 ml of β-mercaptoethanol which was at a final concentration of 0.285M, were added to the residue and the pH adjusted to 8.5 with tetramethylethylene diamine (TEMED) The mixture was then bubbled with nitrogen and left on a metabolic shaker at 37°C for 24 hr. The residue was removed by centrifugation, under nitrogen barrier at 2,500 r.p.m. for 2 hr. at 20°C. The supernatant was treated with x 3 its volume of absolute alcohol for 24 hr. at 4°C. The precipitated protein was collected by centrifugation at 2,700 r.p.m. for 30 min. at 4°C. It was then solvent-dried by washing in absolute alcohol followed by acetone and finally diethylether. The white powder was air-dried at room temperature in a fume cupbaord and stored below 0°C.

For carboxymethylation, an aliquot of the dried powder was dissolved in 5M GuHCl, solubilzed with 0.05M DTE and inhibitors, and left on a metabolic shaker for 24 hr. It was then centrifuged under nitrogen barrier at 12,500 r.p.m. for 30 min. Iodoacetic acid in sodium hydroxide at a 4-fold molar excess over dithioerythritol (DTE) (i.e. 18.6g in 70 ml N NaOH) was added to the supernatant under nitrogen barrier. The vessel was wrapped with aluminium foil, to exclude light and thus prevent iodine reacting with histidine and tyrosine residues, and

pH of the mixture adjusted to and maintained at 8.6 with 5M sodium hydroxide for 45 min. β-mercaptoethanol was then added at a 5-fold molar excess over iodoacetic acid. The solution was then treated with absolute alcohol at 4°C for 24 hr. The precipitated protein was collected by centrifugation and solvent dried as described above. This was the S-carboxymethylated microfibrillin. A schematic representation of the extraction procedure is shown on Table 2.

A similar procedure was carried out without the proteolytic inhibitors, on an aliquot of the starting material. This was done in order to assess the degree of degradation caused in the absence of these inhibitors; as they are fairly expensive. SDS gel electrophoresis in fact, showed no difference in the band pattern of the crude material isolated with and without inhibitors (see Fig. 5).

TABLE 2 PROTOCOL FOLLOWED FOR THE EXTRACTION OF MICROFIBRILLIN FROM BOVINE LIGAMENTUM NUCHAE

MINCED DEFATTED TISSUE (adult bovine ligamentum nuchae)

5M GuHCl–0.1M Tris, 25mM EDTA, 10mM NEM, 1mM PMSF, pH 7.4 at 4°C (2 extractions)

CENTRIFUGATION at 2700 r.p.m. for 2 hr. at 4°C

Supernatant (Collagen and serum proteins) discarded

RESIDUE washed in 0.1M Tris, 25mM EDTA, 1mM PMSF pH 7.4

CENTRIFUGATION at 2700 r.p.m.

Supernatant discarded

RESIDUE treated with 5MGuHCl, 0.1M Tris pH 7.4, 25mM EDTA, 0.285M β-mercaptoethanol pH adjusted to 8.5, mixture bubbled with nitrogen and left at 37°C for 24 hr.

CENTRIFUGATION at 2500 r.p.m. for 2 hr. at 20°C

Residue discarded

SUPERNATANT (microfibrils) treated with 3 vol ethanol and left at 4°C for 24 hr.

CENTRIFUGATION at 2700 r.p.m. for 30 min at 4°C

Supernatant discarded

RESIDUE washed sequentially in ethanol, acetone and diethyl ether

RESIDUE dissolved in 5M GuHCl solution containing 0.05M DTE and inhibitors. Mixture left on metabolic shaker for 24 hr.

CENTRIFUGATION at 12500 r.p.m. for 30 min.

Residue discarded

SUPERNATANT (soluble microfibrils) treated with iodoacetic acid in NaOH. Mixture bubbled with nitrogen, pH adjusted to 8.5 and maintained for 45 min. β-mercaptoethanol added. 3 vol ethanol added and preparation left at 4°C for 24 hr.

CENTRIFUGATION at 2700 r.p.m. for 30 min at 4°C

RESIDUE (S-carboxymethylated microfibrillin) washed in ethanol, acetone and diethyl ether.

Supernatant discarded

2.6. PURIFICATION OF S-CARBOXYMETHYLATED MICROFIBRILLAR PROTEIN.

Attempts were made to purify the crude material by gel chromatography using Sepharose 4B-CL. Assessment of purity was carried out by SDS-acrylamide gel electrophoresis.

2.6.1. Sepharose 4B-CL Gel Chromatography

The procedure was that described by Fish et al., (1970) and involved the following steps:

2.6.1.1. Preparation of protein-SDS complexes

20 mg of crude S-carboxymethylated protein was dissolved in 1 ml of 3% SDS, 8M urea and 3% β-mercapto-ethanol, 25mM EDTA. The solution was left at room temperature with constant stirring for 24 hr. It was then heated at 100°C for 5 min., cooled under tap water and centrifuged at 2,000 r.p.m. to remove undissolved particles. The supernatant was dialysed overnight against phosphate buffer pH 7.2, ionic strength 0.004, containing 0.2% SDS, using a 18/32 visking tubing.

Standard marker proteins: ovalbumin and bovine serum albumin were similarly treated except that the amount of material was 5 mg of each.

2.6.1.2 Preparation of gel column

The eluting solvent was sodium phosphate buffer pH 7.2,(ionic strength 0.004) containing 0.2% SDS. The pre-swollen Sepharose (100-200 mesh) was mixed

with the buffer, degassed and poured into a jacketed column (2 x 95 cm). The column was packed under gravity to a height of 76 cm. It was then equilibrated with the eluting buffer for 24 hr. at a flow rate of about 16 ml/hr. prior to sample application, using a peristaltic pump (Anachem Ltd., Minipuls II).

2.6.1.3 Application of sample and column development

The column flow was momentarily stopped, the buffer on top removed and the dialysed sample solution carefully layered onto the top of gel. Sample was allowed to permeate the gel completely under gravity and column flow started again. Elution was carried out at 20°C. Fractions (3 ml) were collected and monitored for protein content by reading the absorbance at 280nm. The homogeneity of several fractions was monitored by SDS gel electrophoresis. Those selected (see Fig.2) were pooled, dialysed against water, lyophilised and rechromatographed under identical conditions. Fractions from this second chromatography were also monitored for protein content as above and assessment of purity performed by SDS-gel electrophoresis on each appropriate fraction. Those fractions which gave a single band on SDS electrophoresis were pooled, dialysed against water for 24 hr. at room temperature using 18/32 visking tubing and then lyophilised. The dried material was stored below 0°C.

It was found, during preliminary studies, that application of sample without prior removal of salts led to such an increase in viscosity that the rate of sample permeation was greatly reduced. Secondly, there was a large ultraviolet absorption at 280nm caused by the disulfide of β-mercaptoethanol which, although separated perfectly from the standard marker proteins, partly overlapped the microfibrillar component. It was found that the elution position of the microfibrillar component did not change appreciably when the concentration of the sample was increased 3-fold.

2.6.2. Assessment of sample purity

SDS-acrylamide gel electrophoresis has become an invaluable tool both in the determination of molecular weights of polypeptide chains and as a criterion for determining the purity of protein preparations. SDS, an anionic detergent reacts with proteins forming protein-SDS complexes. SDS imparts a constant negative charge per unit mass of the complex the hydrodynamic parameters of which are now dependent, for reduced proteins only, on their molecular weights (Weber and Osborn, 1969; Shapiro et al., 1967; Weber et al, 1972; Reynolds and Tanford, 1970). There are, however, exceptions to this pattern particularly when glycoproteins are involved with a carbohydrate content in excess of 10%. In such

instances, the amount of bound SDS is lowered as well as the electrophoretic mobility of the complex (Schubert, 1970; Pitt-Rivers and Impiobato, 1968). It has also been suggested that water-insoluble membrane proteins and glycoproteins differ from water-soluble proteins in their resistance to dissociating reagents such as SDS which, in the presence of reducing agents can solubilise but not completely dissociate those conjugated proteins (Katzman, 1971). In this work the assessment of the purity of the microfibrillar protein samples involved the following procedures:

2.6.2.1 Preparation of samples and molecular weight markers

This was carried out as described in section 2.6.1.1 except that the sample solvent was 0.1M Tris/HCl pH 7.4 containing 8M urea, 1% (w/v) SDS and 3% (v/v) β-mercaptoethanol. The final protein concentration was 1 mg/ml for the marker proteins and pure samples and 4 mg/ml for crude samples. Samples were used directly without prior dialysis since small volumes were used.

For individual fractions from gel chromatography, aliquots were dried on a watch-glass under an infra-red lamp, the temperature being maintained between 40°C and 60°C. Dried material was allowed to cool and 0.05 ml of sample solvent added. It was found that a high concentration of β-mercaptoethanol (3% v/v) reduced gross aggregation and entrapment of

material at the origin in the case of microfibrillin. For the sake of standardisation the same concentration of β-mercaptoethanol was used for the marker proteins.

2.6.2.2 Preparation of gels

Gels of various porosities were prepared according to the procedure described by Weber et al., (1972), with a few modifications (Table 3), and involved the use of the following solutions:

(a) Acrylamide solution A: 22.2g acrylamide, 0.6g N-N'methylenebisacrylamide per 100 ml distilled water. The solution was filtered through Whatman No.1 paper.

(b) Acrylamide solution B: 30.0g acrylamide, 0.8g N-N'methylenebisacrylamide per 100 ml distilled water.

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

(d) Reservoir buffer: This was prepared from a stock solution of 1M Tris/HCl pH 8.0, containing 2% (w/v) SDS, by making a dilution of 1 in 10 with distilled water.

(e) Ammonium persulphate: 0.03% by volume was used for 7.5% and 10% gel concentrations, 0.036% was used for the 5% gel and 0.023% for the 15% gel concentration. The solution was prepared freshly just before use.

(f) N,N,N',N'-tetramethylethylenediamine (TEMED): 0.07% by volume was used for 10% gels and below, and 0.025% was used for 15% gels.

(g) Staining solution: The staining solution consisted of 2.5g Coomassie brilliant blue (CBB R250) dye dissolved in 454 ml methyl alcohol containing 72 ml glacial acetic acid and made up to 1 litre with distilled water.

(h) Destaining solution: This consisted of 250 ml methyl alcohol, 75 ml glacial acetic acid and 675 ml distilled water.

All solutions except reagents (c), (g), and (h) were kept at 4°C.

TABLE 3 THE COMPOSITION OF ACRYLAMIDE GELS OF VARIOUS POROSITIES

Final acrylamide concentration (%)	5	7.5	10	15
Solution (ml):				
Acrylamide A	6.75	10.1	13.5	-
Acrylamide B	-	-	-	14.8
Distilled water	6.75	3.4	0	0
Gel Buffer	15.0	15.0	15.0	15.0
Ammonium persulphate	1.0	1.0	1.0	7.5mg*
TEMED	0.03	0.03	0.03	0.01

* added as the solid salt.

1% (w/v) SDS in the gel buffer, according to Fairbanks et al., (1971), produced very satisfactory electrophoretic patterns. Pronounced band curvature noted by these workers was not observed except when gels with curved surfaces were used. These authors claimed that electrophoresis carried out at an SDS concentration exceeding the critical micellar concentration produces patterns which are independent of the buffer composition, so that reproducible results and smooth calibration curves can be obtained without dialysing the sample against the electrophoretic buffer to equilibrium. Secondly the tracking dye, pyromin Y, runs as a sharp band, allowing precise mobility measurements and alignment of a series of gels for close comparison. This was not the case with bromophenol blue which ran as a sharp band only when the sample was desalted and a minimum sample load ($\leq$ 50µl) was used. Thirdly, high levels of SDS inhibit proteolytic activity.

To prepare the gels, glass tubes (7.5 cm long, 5mm internal diameter) were soaked in Pyroneg or chromic-sulphuric acid overnight, rinsed thoroughly with tap water, followed by distilled water and oven-dried. One end of each tube was filled to the 6cm mark from the bottom with the gel solution . Trapped air-bubbles were released by gently tapping the bottom end of the tubes. A small volume of water was carefully layered onto the top of the gel using a syringe and a small bevelled hypodermic needle with the bevelled side against the wall of the tube.

In this way turbulence of gel solution was avoided and gel surface curvature prevented. The gels were allowed to set at room temperature for about 20-25 min. After the gel had set completely, as indicated by the appearance of an interface between the gel and the water layer, the water was replaced with reservoir buffer. The tubes were covered with parafilm to prevent dehydration and left at 4°C overnight before use. For 15% gels it was necessary to deaerate the acrylamide solution, before casting the gels, to reduce bubble formation. Gels can keep for up to four weeks if they are well protected from dehydration, in which case replace with fresh reservoir buffer layer from time to time.

2.6.2.3 Electrophoretic procedures

For each gel, 10 µl of 0.05% bromophenol blue in 0.1M Tris buffer pH 7.4 as the tracking dye, 1 drop of glycerol and 10 µl each of reduced protein solution and reservoir buffer were mixed on a square piece of parafilm. Before sample loading, the parafilm covering the bottom of the gel tubes was removed and the buffer layer shaken from the gel surface which was rinsed with a fresh volume of buffer. The tubes were then placed in the electrophoretic tank. About 250 ml of the reservoir buffer was added to the lower chamber. The samples were loaded onto the top of the gels with a micropipette. The reservoir buffer was carefully layered over each sample volume. The upper chamber was filled with buffer and the cover, which in addition

to carrying the cathode, helps to keep out dust particles during electrophoresis, placed over the chamber.

Electrophoresis was carried out at room temperature. A current of 5mA per gel was used and 3mA per gel for 15% gels. The electrophoresis was stopped when the tracker dye was about 6.0 cm from the top of the gel and this took about 3 hr, and 7 hr for 15% gels. Preliminary studies showed that an increase in current from 5 to 8mA did not affect the electrophoretic pattern except 15% gels for which no such studies were carried out.

After the electrophoretic run, the gels were dislodged by squirting water from a syringe between the gel and the tube at both ends. A little pressure from the syringe usually pushed the gel out quite easily except in the case of gels of high concentrations such as 15% or 20% where it was necessary to leave the gel for a few hours at below $0^{\circ}C$ to facilitate its removal from the tube. The distance migrated by the centre of the dye band and the length of the gel were measured.

Staining was carried out in test tubes filled with the staining solution at room temperature for 2 hr. Excess dye was washed off with distilled water and the gels were destained with the destaining solution until backgrounds were clear. Results were hastened and improved by addition of a small amount of DE.52 cellulose ion-exchanger to absorb the dye as it diffused from the gels. Destained gels were photographed through a yellow filter using Pan F type film. Gel densitometry was performed using vitatron densitometer (TLD100 Fisons)

scanning through 0.1mm slit at 570nm. The lengths of the gels and the length from top of the gels to the centre of stained protein band, on the scan, were measured and mobilities calculated from the formula.

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

The log molecular weight of the marker proteins were plotted versus mobilities. Apparent molecular weight of sample proteins were then deduced from the standard curve.

2.7 DETECTION OF CARBOHYDRATES BY PERIODIC ACID-SCHIFF (PAS) STAINING.

The carbohydrate staining reaction is based on the oxidation of diol components by periodic acid. The aldehyde formed reacts with Schiff's leucofuchsin reagent to form a red anil, azomethine or Schiff's base.

$$R\text{-}CHOH\text{-}CHOH\text{-}R^{-} + O_4^{-} \longrightarrow R\text{-}CHO + R^{-}\text{-}CHO + O_3^{-} + H_2O$$

$$R\text{-}CHO + H_2NR^{S} \longrightarrow R\text{-}CH = N.R^{S} + H_2O$$

aldehyde Schiff's leucobase azomethine

(McManus, 1946; Hotchkiss, 1948; Adams and Adams, 1965).

The procedure is essentially as described by Furlan et al., (1975) with only slight modifications. In contrast to other procedures such as that of Zacharius and colleagues (1969) or the much earlier methods of

Keyser (1964), this method was found to be very satisfactory. It involved the use of the following reagents:

(a) 250 ml of isopropyl alcohol in 100 ml of glacial acetic acid and 650 ml of water.

(b) 10% acetic acid (v/v).

(c) 5% acetic acid (v/v).

(d) 0.5% (v/v) periodic acid in water prepared freshly before use. (0.5% periodic acid in 5% acetic acid did not show any difference in the staining characteristics by Schiff's reagent).

(e) 0.5% sodium arsenite in 5% (v/v) acetic acid.

(f) Periodic acid-schiff reagent was prepared by mixing 1g fuchsin red, 2g sodium metabisulfite and 20 ml 1N HCl in 200 ml distilled water and stirring continuously for 18 hr. at 4°C on a magnetic stirrer. 2g of charcoal was added and stirred for a further 4 hr. The mixture was filtered cold under reduced pressure through Whatman No.1 filter paper. The filtrate was stored in a dark bottle at 4°C the stopper wrapped with aluminium foil to prevent atmospheric reoxidation of the basic fuchsin.

(g) 50% (v/v) methanol as destaining solution.

Aliquots of sample preparations containing 40-100 µg protein were loaded on SDS acrylamide gels and electrophoresis carried out exactly as described in section 2.6.2.3. The electrophoretic run was always carried out simultaneously with gels to be stained with Coomassie blue. At the end of the electrophoresis the

TABLE 4 THE PROCEDURE USED FOR THE DETECTION OF CARBOHYDRATES IN SDS GELS

40 ml of each of the following solutions were used	Time in contact with solution(hr)	Temperature
(a) To remove excess SDS which absorbs light significantly at 550nm		
Isopropyl alcohol-Acetic acid-H_2O	1	
Isopropyl alcohol-Acetic acid-H_2O	24	Room temperature
10% (v/v) Acetic acid	2 x 2	
10% (v/v) Acetic acid	1 x 4	
(b)* Periodic acid (0.5%v/v)	2	
(c) Distilled water	30 sec	
(d) To neutralize unreacted periodic acid that may oxidize the leucofuschin		
*Sodium arsenite (0.5%w/v)	1	
(e) To remove excess sodium arsenite		
Distilled water	60 sec	$4^{o}C$
10% (v/v) Acetic acid	3 x 2	
(f)* Schiff reagent	12	
Distilled water	60 sec	
5% (v/v) Acetic acid	2 x 1	
5% (v/v) Acetic acid	24	
Distilled water	24	
*5% (v/v) methanol	24	Room temperature

* with frequent mixing.

carbohydrate was detected following the protocol shown on Table 4.

The gels, which have by now shrunk, are swollen in Coomassie blue destainer overnight and scanned through a slit of 0.1mm at 550nm.

Comparison of the patterns obtain by PAS and protein staining was often a little difficult since the gels stained with PAS often failed to completely swell to the size of those stained with Coomassie blue. Therefore a single gel was sometimes stained with PAS after staining with Coomassie blue. One disadvantage of this method, however, was the loss of weakly-stained bands during the prolonged treatment prior to PAS staining. It was thus necessary to take a photograph of stained gel before superstaining with PAS.

Electrophoretic mobility was calculated as described in section 2.6.2.3.

Bovine serum albumin and lysozyme were used as PAS-negative standards while ovalbumin and trypsin represented PAS-positive standards.

2.8 DETERMINATION OF MOLECULAR WEIGHT OF S-CARBOXYMETHYLATED MICROFIBRILLIN

Apparent molecular weights were determined by (a) SDS-gel electrophoresis and (b) Meniscus depletion sedimentation equilibrium analysis (Chervanka, 1970).

2.8.1 (a) SDS-gel electrophoresis: This was as described in Section 2.6.2.3.

2.8.2 (b) Equilibrium ultracentrifugation

The instrument used was a Spinco model E analytical ultracentrifuge.

GuHCl and SDS were purified before use as described in Sections 2.2 and 2.3 respectively.

Visking tubing (8/32 and 18/32) were obtained from The Scientific Instruments Ltd. (London). Visking 8/32 tubing was acetylated before use employing the method described by Craig and Konisberg (1961). Although it is claimed that insulin with a molecular weight of 6,000 diffuses very slowly through 8/32 (thickness: 0.064mm) and does not diffuse at all through 18/32 (thickness: 0.0175mm) visking tubings Craig et al., (1957) experience in this laboratory has shown that the 8/32 tubing does not retain completely proteins of molecular weight up to 20,000 especially during exhaustive dialyses. Pitt-Rivers and colleagues (1968) even recommend the acetylation of the 8/32 before use for proteins of

molecular weight lower than 25,000.

A suitable length of 8/32 visking tubing was immersed in water and the lower end tied firmly. The open end was then slipped over a suitable glass tube. The water in the membrane was removed by washing with dry pyridine. The membrane was allowed to stand in 10% acetic anhydride in pyridine for 15 hr. at 25°C. The solution was replaced with water and the membrane washed with 0.01N acetic acid until all the pyridine had been removed as indicated by no further reduction in absorption at 260nm in successive washes. This required several hours. The tubing was stored in distilled water at 4°C.

2.8.2.1 Equilibrium dialyses of S-carboxymethylated microfibrillin and ovalbumin (standard protein) in SDS and GuHCl

(a) Equilibrium dialysis in SDS:

1 mg of S-carboxymethylated microfibrillin and 2.3 mg of carboxymethylated ovalbumin were each dissolved in 1 ml of 3% SDS containing 8M urea and 3% βME. The solutions were left at room temperature for 4 hr, and then dialysed against 200 ml of sodium phosphate buffer (ionic strength = 0.004) containing 0.2% SDS (pH 7.2) for 4 days using acetylated 8/32 visking tubing with three buffer changes. A suitable aliquot from each solution was taken for analysis.

(b) Equilibrium dialysis in GuHCl:

Bound SDS was removed from the S-carboxymethylated microfibrillin by the method of Pitt-Rivers and Impiobato (1968).

4 mg of the protein were dissolved in 0.2 ml of water containing a drop of 0.5M acetic acid and shaken in a centrifuge tube with 5 ml of acetone. The precipitated protein was removed by centrifugation at room temperature, resuspended in 0.2 ml of 1M sodium acetate buffer pH 4.0 containing 1M sodium chloride and shaken with a further 5 ml of acetone. The precipitated protein was collected by centrifugation. It was then dissolved in 1 ml of 5M GuHCl and dialysed for 3 days (3 buffer changes) using acetylated 8/32 visking tubing. A suitable aliquot was taken for analysis.

The carboxymethylated ovalbumin was similarly treated except that it did not require the removal of bound SDS as it was not a protein-SDS complex.

The densities of the phosphate buffer and GuHCl were determined by picnometry.

TABLE 5 CONDITIONS FOR ULTRACENTRIFUGATION OF MICROFIBRILLIN AND OVALBUMIN

	Carboxymethylated microfibrillin		Carboxymethylated ovalbumin	
	in SDS	in GuHCl	in SDS	in GuHCl
Temperature (°C)	24.2	25.2	21.2	20.2
Speed (r.p.m.)	27690	33450	20410	27690
$\bar{\nu}$		0.701	0.804	0.749
ρ	1.0005	1.126	1.0005	1.126
Cell	12 mm with double sector centre piece			
Optical system	Rayleigh interference optics			

Fringe displacements were measured by means of a travelling microscope (Projection Cope PQ Ltd.). Readings were taken at 200 microns intervals commencing at the maniscus, along the X-scale, until a deflection of more than 10 microns occurred on the Y-scale between consecutive readings which were then made at 100 microns along the X-scale.

The partial specific volume of carboxymethylated microfibrillin was calculated from compositional data (Zamyetnin, 1972; Gibbon, 1966). The partial specific volume of ovalbumin in GuHCl was taken from Handbook of Chemistry (Sober, H.A. 1970). The partial specific volume of ovalbumin-SDS complex was calculated according to Tanford et al. (1974), using the δ_D estimated by a combination method of Pitt-Rivers and Impiobato (1968) and Hayashi (1975).

The programme of Yphantis and Roark (1972) was utilised in the computation of the molecular weight values from experimental data.

2.9 DETERMINATION OF BOUND SDS BY EQUILIBRIUM DIALYSIS

The amount of bound detergent under the experimental conditions used was determined by a combination of the methods of Pitt-Rivers and Impiobato (1968) and Hayashi (1975). This was done as a counter check for the calculated value from ultracentrifugation analysis data.

1.2 mg of carboxymethylated ovalbumin in 8M urea, 3% SDS and 3% (v/v) β-mercaptoethanol were left overnight at room temperature. The solution was dialysed against phosphate buffer (ionic strength = 0.004) containing 0.2% SDS, pH 7.2 for 10 days at room temperature, using 18/32 visking tubing. At the end of the dialysis period, 0.2 ml aliquot of the solutions inside the dialysis bag was subjected to Pitt-Rivers and Impiobato procedure to extract the bound SDS (see Section 2.8.2.1). After the evaporation of the acetone extracts with a rotary evaporator at 37°C, the SDS was collected by washing the round-bottomed flask several times with 0.66M phosphate buffer pH 6.1. The pooled washings were made up to 25 ml. A similar aliquot of the solution outside the bag was also made up to 25 ml, without undergoing the extraction steps. 1 ml of each solution was further diluted to 5 ml. The

SDS content was determined employing the method of Hayashi (1975) which is based on the formation of a water-insoluble salt between the SDS and methylene blue. The complex so formed is extracted into chloroform and the absorbance at 655nm measured in a spectrophotometer.

For the determination, 1 ml aliquot was added to 0.5 ml of 0.005% methylene blue. The solution was mixed and 3 ml chloroform added and vigorously mixed. It was then centrifuged at 2,000 r.p.m. for 3 min at room temperature. The aqueous phase was removed and the absorbance of the chloroform phase measured at 655nm against a blank, using 'U.V.' cells of 1 cm light path. The determination was carried out in triplicate and the concentration of SDS deduced from a standard curve.

This procedure was repeated for the microfibrillin protein but results showed that there was no SDS still bound after 10 days dialysis, as was postulated on observing that the protein precipitated in the dialysis bag. The estimation was therefore abandoned.

2.10 CHEMICAL ANALYSES

2.10.1 Amino-acid analysis

An aliquot of carboxymethylated microfibrillin was suspended in 2 ml of 6N hydrochloric acid containing a drop of thioglycolic acid in a hydrolysis tube which was flushed with N_2 for 5 min. The tube was sealed and placed in an oil bath set at 110^{o}C for 24 hr.

Hydrolysates were taken to dryness using an Evapomix (Bucher Instrument N.J. USA) with an overhead infra red lamp. The dried material was then left under reduced pressure in the presence of sodium hydroxide pellets.

Amino-acid analyses were performed on a suitable aliquot of diluted solution using a Locarte (single column operation) amino acid analyser. Standard values were obtained from the chromatography of suitable standard solutions of amino acids (Sigma Chemical Co. Ltd.). Norleucine was used as an internal standard. Amino acids were quantitated manually from the chromatograms and corrections for hydrolytic loss were made. Analyses were performed in duplicate.

Hydroxyproline was estimated indepdently on protein hydrolysates by the colorimetric procedure of Serafini-Cessi and Cessi, 1964) in which hydroxyproline is oxidised by hydrogen peroxide in an alkaline medium (Fig. 2). The chromogen so formed, an unsaturated heterocyclic compound, is separated from other non-volatile products by distillation and coupled with p-dimethyl aminobenzaldehyde to produce a chromophore which absorbs maximally at 550 nm and is stable for only 15 min. The concentration of hydroxyproline in the hydrolysate was deduced from a standard curve.

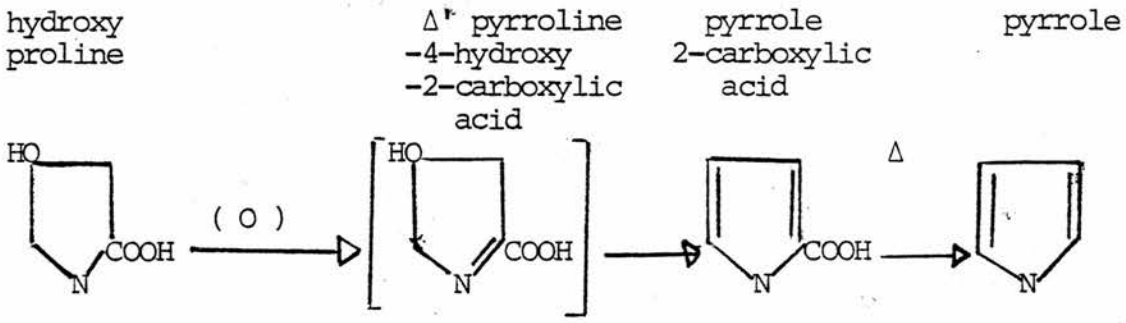

Figure 2: Oxidation and decarboxylation of hydroxyproline to pyrrole (after Prockop and Udenfriend, 1960).

Tryphophan was determined on protein hydrolysed with 3N mercaptoethanesulfonic acid for 24 hr at 110°C. This reagent, it is claimed, in addition to providing excellent tryptophan recoveries from peptides and proteins, offers substantially better values from carbohydrate-containing samples. (Penke et al. 1974). Secondly its use does not necessitate the evaporation of the hydrolysate prior to ion-exchange chromatography. The tryptophan content of the hydrolysate was determined as described in Section 2.10.1.

2.10.2 Measurement of Hexosamine

Samples for hexosamine estimation were prepared as described in Section 2.10.1 except that 4N hydrochloric acid was used in place of 6N hydrochloric acid and the hydrolysis was performed at 90°C for 6 hr and 8 hr. The total hexosamine content was determined on the amino acid

analyses using D-glucosamine and D-galactosamine as standards.

Hexosamine analysis was also performed by the gas chromatographic procedure of Bhatti et al., (1970) as described in the next section.

2.10.3 Estimation of sugars by gas-liquid chromatography (GLC)

Neutral sugars, hexosamines and sialic acid were quantitated by gas chromatography using the technique of Bhatti et al., (1970).

The potential of G.L.C. for the analyses of glycoproteins has only been appreciated about two and a half decades ago - because of the low volatility of the compounds. However, several derivatives have been used - methyl, ethyl and trimethylsilyl (Homing and Vanden-Heuvel, 1963). Increase in volatility is achieved by prevention of hydrogen bonding. Of these derivatives, those obtained with TMS are found to be most widely used in this field because of the ease and rapidity of preparation (Sweeley et al., 1963; Bentley et al., 1963)

Perseitol, standard hexoses and hexosamines and sialic acids were obtained from Sigma Chemical Co. Ltd. (London).

The instrument used was a Pye series 104 equipped with hydrogen flame ionisation detector coupled to a Philips 8000 recorder. Dual column operation was used, the glass columns (250 x 0.32) being packed with 3% SE30 on Diatomite CQ (Pye Unicam).

The operating conditions used were:

column temperature (^{o}C):

programmed from 140-200 (∞) at 0.5^{o}/min, the

upper limit being held until last peak emerged

carrier gas : argon (46 ml/min)

attenuation : 2 to 10 x 10^2

Sample volume : 5 - 10 µl

The technique involved the use of the following reagents:

Dry methanol: 2.5g magnesium turnings and 0.1g iodine were added to 500 ml methanol and the mixture heated under reflux for 1 hr. The dry methanol was then distilled into a clean dry flask.

Methanolic Hydrochloric acid: Hydrochloric acid gas was slowly bubbled into dry methanol until solution was 1.5M.

Trimethylisation (TMS) agent: This consisted of pyridine (proton acceptor), trimethylchlorosilane (TMCS) as a catalyst, and hexamethyldisilasane (HMDS) (5:1:1 by volume). It was prepared freshly before use without centrifugation.

Samples for the analyses were prepared by accurately weighing aliquots of the carboxymethylated microfibrillin into small test tubes. 0.1 ml of perseitol was added to each tube which was then dried in a vacuum desiccator overnight over phosphoric oxide. 0.5 ml of methanolic hydrochloride was added to each tube and N_2 bubbled for 30 sec. The tubes were sealed and placed in an oil bath set at 90°C for 24 hr. The acid was neutralised by the addition of solid silver carbonate. 0.05 ml of acetic

anhydride was added to each ampoule which was left at room temperature overnight. Each ampoule was then centrifuged and the supernatant transferred to a 5 ml pear-shaped flask. The trituration and centrifugation steps were repeated with three further additions of 0.5 ml methanol. Pooled supernatants were evaporated under reduced pressure in a rotary evaporator at 37°C and placed in a vacuum desiccator for 24 hr over phosphoric oxide. 0.05 ml of TMS agent was added to the dried material and the mixture was allowed to stand for 30 min at room temperature. 7 µl of the well shaken mixture was injected into the gas chromatograph.

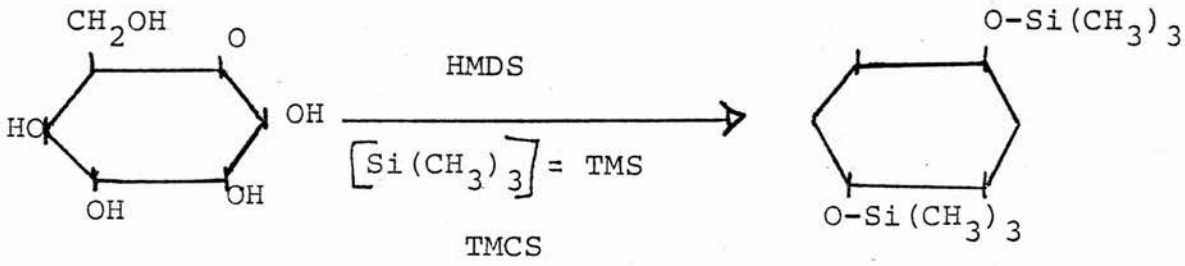

Figure 3: Trimethylsilylation reaction [Nature of reaction is complex and not fully understood but it appears reactive TMS groups are derived from a reaction complex of HMDS or HMDS + pyridine (Holligan, 1971)]

Calculation of results

Quantitative results were obtained by the determination of the Detector Response factor using an internal standard. 0.05 µmole of perseitol was added to a standard solution containing mannose, galactose, glucose, galactosamine and N-acetyl glucosamine in a series of ampoules giving a concentration range of 0.01 to 0.10 µmole for each sugar. After drying thoroughly the ampoules were subjected to the same procedure described above. Peak areas were integrated by transferring chromatogram profiles to a transparent paper of uniform thickness, each peak being cut and accurately weighed. Total peak weight for each sugar was obtained by summing up the peak weights of each isomer. The total peak weight ratio to the peak weight of the internal standard was plotted against the mole ratio of the sugar to the internal standard. The Molar Relative Response Factor was then deduced from the slope of the graph (see Fig. 9). The concentration of each sugar in the sample was calculated from the equation :

$$\frac{\text{Total Peak weight ratio} \times \mu\text{mole internal standard}}{\text{Molar Relative Response Factor}}$$

2.10.4 Determination of sialic acid

Total sialic acid was also determined by the colorimetric method of Jourdian et al., (1971) which involves the oxidation of sialic acid with periodate

to produce a chromogen that reacts with resorcinol and yields a chromophore which absorbs maximally at 630nm. The method measures both free and bound sialic acids and hydrolysis is therefore unnecessary.

Solutions containing N-glycolyl neuraminic acid and N-acetyl neuraminic acid in known concentrations were used to prepare a standard curve.

The method employed the following reagents:

0.04M periodic acid: This was prepared freshly before use from a stock solution of 0.4M periodic acid.

Resorcinol reagent: This consisted of 0.6g resorcinol (prepared freshly from a stock solution of 6% (w/v) resorcinol), 60 ml of 28% (v/v) hydrochloric acid, 40M water and 25 μmoles of copper sulphate.

95% (v/v) tert-butyl alcohol

All reagents were stored at 4°C except tert-butyl alcohol which was kept at room temperature.

To measure total sialic acid, an aliquot of the carboxymethylated microfibrillin, in a total volume of 0.5 ml water, was added to 0.1 ml of periodic acid solution in triplicate. The solutions were thoroughly mixed and kept in an ice bath for 35 min. 1.25 ml of resorcinol reagent was added, mixed and placed in an ice bath for a further 5 min. The solutions were then heated at 100°C for 15 min and cooled under tap water. 1.25 ml of tert-butyl alcohol was added, vigorously mixed and

solutions placed at 37°C in a waterbath for 3 min to stabilize the colour. They were cooled to room temperature and absorbances measured at 630nm against blanks using 1 cm light path cuvettes. Concentrations of sialic acid were deduced from the standard curve.

Standard sialic acids were similarly treated except that the oxidation period was 20 min rather than 35 min as free N-acetyl neuraminic acid produces a chromogen which is unstable after 20 min incubation. N-acetyl neuraminic acid and N-glycolyl neuraminic acid also show a differential stability at 37°C. While the chromogen formed by the latter is stable for up to 100 min that formed by N-acetyl neuraminic acid is almost completely destroyed. A discriminatory test was therefore carried out with another aliquot of the protein as outlined above except that the oxidation was performed at 37°C for 100 min.

SECTION 3

RESULTS

3.1 ISOLATION OF THE MICROFIBRILLAR COMPONENT

The protocol followed for the extraction of the microfibrillar component from ligamentum nuchae is shown in Table 2. The protein was isolated as the S-carboxymethylated derivative with a yield of about 0.1% on a dry weight basis. The sodium dodecyl sulphate electrophoretic pattern of the SDS complex of this crude preparation is shown in Figure 5. The major band designated as M represented about 33% of the loaded material as assessed by densitometry.

3.2 SEPHAROSE 4B-CL GEL CHROMATOGRAPHY

Figure 4 shows the elution profiles of the crude extract from Sepharose 4B-CL gel column. The preparation yielded three fractions A_1, A_2 and A_3 when eluted with sodium phosphate buffer pH 7.2 containing 0.2% sodium dodecyl sulphate. The major peak, A_3, exhibiting a shoulder A_2 had an elution volume of 114 ml. Rechromatography of the fraction 114 - 126 ml (see Figure 4) produced one major peak ($A_{3'}$) with maximum at 116 ml and therefore overlapping peak A_3 of the first chromatographic separation.

3.3 SODIUM DODECYL SULPHATE GEL ELECTROPHORESIS AND CARBOHYDRATE DETECTION BY P.A.S. STAINING.

The band patterns of the crude preparation with and without protease inhibitors, and those of selected fractions from Sepharose 4B-CL are shown in Figure 5.

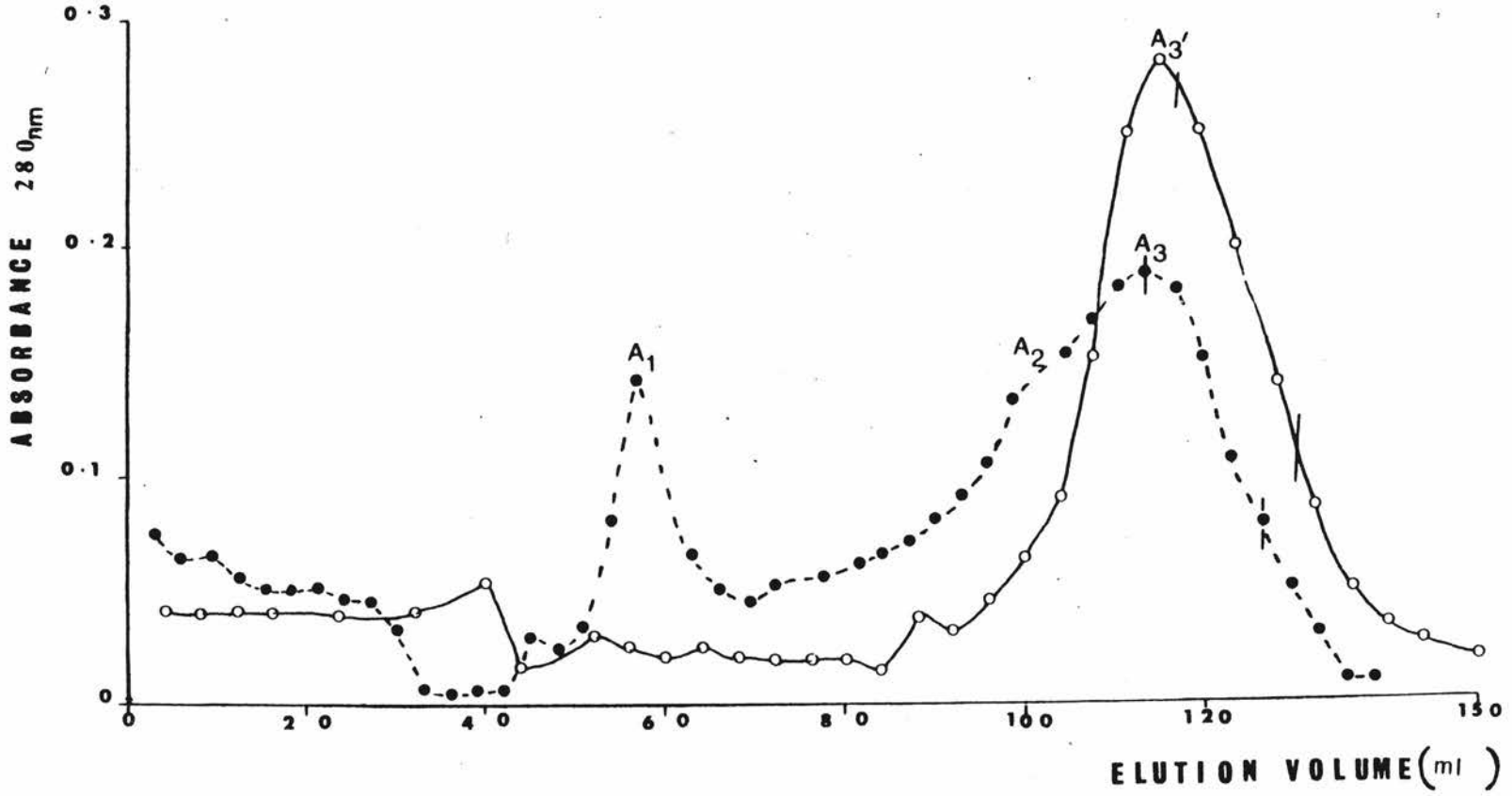
ABSORBANCE 280nm
0·3
0·2
0·1
0
A1
A2
A3
A3'
0
20
40
60
80
100
120
150
ELUTION VOLUME (ml)

Fig. 4

Sepharose 4B-CL gel chromatography of S-carboxymethylated microfibrillin. Elution was with sodium phosphate buffer (ionic strength = 0.004) containing 0.2% SDS (pH 7.2). Details were as described in Section 2.6).

(●) : First chromatographic separation.

(○): Second chromatographic separation of fractions 114-126 ml. (Fractions 117.5-130 were pooled, dialysed and lyophilised. This represented pure microfibrillin).

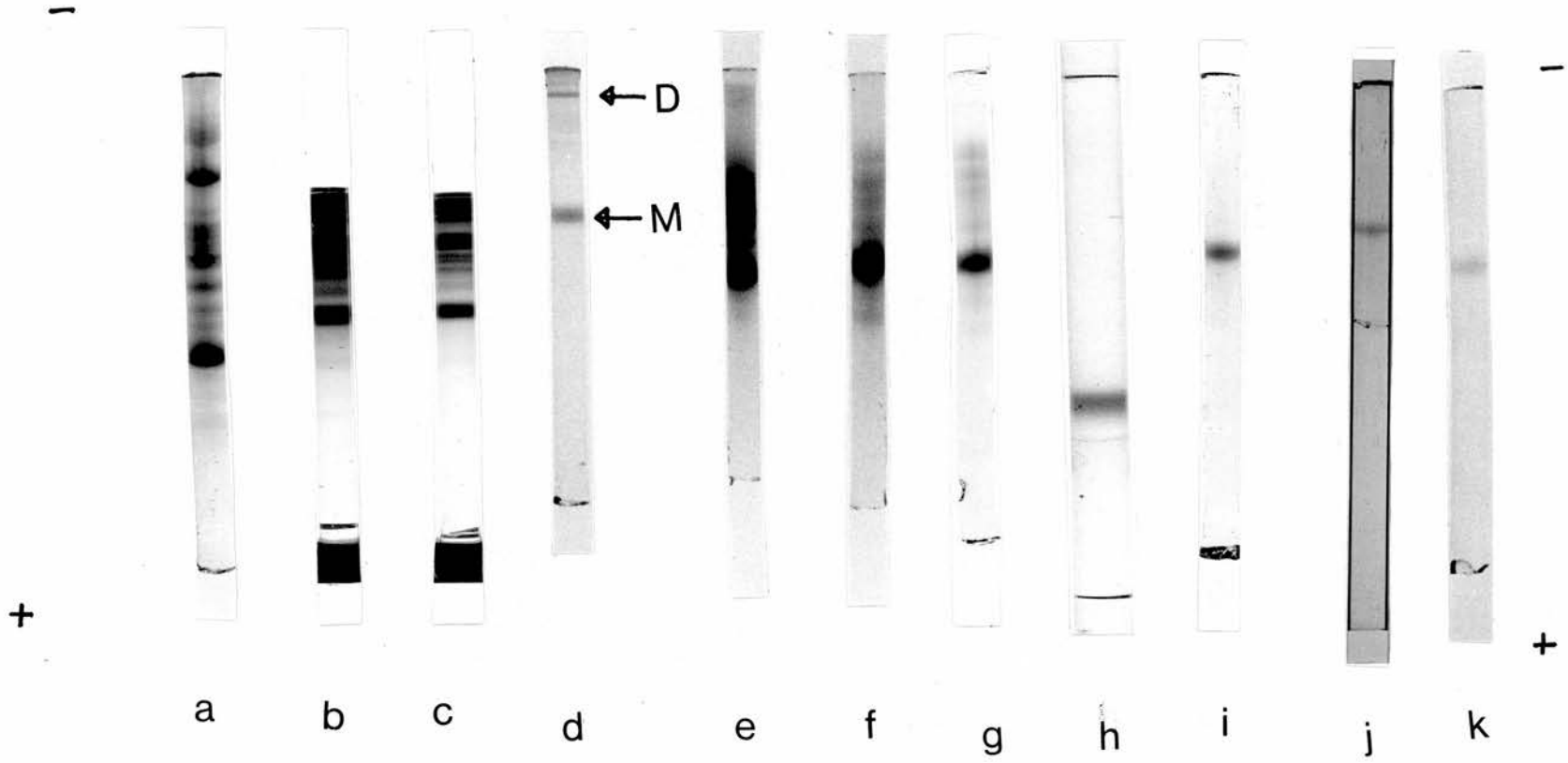
−
+
←D
←M
−
+
a
b
c
d
e
f
g
h
i
j
k

Fig. 5.

SDS-acrylamide gel electrophoretic patterns of various preparations of S-carboxymethylated microfibrillin (electrophoretic details were as described in Section 2.6.2).

Samples:

(a) and (b) 80µg of crude preparations obtained in the presence of proteolytic inhibitors using 5% and 7.5% acrylamide gel respectively.

(c) 80µg of crude preparation obtained in the absence of proteolytic inhibitors using 7.5% acrylamide gel.

(d) P.A.S staining of (b).

(e) and (f) Peak A_2 and Peak A_3, eluted after first chromatographic separation.

(g) Peak A_3', eluted after second chromatographic separation.

(h) 20µg of purified microfibrillin using 5% acrylamide gel and superstained with P.A.S.*

(i) and (j) 20µg of purified microfibrillin using 7.5% and 10% acrylamide gel respectively.

(k) P.A.S staining of (i).

*The leading side of peak A3', as stated elsewhere, contains microfibrillin associated with other contaminants. Aliquot h (pooled fractions 117.5-130 ml), only a part of the total material in peak A3', was shown to be only pure microfibrillin.

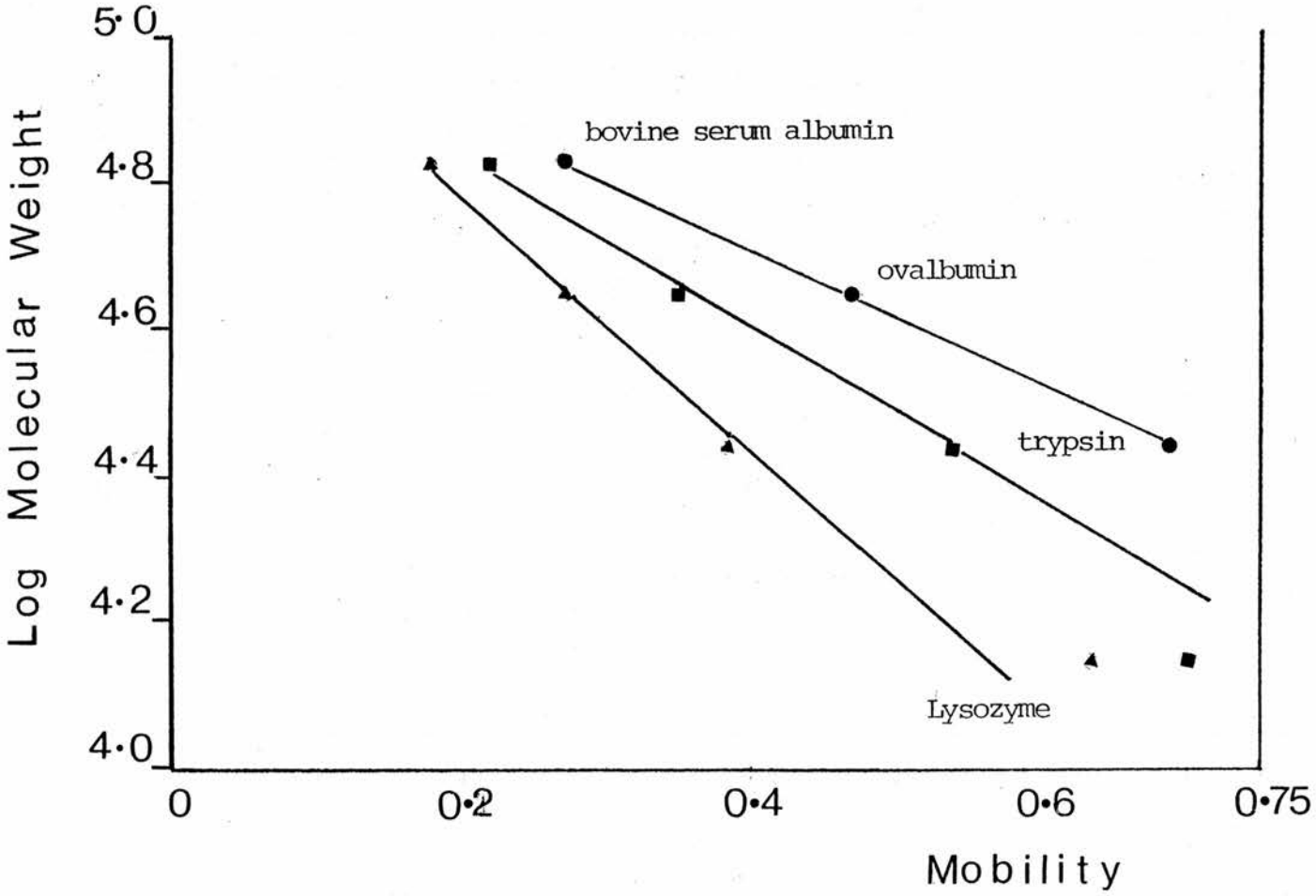
5·0
4·8
4·6
4·4
4·2
4·0
Log Molecular Weight
bovine serum albumin
ovalbumin
trypsin
Lysozyme
0
0·2
0·4
0·6
0·75
Mobility

Fig. 5B

Plots of log molecular weight against mobility by SDS-gel electrophoresis of standard marker proteins. 10µg each of bovine serum albumin, ovalbumin, trypsin and lysozyme was applied. (Details were as described in Section 2.6.2).

(●): 5% gel

(■): 7.5% gel

(▲): 10% gel

The pattern of the crude material isolated in the presence of proteolytic inhibitors revealed a major band (M) and several discrete bands of apparent higher molecular weight. There were also two very weakly stained bands migrating ahead of band M. No difference in the band patterns of the crude material isolated with and without inhibitors was observed. Of these bands, those indicated as M and D were found to be P.A.S. positive. The apparent molecular weight of these two proteins was determined to be about 38,000 and 78,000 respectively using 7.5% acrylamide cross-linking. Aliquots of the three peaks A_2, A_3 and A_3' obtained from first and second Sepharose chromatography showed a similar pattern and only the fractions containing 117.5 $\pm$ 130 ml from the second chromatography gave a single band pattern corresponding to the 38,000 molecular weight protein. After isolation, the pure protein gave a single band pattern on acrylamide sodium dodecyl sulphate gel electrophoresis and the apparent molecular weight varied according to the gel porosity - the highest value being 37,000 on 5% acrylamide and the lowest 35,000 on 10% acrylamide.

3.4. ANALYTICAL ULTRACENTRIFUGATION

The molecular weight data derived from the ultracentrifugation analyses of purified *S*-carboxymethylated microfibrillar component and ovalbumin in (a) sodium phosphate buffer (ionic strength = 0.004)

containing 0.2% sodium dodecyl sulphate pH 7.2 and (b) 5M guanidinium hydrochloride in 0.1M Tris pH 7.4 are reported on Figures 6a, 6b, 7a and 7b. At vanishing concentration, the two standard moments Sigma-N and Sigma-W of the microfibrillar component in guanidinium hydrochloride converged to a value corresponding to a molecular weight of about 21,000 obtained using a partial specific volume of 0.701 ml g^{-1}. The accuracy of these extrapolations is supported by the coincidence of the intercepts of the standard moments with the values exhibited at low concentrations by the ideal moment $M\text{-}Y_8$ which is a combined M_n, M_w and M_z average ideal molecular weight moment in which corrections have been carried out for pertubations affecting the second, third and fourth virial coefficients.

Assessment of homogeneity was carried out by assessing the molecular weight of microfibrillin in sodium dodecyl sulphate solution. As in the previous experiments, the standard moment showed intercepts, at zero concentration, which were in agreement with the fluctuations of $M\text{-}Y_8$ at low concentration. Molecular weight obtained in sodium dodecyl sulphate solution could be reduced to the values assessed in guanidinium hydrochloride solution assuming that the detergent was bound to the glycoprotein at a ratio of 0.957g/g.

Since the δ_D could not be assessed independently by equilibrium dialysis as the glycoprotein precipitated

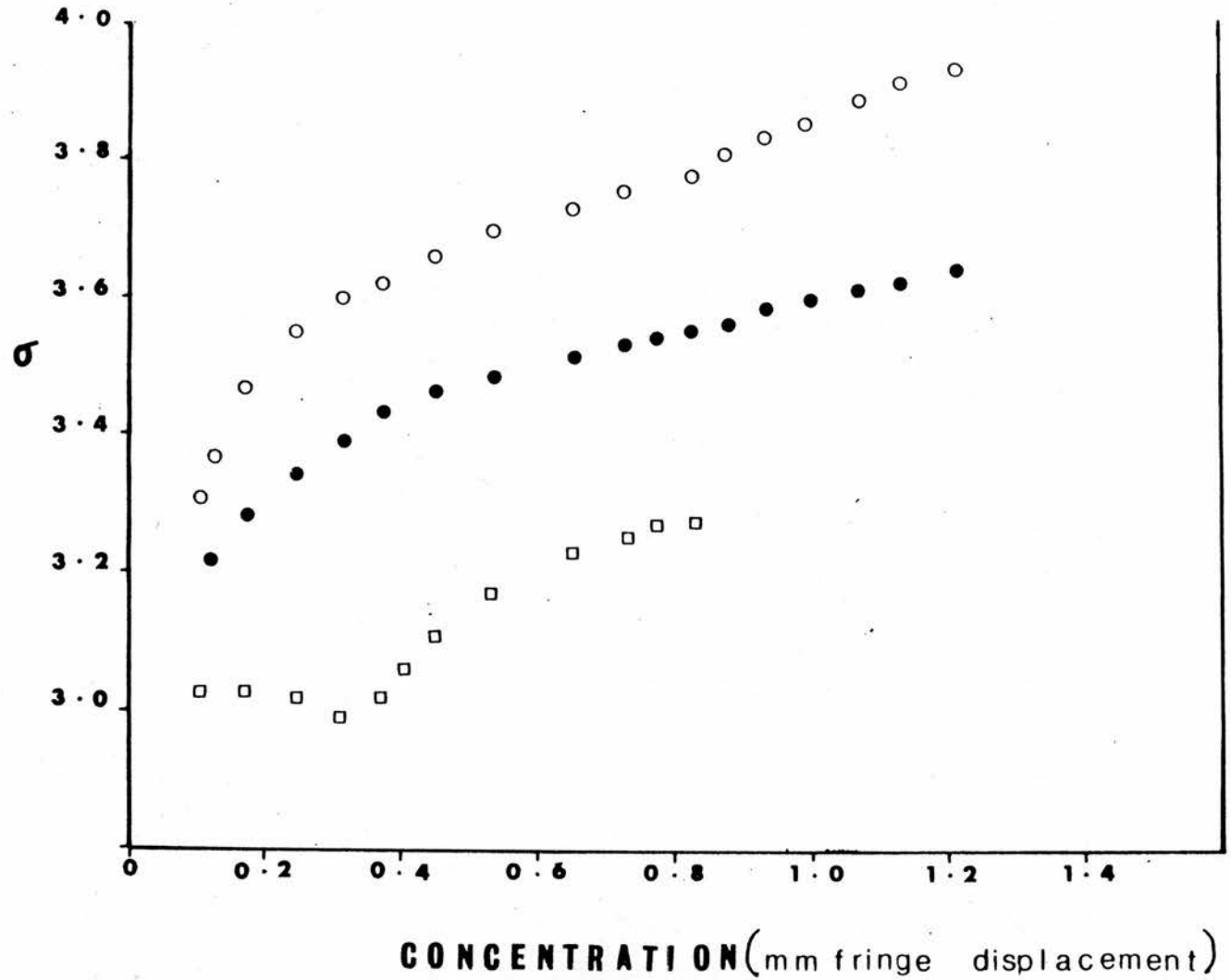
4·0
3·8
3·6
3·4
3·2
3·0
σ
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
CONCENTRATION (mm fringe displacement)

Fig. 6 (a):

Plots of Sigma (σ) moments of S-carboxymethylated microfibrillin in sodium phosphate buffer (ionic strength = 0.004), 0.2% SDS (pH 7.2) against the concentration in the cell.

σ-y values represent ideal molecular weight values to which various correction values have been applied.

$\sigma.y_8$ supplies the most realistic estimate of molecular weight having been corrected for effects of concentration and non-ideality in the system.

(●): σ-N

(⊙): σ-w

(□): σ-Y_8

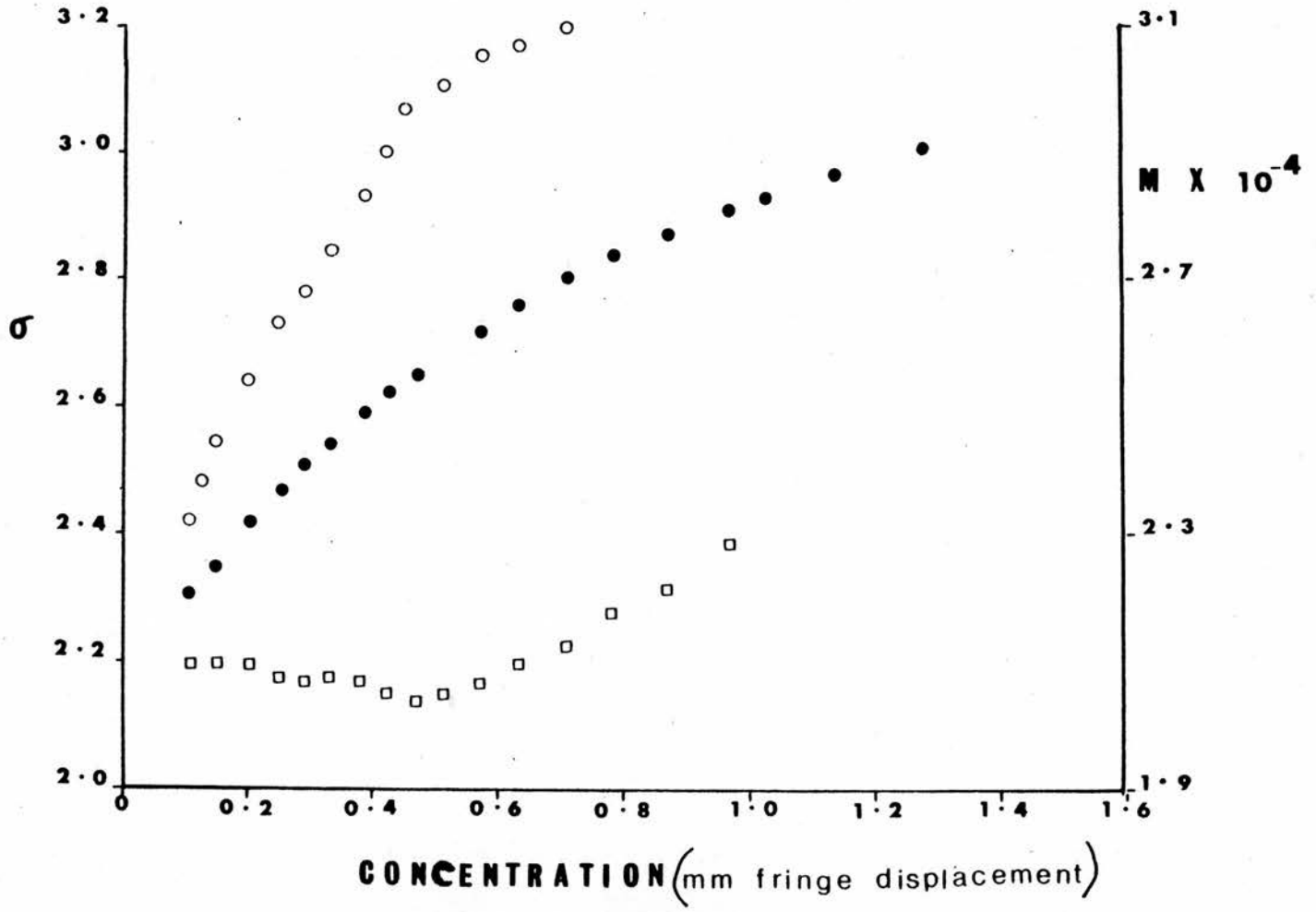
3·2
3·0
2·8
2·6
2·4
2·2
2·0
σ
3·1
2·7
2·3
1·9
M X 10⁻⁴
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
CONCENTRATION (mm fringe displacement)

Fig. 6 (b)

Plots of σ moments of S-carboxymethylated microfibrillin in 5M guanidinium hydrochloride 0.1M Tris pH 7.4 against the concentration in the cell.

Partial specific volume used for the calculation of molecular weight was 0.701.

See Fig. 6 (a) for the explanation of σ_y values.

(●): $\sigma - N$

(○): $\sigma - w$

(□): $\sigma - y_8$

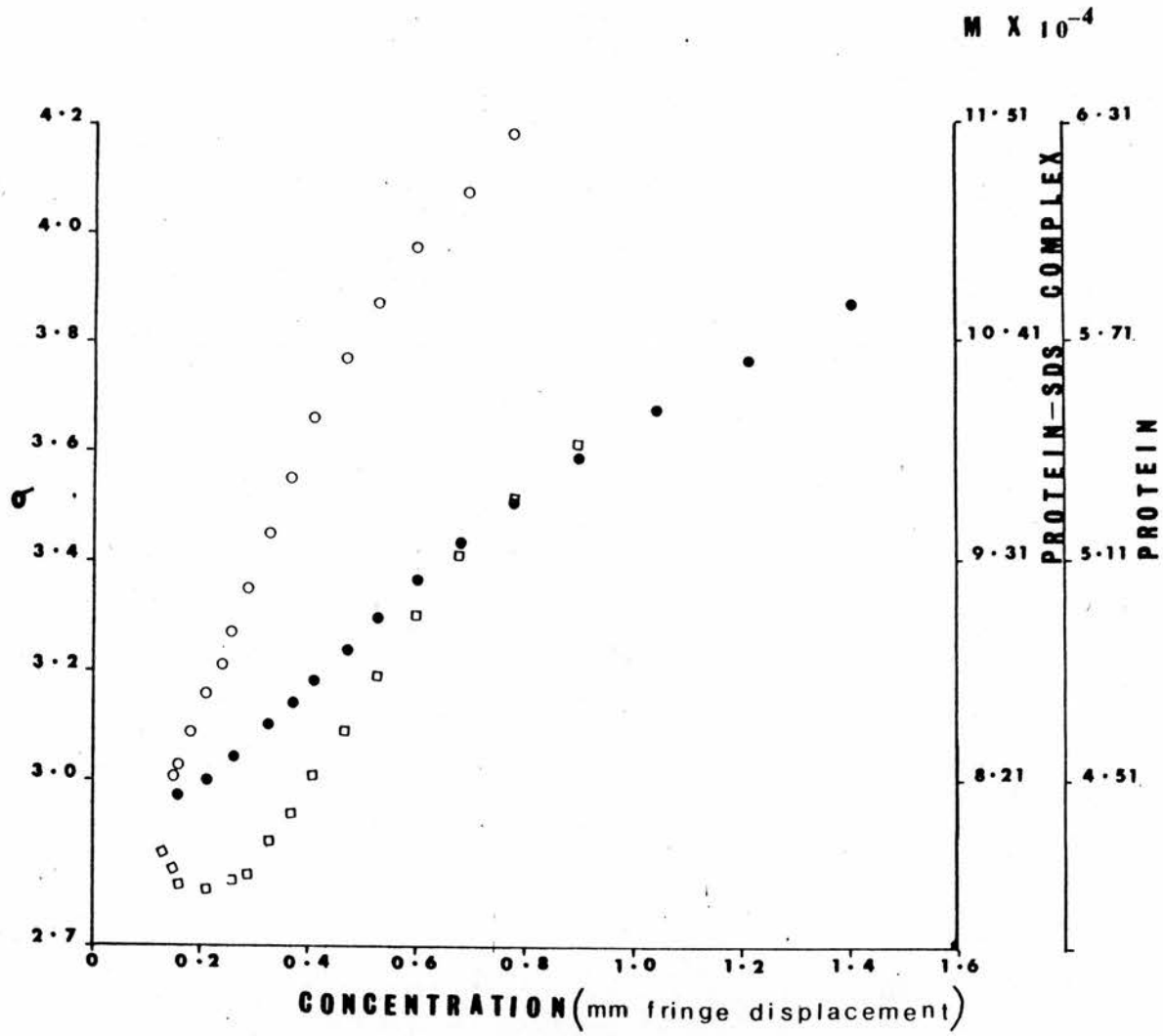
M X 10⁻⁴
4·2
4·0
3·8
3·6
3·4
3·2
3·0
2·7
σ
11·51
10·41
9·31
8·21
6·31
5·71
5·11
4·51
PROTEIN—SDS COMPLEX
PROTEIN
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
CONCENTRATION (mm fringe displacement)

Fig. 7 (a)

Plots of σ moments of S-carboxymethylated ovalbumin in sodium phosphate buffer (ionic strength = 0.004) - 0.2% SDS (pH 7.2) against concentration in the cell. Partial specific volume used for the calculation of molecular weight was 0.804. (See Fig. 6 (a) for the explanation of σ-y values).

(●): $\sigma - N$

(⊙): $\sigma - w$

(□): $\sigma - y_8$

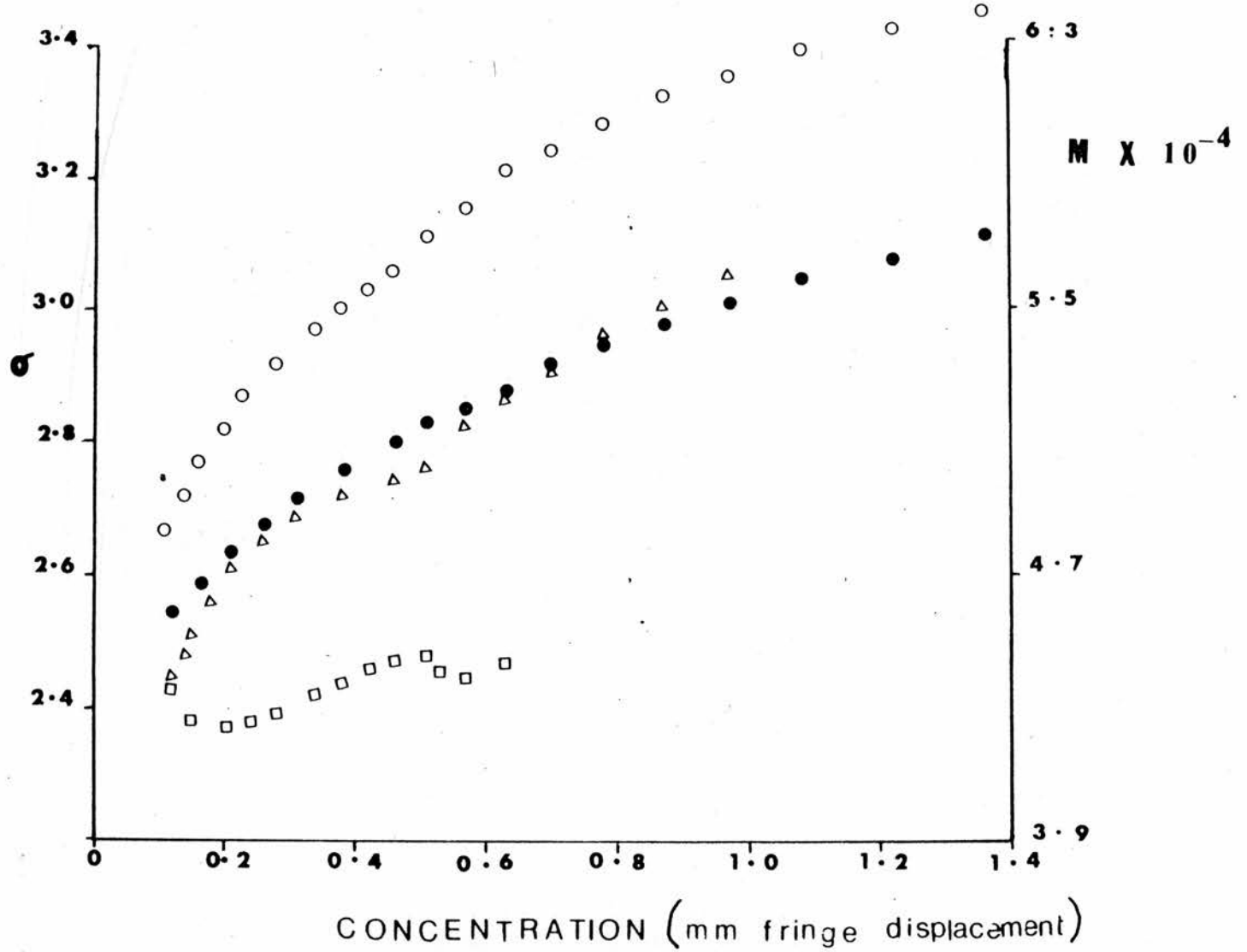
3·4
3·2
3·0
2·8
2·6
2·4
σ
6·3
5·5
4·7
3·9
M X 10⁻⁴
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
CONCENTRATION (mm fringe displacement)

Fig. 7 (b)

Plots of σ moments of S-carboxymethylated ovalbumin in 5M guanidinium hydrochloride 0.1M Tris (pH 7.4) against the concentration in the cell.

Partial specific volume used for the calculation of molecular weight was 0.749. (See Fig. 6 (a) for the explanation of σ.y values).

(●): $\sigma - N$

(○): $\sigma - w$

(□): $\sigma - y_8$

(△): $\sigma - y_2$

after a few days, the validity of δ_D evaluation by equilibrium ultracentrifugation was checked using ovalbumin for which a δ_D of 0.82 was estimated by equilibrium dialysis. The data obtained for ovalbumin in the presence and absence of SDS by analytical ultracentrifugation yielded a δ_D of 0.85. The formula used for this calculation is that proposed by Tanford et al., (1970):

$$M(1-\Phi\rho) = M(1-v_P\rho) + \delta_D(1-v_D\rho)$$

where Φ' = effective partial specific volume of complex

$\bar{v}_P$ and $\bar{v}_D$ = partial specific volumes of protein and detergent respectively.

ρ = density of solution (1.0005)

M = molecular weight.

$(1-\Phi\rho)$ can be assessed using the formula:

$$M = \frac{RT}{(1-\Phi\rho)\omega^2} \times \sigma$$

where M = 43000

σ = value of σy_8 moment at low concentration (see Fig. 5 for explanation of σy_8 moment)

and R,T, ω have their usual meanings.

TABLE 6

Composition of the microfibrillar component isolated from adult bovine ligamentum nuchae.

(Values are expressed as residues per 1000 amino acid residues)

Hydroxyproline	0.0
Aspartic Acid	117.9
Threonine	52.6
Serine	71.4
Glutamic acid	108.4
Proline	42.4
Glycine	113.3
Alanine	69.1
Valine	46.0
½ Cystine(x)	33.9
Methionine	10.2
Isoleucine	35.7
Leucine	83.9
Tyrosine	42.7
Phenylalanine	46.4
Tryptophan(y)	16.4
Lysine	40.7
Histidine	18.8
Arginine	51.9
	1000.0
N-acetyl glucosamine	17.6
Mannose	22.0
Galactose	9.0
Sialic acid	10.0

x = quantitated as CM-cysteine

y = quantitated on a different hydrolysate

3.5 CHEMICAL ANALYSES

Amino acid analysis

The amino acid composition of the purified S-carboxymethylated microfibrillar protein is summarised on table 7. The dicarboxylic amino acids account for about 1/5 of the total amino acid residues. This material contained no hydroxyproline.

3.6 CARBOHYDRATE COMPOSITION

The GLC analysis profile of the purified carboxymethylated microfibrillar protein is shown in Fig. 8. It contained mannose, galactose, N-Acetyl glucosamine as the only hexosamine present, and sialic acid (Table 7). The sugars were present in the molar ratios of 2:1:2:1(mannose:galactose:N-acetyl glucosamine: sialic acid) and represented about 9.4% of the dry weight of the glycoprotein. GLC analysis also revealed the presence of glucose the galactose:glucose ratio being about 1.5:1. This was considered to be an artefact because the material had been chromatographed on Sepharose during the preparations. Several reasons justified this assumption. Firstly, the amount of glucose in the crude preparation and in the purified material was variable, being higher in the latter.

Secondly, in the crude preparation, the amount of glucose was matched by an equal increment of galactose relative to N-acetyl glucosamine. This suggests that, in the crude material, glucose could arise from collagen contamination a glycoprotein which has a 1:1 ratio of glucose and galactose.

TABLE 7

The influence of temperature on chromogen production by periodate oxidation of sialic acid.

Compound (μ mole)	Absorbance at 630nm		
	0°C (20 min)	0°C (35 min)	37°C (100 min)
N-Acetyl Neuraminic Acid (0.076)	0.469	-	-
*N-Glycolyl-Neuraminic Acid (0.154)	0.80	-	-
N-Acetyl-Neuraminic and N-Glycolyl-Neuraminic Acid (0.038 + 0.077)	-	-	0.015
Carboxymethylated micro-fibrillin (0.037)	-	0.469	0.500

The details of assay were described in Section 2.10.4.

*It was discovered, from this experiment and later confirmed by GLC, that the commercially obtained standard N-Glycolyl Neuraminic Acid was in fact, N-Acetyl Neuraminic Acid.

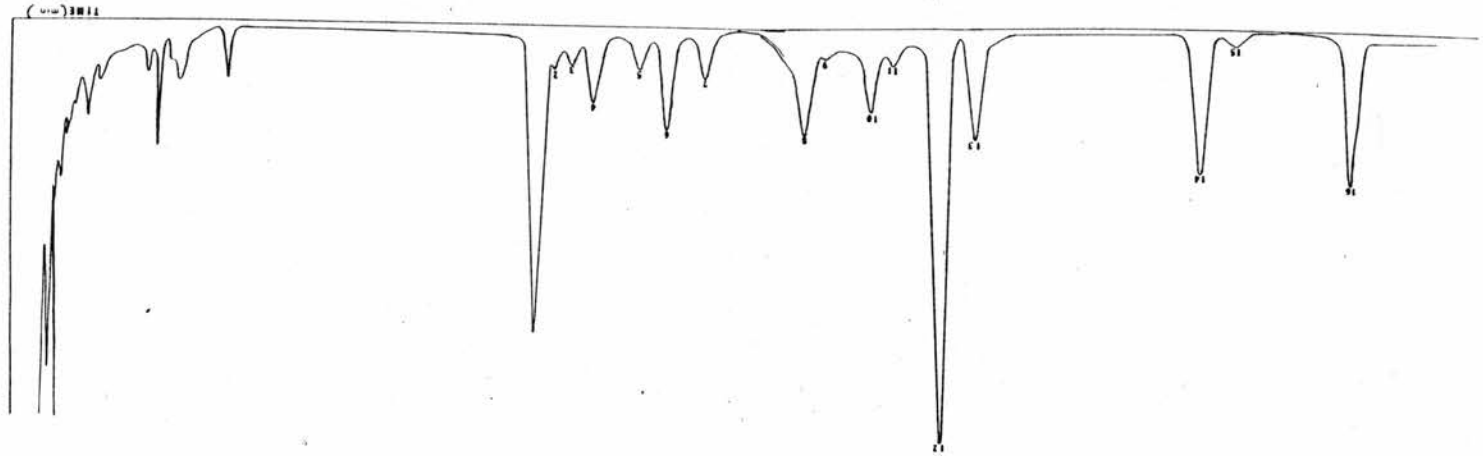

Fig. 8

GLC separation of microfibrillin sugar components as TMS methyl glycosides as described in Section 2.10.3. Peaks were numbered in their order of emergence and are identified as follows:

Mannose	1,3
Galactose	2,4,5
Glucose	6,7
N-acetylglucosamine	8,9,10,11,12,13
Perseilol	14,15 (internal standard)
N-glycolyl neuraminic acid	16

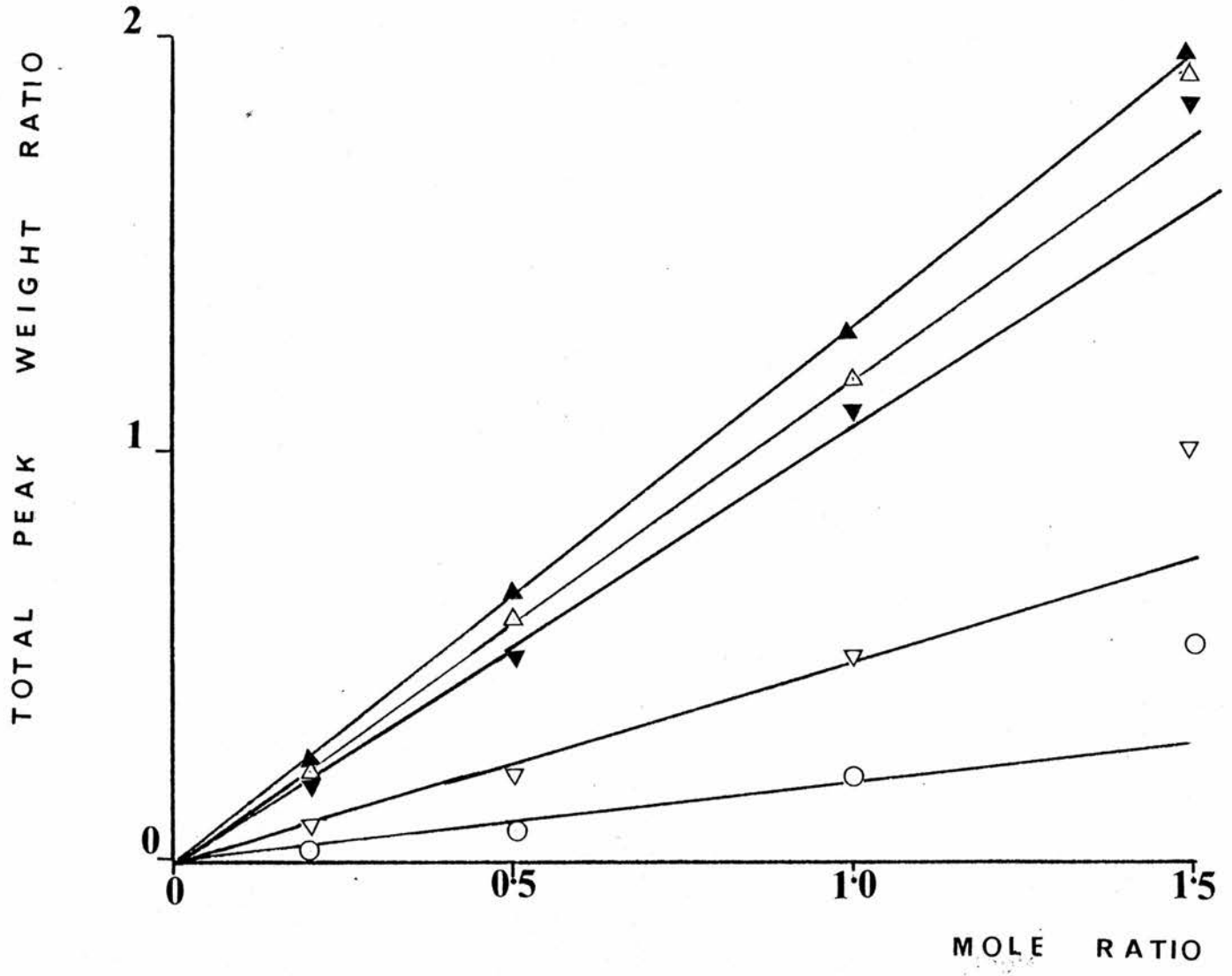
TOTAL PEAK WEIGHT RATIO
2
1
0
0
0·5
1·0
1·5
MOLE RATIO

Fig. 9

Graph showing relationship of total peak weight ratio to mole ratio of mannose, glucose, galactose, N-acetyl glucosamine and sialic acid.

Internal standard was perseitol

Molar Relative Response factor (MRRF) is given by the slope.

Assay details are as described in Section 2.10.3.

(▲): Glucose

(△): Mannose

(▼): Galactose

(▽): N-Acetyl glucosamine

(○): Sialic acid

Analyses carried out, both, on hexosamines after separation by column chromatography, and on sialic acid by a colorimetric procedure confirmed the validity of the values obtained for these sugars by GLC. In the case of the hexosamines, the sugar residues were calculated relative to the concentration of the aspartic acid - this amino acid being the first to be completely released from the protein after a few hours of hydrolysis. From the results obtained for sialic acid, using the procedure of Jourdian et al., (1971), it appeared that it was present primarily as N-glycolyl-Neuraminic acid (Table 6) as the microfibrillin protein retained its absorbance at 630 nm after 100 min incubation at 37°C. This finding was confirmed by the coincidence of the retention times, on GLC, of both the sialic acid peak from microfibrillin protein and that of the standard N-glycolyl-Neuraminic acid.

SECTION 4

DISCUSSION

Several attempts have been made to isolate and purify the microfibrillar component from various tissues and species (Ross and Bornstein, 1969; Fracassini et al., 1975; Moczar et al., 1977; Muir et al., 1976). In this work the protein was isolated from adult bovine ligamentum nuchae by a non-enzymic procedure developed in this laboratory and described in Section 2.

A very low yield of about 0.1% of dry weight of the ligament was obtained. This is in contrast to the value of 5-10% of dry weight, so far the only value reported in the literature for the yield, found in at-term foetal ligamentum nuchae by Ross and Bornstein. This discrepancy could be age-related, since the microfibrils are the first structures to appear in the morphogenesis of elastic fibres (Usuku, 1958; Low, 1962; Gotte and Serafini-Fracassini, 1963; Greenlee et al., 1966; Ross and Bornstein, 1969, 1971; Fahrenbach et al., 1966). However the two isolation methods differ considerably as Ross and Bornstein adopted an enzymic procedure which employed collagenase to remove contaminating collagen.

Another explanation to this low yield could be found in the incomplete solubilisation of the protein as we used β-mercaptoethanol to cleave the disulfide bonds resulting in incomplete carboxymethylation and decreased recovery of soluble microfibrillin. Fracassini et al. (1975) adopted a similar method to that of Ross and Bornstein but

employed dithioerythritol which is more efficient, and obtained a yield of 0.7%.

Purification of the microfibrillar component has always been a major problem in the investigation of this protein due to numerous reasons. One of these and the most important one is intricate association of microfibrillin with other structural glycoproteins, collagen and even tropoelastin (Wolff et al., 1971; Anderson et al., 1972; Fracassini and Smith, 1974). In the present investigation attempts to purify the crude material by gel chromatography on Sepharose 4B-CL revealed that each of the two peaks A_2 and A_3 eluted were almost identical with respect to their SDS-gel electrophoretic patterns (Fig. 5) and when a portion of peak A_3 which contained the microfibrillar protein was rechromatographed on the same column, it produced a single broad peak, the SDS-gel electrophoretic pattern of which was again almost identical to those of the previous two peaks.

Self-aggregation of the microfibrillar protein has also been implicated in this phenomenon (Kadar et al., 1973; Moczar et al., 1977). Moczar et al. reported that self-aggregation was most evident when disulfide bonds were incompletely reduced. In our experiments although the protein was S-carboxymethylated, aggregation and entrapment of P.A.S. positive material at the surface of acrylamide gels was observed. This aggregation of material was independent of the amount of protein loaded.

Increasing the concentration of the β-mercaptoethanol from 2% to 3% (v/v) during sample preparation (Section 2) decreased the aggregation considerably and eliminated it completely when pure microfibrillin was examined. However, band D (see Fig.5) which in terms of its molecular weight could be considered to represent the dimer of band M, was always present at all stages of the purification except in the purified material. It, therefore, appears as postulated in paragraph 3 that the initial solubilisation and S-carboxymethylation was incomplete as fully S-carboxymethylated proteins do not usually require further reduction before application on acrylamide gels.

The purity of the final preparation is evident from the SDS-acrylamide electrophoretic patterns which gave a single P.A.S. positive band which overlapped that stained by Coomassie blue and exhibited an apparent molecular weight of about 35,000 using gels of various porosities. Analytical ultracentrifugation data yielded a molecular weight of about 21,000 in guanidinium hydrochloride after correction for non-ideality.

The lack of correlation of the molecular weight values determined by the two procedures could be attributed to the well-known anomalous behaviour which glycoproteins with significant amounts of carbohydrate exhibit during electrophoresis on SDS-acrylamide gels (Weber et al., 1972; Schubert, 1976; Pitt-Rivers and Impiobato, 1968). The cause of this phenomenon is not well understood, but it has been

suggested that an interaction between carbohydrate and polyacrylamide may lead to a disproportionate retardation of glycoproteins relative to proteins containing little or no carbohydrate. Furthermore there may be an increase in the hydrodynamic volume of glycoproteins due to the hydration of the carbohydrate moiety and/or branching of the carbohydrate chain which reduce migration (Schubert, 1976; Segrest and Jackson, 1972).

Proteins and glycoproteins also exhibit differential detergent binding capacity. It has been suggested that glycoproteins may not be completely dissociated in SDS solution containing a reducing agent such as β-mercaptoethanol. And since the degree of binding depends to a certain extent on the unfolding and complete dissociation of the molecule, glycoproteins with branched carbohydrate chains and unfolded proteins may be expected to show lowered SDS binding (Nelson, 1971; Pitt-Rivers and Impiobato, 1968; Katzman, 1972). In this respect it is worth noting that increasing the ionic strength results in an increase of the amount of detergent bound (Nelson, 1971). And as SDS-protein binding is ionic, highly negatively charged proteins may exhibit lowered binding particularly if they carry a considerable number of sialic acid residues.

Of these hypotheses those invoking interaction of carbohydrate with the acrylamide and/or variation of the hydrodynamic volume appear to be more appropriate in our case as the amount of SDS bound by microfibrillin exceeded, under the experimental conditions used, that of ovalbumin.

As far as the variability in the apparent molecular weight on gel electrophoresis is concerned, this follows again, a well-known pattern. The apparent molecular weight of glycoprotein, in fact, varies with the gel porosity decreasing with increasing acrylamide gel cross-linking. It has been suggested that molecular sieving - one of the two factors involved in electrophoresis - predominates with increasing acrylamide concentration. Under these conditions the anomalously high apparent molecular weight decreases, approaching in an asymptotic manner values close to the real molecular weight (Segrest and Jackson, 1972).

Neither the value obtained in guanidinium hydrochloride nor that estimated in SDS by ultracentrifugation agreed with that (~15,000) obtained by Fracassini et al., (1975) using a preparation treated with collagenase. It is possible as it has been pointed out earlier in Section 1, that collagenase may have induced some degradation. The value of 35,000 obtained by SDS electrophoresis however agreed with that recently reported by Moczar et al., (1977) although different gel porosities were used in the two experiments. The value of 270,000 reported by Muir et al., is far from those given by all other authors. As it has already been postulated in Section 1, there could have been gross contamination by other tissue proteins and glycoproteins particularly by fibronectin as microfibrillin has a strong tendency to form aggregates with other structural glycoproteins (Moczar et al., 1977; Kadar et al., 1973).

The amino acid composition of the preparation (see Table) is very similar to those reported for different preparations of different tissues like bovine ligamentum nuchae (Ross and Bornstein, 1969; Fracassini et al., 1975); pig aorta (Moczar et al., 1977); monkey smooth muscle cells from tissue cultures (Muir et al., 1976), except with respect to glycine, ½ cystine and methionine residues. The glycine and ½ cystine contents were higher in the present preparation than those reported by Fracassini et al., (1975). The methionine and ½ cystine contents were also higher than those reported by Moczar et al., (1977) and surprisingly the overall amino acid composition was closest to that of Muir et al., (1976).

The preparations differ also with regard to their carbohydrate contents. These variabilities could be attributed to age, tissue and/or species specificity.

Sialic acid in this preparation was identified as N-glycolyl-Neuraminic acid. Although fucose was present in the crude preparation, it was not detected in the purified microfibrillin and is therefore presumably located in the protein originating in the D band (Fig. 5). This finding is of some relevance when considered in conjunction with the work carried out by Sear and colleagues (1978) on tissue cultures of ligamentum nuchae, as they have used L-$\left[6\text{-}^{3}H\right]$ Fucose for the identification of newly synthesized microfibrillin.

Although no conclusive evidence is presented to show that the material investigated here is microfibrillin, its close similarity to preparations isolated by collagenase digestion implies that it is in fact connective tissue microfibrillin. (Ross and Bornstein, 1969; Fracassini et al., 1975). More conclusive evidence could be gained by (a) reconstituting the purified preparation for examination under the electron microscope and (b) testing for reaction with microfibrillin-specific antibodies.

BIBLIOGRAPHY

ADAMS, C.E.M. and ADAMS, C.W.M. (1965). Neurochemistry (editors). American Elsevier Publishing Company Inc. N.Y. p.67.

AMINOFF, D. (1961). Biochemical Journal 81, 384.

ANDERSON, M.C. and JACKSON, D.S. (1972). Biochemical Journal 127, 179-186.

BENTLEY, R., SWEELEY, C.C., MAKATA, M. and WELLS, W.W. (1963). Biochemical and Biophysical Research Communications 11, 14.

BHATTI, T., CHAMBERS, R.E. and CLAMP, J.R. (1970). Biochimica et Biophysica Acta 222, 339-347.

BIRDI, K.S. (1976). Analytical Biochemistry 74, 620-622.

BRAUN, P.E. and RADIN, N.S. (1969). Biochemistry 8, 4310.

CLAMP, J.R., BHATTI, T., CHAMBERS, R.E. in Methods of Biochemical Analysis, Vol. 19

CHERVENKA, C.H. (1970). Analytical Biochemistry 34, 24-29.

CRAIG, L.C., KING, T.P. and STRACHER, A. (1957). Journal of American Chemical Society 79, 3729.

CRAIG, L.C. and KONISBERG , W. (1961). Journal of Physical Chemical 65, 166.

DOHNAL, C.J. and GARVIN, J.E. (1979). Biochimica et Biophysica Acta 576, 393-403.

FAHRENBACH, W.H., SANDBERG, L.B. and CLEARY, E.G. (1966) Anatomical Record 155, 563-575.

FAIRBANKS, G., STECK, T.L. and WALLACK, D.M.F. (1971). Biochemistry 10, 13, 2606-2617.

FISH, W.W., REYNOLDS, J.A. and TANFORD, C. (1970). Journal of Biological Chemistry 245, 19, 5166-5168.

FURLAN, M., SEELICH, T. and BECK, E.A. (1975). Biochimica Biophysica Acta 400, 112-120.

GIBBON, R.A. (1966) in Gottschalk, Alfreds (editor). Glycoproteins Vol. 5, p.61 Elsevier Publishing Company.

GOTTE, L. and SERAFINI-FRACASSINI, A. (1963). Journal of Atherosclerosis Research 3, 247.

GREENLEE, T.K. Jnr., ROSS, R. and HARTMAN, J.L. (1966). Journal of Cell Biology, 30, 59.

HAUST, M.D. (1965). American Journal of Pathology 47, 1113.

HAYASHI, K. (1975). Analytical Biochemistry 67, 503-506.

HOLLIGAN, P.M. (1971). New Phytology 70, 239-269.

HORNING, E.C. and VANDEN-HEUVEL, W.J.A. (1963). Annual Review of Biochemistry 32, 709.

HOTCHKISS, R.D. (1948). Archives of Biochemistry 16, 131-141.

JI, T.H. and BENSON, A.A. (1968). Biochimica Biphysica Acta 150, 686-693.

JOURDIAN, G.W., DEAN, L. and ROSEMAN, S. (1971). Journal of Biological Chemistry 246, 2, 430-435.

KADAR, A., ROBERT, B. and ROBERT, L. (1973). Pathology Biology Supplement 21, 80-88.

KARRER, H.E. (1961). Journal of Ultrastructural Research 5, 1.

KATZMAN, R.L. (1972). Biochimica et Biophysica Acta 266, 269-272.

KEYSER, J.W. (1964). Analytical Biochemistry 9, 249.

LAEMMLI, U.K. (1977). Journal of Biological Chemistry 252, 1102-1106.

LOW, F.N. (1962). Anatomical Record 142, 131.

MATHEKA, H.D., ENZMANN, P.J., BACHRACH, H.L. and MIGI, B. (1977). Analytical Biochemistry 81, 9-17.

McMANUS, J.F.A. (1946). Nature 158, 202.

MOCZAR, M., MOCZAR, E. and ROBERT, L. (1977). Biochimie 59, 141-151.

MUIR, L.W., BORNSTEIN, P. and ROSS, R. (1976). European Journal of Biochemistry 64, 105-114.

NELSON, C.A. (1971). Journal of Biological Chemistry 246, 12, 3895-3901.

NOZAKI, Y. (1972). in S.P. Colowick and N.O. Kaplan (editors) Methods in Enzymology. Vol. XXVI, p.43.

PENKE, B., FERENCZI, R. and KOVACS, K. (1974). Analytical Biochemistry 60, 45-50.

PERRY, M.B. (1964). Canadian Journal of Biochemistry 42, 451.

PITT-RIVERS, R. and AMBESI-IMPIOBATO, F.S. (1968). Biochemical Journal 109, 825-830.

PROCKOP, D.J. and UDENFRIEND, S. (1960). Analytical Biochemistry 1, 228-239.

RADHAKRISHNAMURTHY, B., DALFRERES, E.R.Jnr., and BERENSON,G.S. (1966) Analytical Biochemistry 17, 545-550.

REYNOLDS, J.A. and TANFORD, C. (1970). Journal of Biological Chemistry 245, 5161-5165.

RICHEY, J.M., RICHEY, H.G. Jnr., and SCHRAER, R. (1964) Analytical Biochemistry 9, 272.

ROSS, R. (1968) Treatise on Collagen. B.S. Gould (editor) Academic Press Inc. London p.1.

ROSS, R. and BORNSTEIN, P. (1969) Journal of Cell Biology 40, 366-381.

ROSS, R. and BORNSTEIN, P. (1970). Chemistry and Molecular Biology of the Intercellular Matrix, Vol. 1. pp.641-655. New York.

ROSS, R. and BORNSTEIN, P. (1971). Scientific American 244, 44-52.

ROWE, S. and STEINDHARDT, J. (1976) Biochemistry 15, 2579-2585.

SANDBERG, L.B. (1976) International Review of Connective Tissue Research.

SCHUBERT, D. (1970). Journal of Molecular Biology 51, 287-301.

SEAR, C.H.J., KEWLEY, M.A., JONES, C.J.P., GRANT, M.E. and JACKSON, D.S. (1978). Biochemical Journal 170, 715-718.

SEGREST, J.P. and JACKSON, R.L. (1972) in Methods in Enzymology, Grinsburg, V. (editor) Vol. XXVIII, Part B, pp. 54-63.

SERAFINI-CESSI, F. and CESSI, C. (1964) Analytical Biochemistry 8, 527-528.

SERAFINI-FRACASSINI, A., FIELD, J.M. and ARMITT, C. (1975) Biochemical and Biophysical Research Communication 65, 1146-1152.

SERAFINI-FRACASSINI, A. and SMITH, J.W. (1974) The Structure and Biochemistry of Cartilage, Churchill Livingstone. Edinburgh and London.

SHAPIRO, L.A., VINUELA, E. and MAIZEL, V.J. (1967) Biochemical and Biophysical Research Communication 28, 5. 815-820.

SHIPP, W.D. and BOWNESS, J.M. (1974). Biochimica et Biophysica

STEINHARDT, J. and REYNOLDS, J.A. (1970). Multiple Equilibra in Proteins. New York, Academic Press.

STIMSON, W.H. (1971). FEBS Letters 13, 1, 17-20.

SWEELEY, C.C., BENTLEY, R., MAKHA, M. and WELLS, W.W. (1963) Gas-Liquid Chromatography of Trimethylsilyl derivatives of Sugars and Related Substances. Journal of American Chemical Society 85, 2497.

TANFORD, C. (1973). The Hydrophobic Effect. Wiley, New York.

TANFORD, C., NOZAKI, Y., REYNOLDS, J. and MAKINO, S. (1974) Biochemistry 13, 11, 2369-2376.

USUKU, G. (1958). Kumamoto Medical Journal 11, 84.

WEBER, K. and OSBORN, M. (1969). Journal of Biological Chemistry 244, 16, 4406-4412.

WEBER, K., PRINGLE, J.R. and OSBORN, M. (1972) in S.P. Colowick and N.O. Kaplan (editors) Methods in Enzymology. Vol. 26, pp.3-27.

WOLFF, I., FUCHSWANS, W., WEISER, M., FURTHMAYER, H. and TIMPL, R. (1971). European Journal of Biochemistry 20, 426-431.

YPHANTIS, D.A. and ROARK, D.E. (1972). Biochemistry 11, 2925-2934.

ZACHARIUS, R.M. and ZELL, T.E. (1969). Analytical Biochemistry 30, 148-152.

ZAMYATNIN, A.A. (1972). Progress in Biophysical and Molecular Biology 24, 109-123.