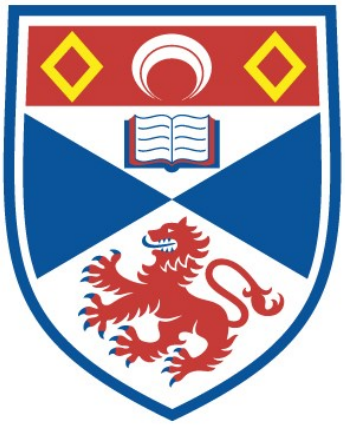
CHARACTERIZATION OF A STRUCTURAL GLYCOPROTEIN FROM BOVINE LIGAMENTUM NUCHAE EXIBITING DUAL AMINE OXIDASE ACTIVITY

Giambattista Ventrella

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1981

CHARACTERIZATION OF A STRUCTURAL GLYCOPROTEIN FROM BOVINE LIGAMENTUM NUCHAE EXIBITING DUAL AMINE OXIDASE ACTIVITY

by

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A thesis presented to the
University of St. Andrews
for the Degree of Doctor of Philosophy.

Department of Biochemistry,
University of St. Andrews.

1981

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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, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Professor A. Serafini-Fracassini.

CERTIFICATE

I hereby certify that G. Ventrella has spent nine terms in research under my direction and that he has fulfilled the conditions of Ordinance General No.12, and Resolution of the University Court 1967, No.1, and that he is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

ACADEMIC RECORD

I matriculated at the University of Bologna (Italy) in October 1971, and obtained the degree of Doctor of Chemistry (Laurea) in July 1977.

I matriculated as a research student in the Department of Biochemistry, University of St. Andrews in October 1977.

ACKNOWLEDGEMENTS

I should like to express my sincere gratitude to Professor A. Serafini-Fracassini for all his help and patient guidance in the course of this work.

I am also grateful to Dr. J. Hinnie for his additional advice and to Dr. R. Griffiths for his assistance during the enzymic assays. My thanks also to Mr. J.C. Hunter for the operation of the amino acid analyzer, to Mr. C. Armitt for the operation of the ultra centrifuge, to Mr. W. Blyth for the photographic work and to all the other members of the technical staff of the Biochemistry Department.

CONTENTS

ABBREVIATIONS:

Allysine	α-Amino-adipic-δ-semialdehyde
BAPN	β-Aminopropionitrile
CMC	Critical micelle concentration
DMAB	p-Dimethylaminobenzaldehyde
DTE	Dithioerythreitol
EDTA	Ethylenediaminetetracetic acid disodium salt
FMD	Fraction of maximum deviation
Gal	Galactose
GLC	Gas-liquid chromatography
Glc NAc	N-Acetyl Glucosamine
Gu-HCl	Guanidine hydrochloride
Man	Mannose
NEM	N-ethylmaleimide
PAGE	Polyacrylamide gel electrophoresis
Macro-PAGE	Preparative polyacrylamide gel electrophoresis
PAS	Periodic acid Schiff
PMSF	Phenyl methyl sulphonyl fluoride
SDS	Sodium dodecyl sulphate
SGP	Structural glycoprotein
TEMED	Tetramethylethylenediamine
Tris	Tris-(hydroxy-methyl)-amino ethane

ABSTRACT: A structural glycoprotein has been extracted from bovine ligamentum nuchae, using 5M guanidine hydrochloride containing a disulphide bond reducing agent, and purified by preparative gel electrophoresis. The isolated material appeared to be monodisperse with a molecular weight of ∿ 34 000 as shown by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and by analytical ultracentrifugation. It contains 10% carbohydrate comprising mannose, N-acetyl-glucosamine, galactose and sialic acid in a 6:5:3:3 molar ratio. The glycoprotein has been assayed for peptidyl-lysine oxidase activity using ^{3}H lysine-aortic elastin, prepared from 15- to 17-day-old chick embryos, as a substrate. In the absence of free lysine, the specific activity of the preparation over a 2h incubation was ∿ 60 x 10^{4} dpm/mg purified protein. Addition of 10mM lysine resulted in an ∿ 50% decrease in the specific activity. Free lysine was shown to act as a substrate for the glycoprotein preparation as indicated by control experiments using ^{3}H lysine in place of the aortic substrate. These results demonstrate that the glycoprotein exhibits a dual amine oxidase activity. In the presence of 0.27mM β-aminopropionitrile fumarate, a concentration which completely inhibits peptidyl-lysine oxidase activity in other lysyl oxidases, the glycoprotein preparation was inhibited by ∿ 14%. In the absence of 5M guanidine hydrochloride and a reducing agent, the

glycoprotein undergoes aggregation which in the presence of copper ions results in the formation of cylindrical tactoids, the diameter of which (11nm) corresponds closely to that of the fibrils which in the majority of connective tissue matrices constitute the microfibrillar component mainly associated with elastic fibres.

SECTION ONE

INTRODUCTION

INTRODUCTION

Electron microscopic studies on elastic tissue have shown that most vertebrate elastic fibres consist of two morphologically distinct components: elastin, appearing as a centrally located amorphous structure and tubular appearing microfibrils, of about 11nm in diameter, which surround the elastic core (Greenlee et al., 1966; Fahrenbach et al., 1966).

The staining properties of these structures vary considerably: the microfibrils show marked affinity for both cationic lead and uranyl acetate while the amorphous component is stained by anionic phosphotungstic acid (Greenlee et al., 1966).

The fact that the microfibrillar component can be solubilized only by treatment with chaotropic solutions containing reducing agents such as 2-mercaptoethanol or dithioerythreitol lead Robert et al. (1971a) to suggest that these microfibrils could be closely related to a group of glycoproteins that because of their relatively high content of disulphide bonds have been classified by Moczar and Robert (1970) as structural glycoproteins.

Studies on these structural glycoproteins (SGP) have been mainly carried out on porcine aorta and on bovine ligamentum nuchae where they have been reported to represent a variable proportion of the tissue dry weight.

SGP preparations described in the literature are often heterogeneous (Downie et al., 1973; Timpl et al., 1968; Wolff et al., 1971) and it is not clear whether this heterogeneity is due to aggregation or polymerization of subunits, to degradation of larger polypeptides, or to the presence of a large number of different

proteins. Often the techniques used for the isolation of SGP are relatively harsh: autoclaving was used by Keeley et al. (1972), hot trichloroacetic acid by McCullagh et al. (1973), and Moczar and Robert (1970) while digestion with collagenase of unspecified purity was performed by Timpl et al. (1968). Some SGP has been extracted with cold or even hot dilute NaOH (Barnes and Partridge, 1968; Timpl et al., 1969). Some, if not all of these techniques could result in degradation of the polypeptide chains.

SGP has been found in large amounts in aorta and it has been suggested that it could play a role in the pathogenesis of atherosclerosis (McCullagh et al., 1973; Ouzilou et al., 1973; Robert et al., 1971b).

So far the only available data on the composition of homogeneous preparations of SGP are those published (a) by Ross and Bornstein (1969) on the microfibrillar component isolated from foetal bovine ligamentum nuchae and purified by collagenase treatment, and (b) by Moczar et al. (1977) on a structural glycoprotein isolated from pig aorta. The latter behaved on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as a single 35000 molecular weight species and contained mannose, galactose, N-acetylglucosamine and sialic acid in 4:3:3:1 molar ratios. It showed strong tendency to form aggregates by self-association and by interaction with collagen.

The protein moieties were rich in polar and sulphur containing amino acids but did not contain any of the polyfunctional amino acids characteristic of elastin.

Recently the existence of SGP as a major component of the aortic

wall has been questioned by Bach and Bentley (1980) in an article showing that most of the 35000 molecular weight material extracted from aorta and considered to be SGP is in fact actin.

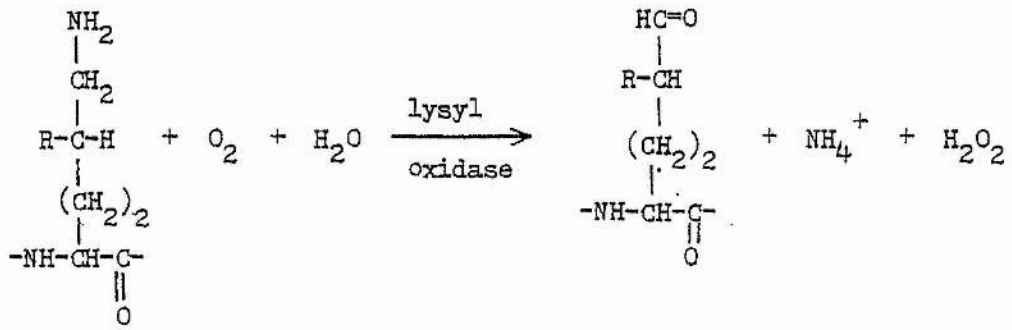
The present investigation was carried out on a structural glycoprotein extracted from bovine ligamentum nuchae (that will be referred to as ligamentum nuchae SGP) as a result of the low cellular content of this tissue and therefore avoiding gross contamination by actin.

Once the general characteristics of SGP had been determined, it became apparent that similarities in amino acid composition and molecular weight existed with lysyl oxidases isolated from bovine tissues (Jordan et al., 1977; Kagan et al., 1979) (Table IV).

These enzymes are present in connective tissues (Stassen, 1976; Vidal et al., 1975; Harris et al., 1974. Narayanan et al., 1972; Kagan et al., 1979; Jordan et al., 1977) where there is evidence for a multiplicity of enzyme species since at least two peaks (Vidal et al., 1975) and in some cases four peaks (Stassen, 1976; Jordan et al., 1977; Kagan et al., 1979) of enzyme activity can be resolved on DEAE cellulose. Since these multiple species do not seem to originate from proteolysis in vitro of a common parent molecule, it is possible that proteolysis in vivo is the basis of heterogeneity, possibly as an important processing event in the biosynthesis and/or secretion of lysyl oxidase.

Lysyl oxidases are responsible for the initiation of the biogenesis of cross-links in both collagen and elastin. They catalyze the oxidative deamination of ε-amino groups of certain lysyl and hydroxylysyl residues in these molecules (Pinnell and Martin, 1968). As shown by the following equation, peptidyl

lysine is oxidized to form allysyl residues which are then involved in a series of non enzymic reactions yielding several polyfunctional amino acids via aldol condensation and Schiff-base formation.



Lysyl (R=H) or hydroxylysyl (R=OH) residue.

Allysine (R=H) or Hydroxy-allysine (R=OH).

The enzyme is copper dependent and utilizes molecular oxygen as cosubstrate and hydrogen acceptor.

The assay system I used to measure amino oxidase activity is essentially that originally developed by Pinnell and Martin (1968). In this procedure the substrate is represented by chick aorta incubated for 48h in the presence of L- 4,5-^{3}H -lysine. The rate of convertion of peptydyl-lysine into allysine by the enzyme is assessed by the amount of tritium released, which is recovered by microdistillation.

The effect of β-aminopropionitrile (BAPN) on SGP enzymic activity was also investigated. BAPN is known to inhibit lysyl oxidases and to suppress the formation of cross-links in both collagen and elastin <u>in vivo</u> (Gross <u>et al</u>., 1960; Miller <u>et al</u>., 1967). Its action on amine oxidases is less pronounced.

Page and Benditt (1967b) proposed two mechanisms by which the

reagent may cause inhibition of oxidative deamination and cross link formation of collagen and elastin: (a) a highly reactive aldehyde derived from BAPN by the action of the enzyme may interact with the ε-amino function of strategic lysyl residues of collagen and elastin and block their conversion into allysine, and/or (b) BAPN may compete with native substrate molecules for the active site of the amino oxidase and therefore reduce the rate of metabolism (Bird et al., 1966).

However lysyl oxidase activity is irreversibly inhibited when the concentration of BAPN is brought up to 4-10 μM which is approximately 100-fold less than concentrations required for the competitive inhibition of amine oxidases reported by Tage and Benditt (1967a). Therefore Walsh (1978) and Narayanan et al. (1972) proposed an alternative mechanism by which BAPN forms a covalent linkage with the enzyme itself.

To assess whether SGP exhibits lysyl oxidase activity exclusively or a more general amine oxidase function, free lysine (10mM) was added to the aortic substrate in order to suppress any activity not specific for peptide-bound lysyl residues (Siegel 1979).

Attempts were also made to elicit the fibril formation of SGP in vitro. In the presence of Cu^{++} the glycoprotein formed precipitates which under the electron microscope appeared to be constitued by fibrils exhibiting lateral dimensions identical with those of the microfibrils observed in the embryonic and mature tissues by Greenlee et al. (1966).

Most electron microscopists agree that the microfibrils are the first structures to appear during the morphogenesis of the elastic

tissue (Karrer, 1961; Low, 1962; Fahrenbach et al., 1966; Greenlee et al., 1966) with elastin forming later at the centre of bundles of microfibrils.

The observation that this fibrillar envelop of the elastic fibril consists of a polymeric form of a lysyl oxidase may be of interest in the understanding of the morphogenesis of elastic tissues.

SECTION TWO

MATERIALS AND METHODS

2.1 CHEMICALS

All reagents (Analar or Aristar grade) were obtained from B.D.H. Chemicals Ltd. Proteolytic inhibitors, enzymes, molecular weight marker proteins, carbohydrate standards and guanidine hydrochloride were purchased from Sigma Ltd.

2.2 PURIFICATION OF CHEMICALS

Of all the chemicals used only guanidine hydrochloride and SDS were further purified.

Guanidine hydrochloride (Practical grade - Sigma Ltd) contains some impurities such as biguanidine and guanylurea. For most procedures (i.e. extraction purposes) it was only necessary to filter the insoluble materials from the solutions, but for ultracentrifugation analyses contaminants were removed by recrystallization.

SDS (BDH, special grade for electrophoresis) was also purified since Birdi (1976) showed that commercially available SDS of 99.5% purity contains a high proportion (up to 31%) of higher chain-lengths molecules. The main contaminants of SDS are mainly tetradecyl sulphate and decyl sulphate plus small amounts of inorganic salts and organic compounds such as unsulphated alcohols (Birdi, 1976).

2.2.1 Purification of Guanidine hydrochloride

The purification was performed according to the Nozaki (1972) procedure.

Aliquots (250g) of Gu-HCl (Practical grade) were dissolved in 1 litre of cold ethanol and heated under reflux in a round-bottomed flask with overhead stirrer. The solution was then filtered through a Buckner funnel and 500ml of benzene was gradually added to the hot ethanol-Gu-HCl solution that was then allowed to cool at $4^{\circ}C$ for 12 hours. The crystalline needles were collected and rinsed with small volumes of the ethanol-benzene mixture. At this stage the yield was about 70%.

A further recrystallization from methanol was performed on these crystals by dissolving them in 320ml of near-boiling methanol, cooling the solution in a dry ice-acetone mixture for several hours and collecting the crystals on a cooled Buckner funnel. The crystals were moistened with chilled methanol and finally dried by rotary evaporation at 37°C.

The final yield was approximately 38%.

2.2.2 Purification of SDS

Aliquots (20g) of sodium dodecylsulphate were suspended in 200ml of absolute ethanol and heated under reflux in a 1 litre round-bottomed flask. When SDS was completely dissolved, the solution was allowed to cool at room temperature and centrifuged at 23 000g (at 4°C). The solid residue that formed was rinsed with cold ethanol (approx. 200ml), filtered through a Buckner funnel and dried by rotary evaporation at 37°C.

2.3 DETERMINATION OF THE CRITICAL MICELLE CONCENTRATION (CMC) OF SDS.

The purity of sodium dodecyl sulphate recrystallized according to the procedure previously described was checked by determining its critical micelle concentration in distilled water by the method of dye solubilization, employing naphthalene.

Since log CMC varies linearly with the alkyl hydrocarbon chain length, accurate CMC measurements of SDS samples should, in principle, show different values if the content of decyl sulphate or tetradecyl sulphate is rather high.

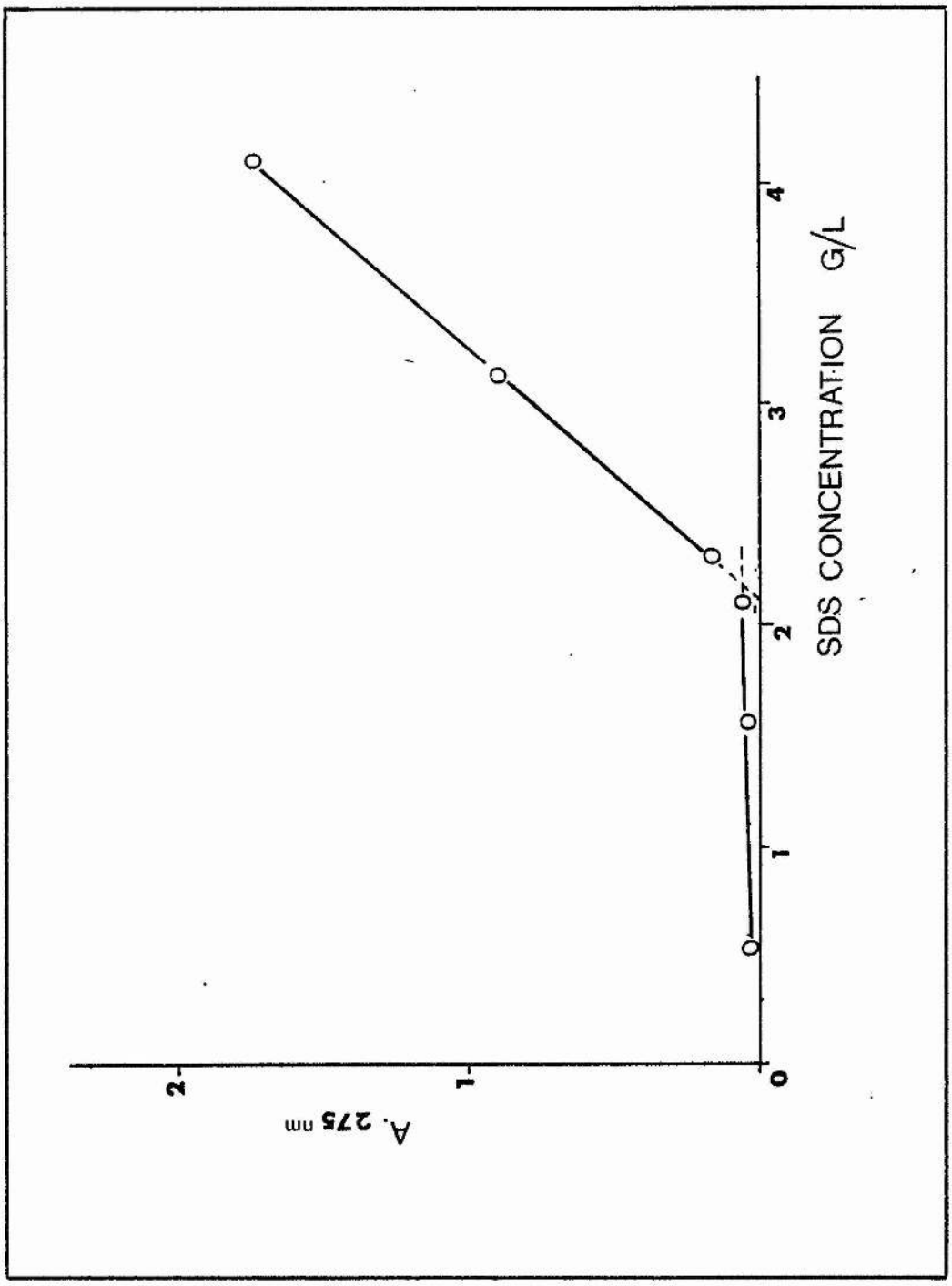
Aqueous solutions containing 0.5g/litre to 4.0g/litre of SDS were prepared. 20mg of naphthalene was added to 5ml of each SDS solution in a test-tube capped with parafilm and the suspensions shaken for 48hrs to reach equilibrium. After centrifugation of the excess naphthalene, the amount dissolved in the micellar solution was determined by the absorption at 275nm.

A plot of absorbance versus concentration of SDS is given in Fig 1. The value of CMC was determined from the break in the curve and corresponded to 2.15g/litre. (The CMC value of highly pure sodium dodecyl sulphate has been reported by many workers (Mukerjee, 1958; Emerson and Hotzer, 1967) to be between 2.32 and 2.34g/litre at 25°C in water.)

A CMC value of 1.75 g/litre was obtained under analogous experimental conditions for SDS before recrystallization.

Fig 1:

Determination of the critical micelle concentration of SDS in distilled water.


A. 275 nm
0
1
2
0
1
2
3
4
SDS CONCENTRATION G/L

2.4 ACETYLATION OF VISKING DIALYSIS TUBING

Visking tubings (8/32 and 18/32) were obtained from Scientific Instruments Ltd. (London).

Although it is claimed that insulin with a molecular weight of 6000 diffuses very slowly through 8/32 membranes (thickness: 0.064mm) and does not diffuse at all through 18/32 membranes (thickness: 0.0175mm) personal experience has shown that 8/32 tubing does not retain completely proteins of molecular weight up to 40 000 especially during exhaustive dialysis in the presence of 8M urea or guanidine hydrochloride, and therefore it proved necessary to reduce the porosity of the membranes by acetylation.

The technique used was essentially that described by Craig and Konigserg, 1961.

A suitable length of Visking tubing was soaked in water and the lower end tied while the upper opening was slipped over a glass collar.

After rinsing with dry pyridine the membrane was hung inside a glass cylinder of the same length that was then filled with a 10% solution of acetic anhydride in pyridine.

After 15hr 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 i.e. no further absorption at 260nm was monitored.

2.5 ISOLATION OF SGP FROM BOVINE LIGAMENTUM NUCHAE

Ligamentum nuchae from three-year-old cattle was freed from adhering tissue and minced at 4°C. Aliquots (250g) were stirred at 4°C for two 24hr periods in 1% NaCl containing 25mM EDTA, 10mM N-ethylmaleimide (NEM) and 1mM phenylmethanesulphonylfluoride (PMSF). The concentration of PMSF (half life of about 6hr) in this and subsequent manipulations was maintained by addition of fresh reagent once every 12hr. After the second treatment with NaCl, the pellets were washed with distilled water and defatted with a 1:3 (V/V) mixture of chloroform-methanol followed by a second treatment with a 1:2 (V/V) mixture of the same solvents.

The dried material was suspended in 1 litre of 5M guanidine HCl -0.1M Tris (pH 7.4) containing 25mM EDTA, 10mM NEM and 1mM PMSF and extracted for 24hr at 4°C with continuous stirring. The residue was collected by centrifugation and washed exhaustively with 5M GuHCl-0.1M Tris (pH 7.4) containing 25mM EDTA and 1mM PMSF prior to a further extraction at 37°C under N_2 with 1 litre of the same medium containing 20ml of 2-mercaptoethanol (0.285M) adjusted to pH 8.5 with tetramethylethylene diamine (TEMED). After 24hr, the solution was centrifuged at 23 000g for 30min under N_2 and the supernatant was added to three volumes of absolute ethanol at 4°C.

After 12hr the precipitate that formed was collected by centrifugation at 4°C, dried by serial washing with ethanol, acetone and ether and stored at -10°C.

An aliquot of tissue was extracted as above, outwith the presence of protease inhibitors to determine the degree of

proteolytic degradation in the absence of these inhibitors. SDS disc gel electrophoresis was used to compare the composition of the crude extracts isolated with and without inhibitors.

2.6 PURIFICATION OF LIGAMENTUM NUCHAE

Purification of SGP was performed by a combined use of ultrafiltration and preparative polyacrylamide-gel electrophoresis (Macro-PAGE), since satisfactory results could not be achieved by gel filtration or ion-exchange chromatography.

2.6.1 Ultrafiltration

An aliquot (200mg) of the crude non-carboxymethylated protein preparation was dissolved in 20ml of 8M urea -0.1M Tris buffer pH 7.4 containing 3% 2-mercaptoethanol under N_2.

After 12hr the suspension was centrifuged at 23 000g for 30min and the supernatant filtered through a Diaflo XM-100 membrane (Amicon Ltd., Holland) fitted on a 50ml cell (Amicon Ltd., Holland) under a 3 psi N_2 pressure prior to dialysis against water using acetylated Visking tubing 18/32 (Craig and Konigsberg, 1961). The resulting precipitate was freeze dried and stored at -10°C.

2.6.2 Preparative SDS-acrylamide gel electrophoresis (Macro-PAGE)

This method is basically an extension on a preparative scale of the analytical SDS-PAGE described in section 2.10.

The cylindrical 6x5cm gel for the electrophoresis was prepared in a separate perspex mould. 13,3g of acrylamide and 0.7g of N-N'methylenebisacrylamide were dissolved in 170ml of 8M urea-0.1M Tris buffer pH 7.4 containing 1% SDS. The catalysts (0.21g of ammonium persulphate dissolved in 5ml of the same buffer) and 0.21ml of TEMED were added and the solution rapidly poured into the gel

mould where it was left to set at 4°C for at least 24hr to ensure complete polymerization. The final acrylamide concentration of the gel was 8%.

After polymerization, the gel was transferred from the mould to the support tube of the Brownstone tank of the electrophoresis apparatus (Macropage, Birchover Instruments Ltd.) and the tank filled with 7.5l. of tank buffer (0.1M Tris-HCl pH 8.0 containing 0.2% SDS).

Before loading the protein sample, ionic impurities were removed from the gel by a period of pre-electrophoresis.

A solution of 0.01% bromophenol blue in a 8M urea-0.1M Tris-1% (W/V) SDS buffer plus 5% (W/V) sucrose, was loaded onto the gel surface under the tank buffer by inserting a syringe through the top electrode assembly to form a layer 1mm thick and the power turned on. After the band of dye had passed through the gel and the tank been refilled with fresh buffer, the protein sample, prepared as follows, was loaded.

Aliquots (35mg) of material partially purified by ultrafiltration (Section 2.6.1), and 105mg of SDS were dissolved in 2ml of 8M urea-0.1M Tris-3% mercaptoethanol pH 7.4 buffer and left stirring at room temperature. After 12hr, the solution was dialyzed overnight against the same urea buffer but with only 0.1% of 2-mercaptoethanol.

After dialysis, the solution was made 5% in sucrose and applied onto the gel as previously described. The Brownstone tank was specially modified to allow the use of urea buffers and reduce to a minimum the electrolytical decomposition of urea that would otherwise give rise to a strong U.V. absorption. The temperature

of the tank buffer was kept at 12°C to avoid precipitation of SDS and the conditions used for the electrophoresis were: 60.5 Volts and 0.23 Amp. (with a resulting power dissipation of 13.91 Watts).

Fractions (6ml) were collected every hour and their U.V. absorbance monitored at 280nm. Fractions 27 to 33 (see Fig 3) were pooled together, dialysed against distilled water for three days using acetylated Visking tubings, lyophilized and stored at -10°C.

2.7 REMOVAL OF BOUND SDS FROM THE PROTEIN

SGP purified by preparative SDS electrophoresis was obtained as a protein-SDS complex. To remove the bound SDS, the protein-SDS complex was treated with acetone according to the procedure of Hunter and Duffie (1959).

To 5ml of the SDS-protein solution in water were added at room temperature, 2ml of 1 M NaCl, 10.5ml of acetone and a few drops of 1M acetate buffer pH 3.9 to bring the pH between 5.0 and 5.3. This precipitated the protein while the SDS remained in solution.

The suspension was centrifuged and the precipitate washed with 5ml of water, 2ml of 1N NaCl and 10.5ml of acetone. The suspension was centrifuged again, the supernatant discarded and the precipitated protein solvent-dried.

While this procedure extracted most of the bound SDS, the last traces of detergent had to be removed by exhaustive dialysis. The glycoprotein was therefore dissolved in 8M urea-0.1 Tris-3% mercaptoethanol pH 7.4 and dialysed against frequent changes of 8M urea -0.1M Tris pH 7.4 for one week and finally against distilled water.

2.8 CARBOXYMETHYLATION OF SGP

For carboxymethylation (performed according to the method of Crestfield et al., 1963) an aliquot of the dried glycoprotein was dissolved in 5M GuHCl, containing 0.05M dithioerythreitol (DTE) and left on a metabolic shaker (37°C) for 24hr.

Iodoacetic acid in 1M sodium hydroxyde at a 4-fold molar excess over DTE 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 the pH of the solution adjusted to and maintained at 8.6 with 5M sodium hydroxide for 45 min. 2-Mercaptoethanol was then added to a 5-fold molar excess over iodoacetic acid.

The carboxymethylated protein was then precipitated by pouring the solution into three volumes of absolute ethanol at 4°C. After 24hr the precipitated protein was collected by centrifugation and solvent dried.

2.9 TREATMENT OF CRUDE SGP WITH COLLAGENASE

An aliquot of the partially purified SGP (i.e. after ultrafiltration) was digested with collagenase purified by affinity chromatography as described by Serafini-Fracassini et al. (1975).

Crude collagenase (collagenase type 1, Sigma, Ltd.) was first purified by dissolving an aliquot (300mg) of the crude preparation in 30ml of 5mM Tris-HCl (pH 7.5) containing 4mM $CaCl_2$ and 100mM NaCl and by adding the resulting solution to 20g of pre-swollen DEAE-cellulose (DE52-cellulose, Whatman Biochemicals Ltd.) suspended in 20ml of the same buffer.

The suspension was left stirring at 4°C and after 1hr the ion-exchanger was removed by centrifugation, the supernatant added to a fresh DEAE-cellulose suspension and the process repeated.

The resulting collagenase solution was dialyzed exhaustively against 0.1mM $CaCl_2$ at 4°C and lyophilized. An aliquot of this preparation was dissolved in 5ml of 15mM Tris (pH 7.5) containing 1mM $CaCl_2$, and applied under gravity to a column (1.3cm x 6cm) of finely milled, collagen-free elastin, prepared from bovine ligamentum nuchae by alkali treatment (45min in boiling 0.1N NaOH), equilibrated with the same buffer at 4°C. Elution was carried out at a flow rate of 20ml/hr and the enzyme contained in the first 18ml was collected and stored at 4°C. To test collagenolytic activity aliquots (0.5ml) of enzyme solution, containing a suitable amount of enzyme, were added to 9.5ml of 0.1M Tris (pH 7.5) containing 10mM $CaCl_2$ and 10mg of insoluble collagen (isolated from calf skin). The mixture was incubated at 37°C for 2hr after which time it was filtered. In the case of the control, 0.5ml of the

enzyme solution was added to a collagen suspension which had undergone a 2hr incubation at 37°C. The suspension was filtered immediately after the addition of the enzyme. The amount of soluble material that had been released by the action of collagenase was then estimated using ninhydrin to detect free amino groups. To 0.5ml of filtrate, 2ml of ninhydrin solution (1.2% solution of ninhydrin in methoxy-ethanol-sodium acetate (pH 5.5) containing 0.033% stannous chloride as antioxidant was added and the solutions mixed thoroughly for 2min. using a Vortex mixer. After 15min. at 100°C the reaction mixture was cooled, diluted with 10ml of 50% (V/V) aqueous n-propanol and the absorbance of the solution read at 578nM after 10min. at room temperature. Solutions of leucine of known concentration were used to prepare a calibration curve.

The digestion of crude SGP with this purified collagenase preparation was carried out in pH-stat at 37°C in $CaCl_2$ (10mM) keeping the pH at 7.5. When there was no indication of further digestion, the suspension was centrifuged at 38 000g for 90min, the residue resuspended in 0.1M $CaCl_2$ and the treatment with collagenase repeated. SGP was collected by centrifugation, washed with water and lyophilized.

2.10 SDS-ACRYLAMIDE DISC-GEL ELECTROPHORESIS (PAGE)

This technique has been used throughout the work to assess the purity and homogeneity of protein preparations and for molecular weight determinations.

Preparation of samples and molecular weight markers.

The protein samples were converted into SDS-protein complexes by dissolving them in 1ml of 8M urea, 0.1M Tris, 3% 2-mercapto-ethanol containing enough detergent to keep the weight ratio SDS to protein at least at 3:1. The solution was left at room temperature for 24hr and heated at 100°C for 5min.

The final protein concentration was 1mg/ml for the marker proteins and pure samples and 4mg/ml for crude samples. Samples were used directly without prior dialysis since small volumes were used.

Preparation of the gels

Gels of various porosities were prepared according to the procedure described by Weber et al, (1972). The following solutions were prepared:

a) Acrylamide solution A: 22.2g acrylamide, 0.6g N-N'-methylene-bisacrylamide per 100ml distilled water. If necessary the solution was filtered through Whatman No.1 paper (stored at 4°C).

b) Acrylamide solution B: 30g acrylamide, 0.8g N-N'-methylene-bisacrylamide per 100ml distilled water (stored at 4°C).

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

d) Reservoir buffer: prepared by dilution 1:10 with distilled water of a stock solution of 1M Tris/HCl pH8.0, containing 2% (W/V) SDS.

e) Ammonium persulphate: 0.03% (V/V) was used for 7.5% and 10% gel concentrations; 0.036% for the 5% gel and 0.023% for the 15% gel concentration. Prepared fresh before use.

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

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

h) Destaining solution: 250ml methanol, 75ml glacial acetic acid and 675ml distilled water.

Final Acryl. conc. %	5	7.5	10	15
Solution (ml)				
Acryl. A.	6.75	10.1	13.5	-
Acryl. B.	-	-	-	14.8
Dist. Water	6.75	3.4	0	0
Gel buffer	15.0	15.0	15.0	15.0
Ammonium persulph. ml from a stock sol. of 0.9g in 100cc.	1.0	1.0	1.0	0.01
TEMED	0.03	0.03	0.03	0.01

composition of acrylamide gels of various porosities

To prepare the gels, glass tubes (7.5cm long, 5mm internal diameter) were filled to a mark 6cm from the bottom with the gel solution and gently tapped to eliminate air bubbles.

A small volume of water was carefully layered onto the top of the gel using a syringe. The gels were allowed to set at room temperature for about 20-25min. After the gels 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, covered with parafilm to prevent dehydration and stored at $4^{\circ}C$.

Electrophoresis procedures

For each gel, 10µl of tracking dye (0.05% bromophenol blue in 0.1M Tris buffer pH 7.4), 1 drop of glycerol, 10µl of reservoir buffer and 10µl of reduced protein solution were mixed on a piece of parafilm and electrophoresis was carried out at room temperature with a current of 5mA per gel (3mA per gel for 15% gels) and stopped when the tracking dye was approaching the bottom of the gels. Gels were removed from the glass tubes by squirting water from a syringe with a long, fine needle between the gel and the tube wall. The distance migrated by the centre of the dye band and the length of the gels were measured before staining (2hr at room temperature).

Excess dye was washed out by rinsing the gels with distilled water and the gels left in the destaining solution, with the addition of small quantities of DE52 cellulose to adsorb the dye diffusing from the gels. When the background was cleared, the gels were photographed through a yellow filter and scanned through a Vitatron densitometer (TLD 100 Fisons) at 570nm using a 0.1mm slit. Mobilities were then calculated using the following formula

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

The log of molecular weight of the marker proteins were plotted versus mobilities, and molecular weights of sample proteins were deduced from the standard curve.

Detection of carbohydrates by Periodic Acid-Schiff (PAS) Staining

This is an interesting application of the Schiff's reaction to the staining of acrylamide gels to visualize the presence of carbohydrates and is therefore a valid complement of the Coomassie blue staining procedure for the detection of glycoproteins. The reaction is based on the oxidation of diol components by periodic acid.

The aldehyde formed reacts with the Schiff's reagent (leucofuchsin) to form a red anil (azomethine or Schiff's base).

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

$$RCHO + H_2NR'' \longrightarrow R\text{-}CH = N\text{-}R'' + H_2O$$

Schiff's reagent — Schiff's base

(Adams and Adams, 1965)

For the staining procedure the method proposed by Furlan et al, (1975) has been followed with only slight modifications.
The reagents used were:

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

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

f) periodic acid-Schiff reagent prepared by mixing 1g fuchsin red,

2g sodium metabisulfite and 20ml 1N HCl in 200ml distilled water, stirring continuously for 18hr at 4°C on a magnetic stirrer. Charcoal (2g) was added and stirred for a further 4hr. The mixture was then filtered at 4°C under reduced pressure through Whatman No 1 filter paper. The filtrate was stored at 4°C in a dark bottle carefully stoppered to prevent oxidation of the basic fuchsin.

g) 50% (V/V) methanol as destaining solution. The SDS-acrylamide gels underwent electrophoresis under exactly the same conditions used for the gels to be stained with Commassie blue with the exception of the load that was increased to 100µg of protein.

The electrophoresis run was always carried out simultaneously with gels to be stained with Coomassie blue.

The staining procedure is resumed in the following table. Since such treatment caused the gels to shrink it was found necessary to soak the gels overnight in the Coomassie blue destainer to allow them to swell back to their original size.

The marker proteins used for the PAS staining were ovalbumin and trypsin (PAS positive) and bovine serum albumin (PAS negative).

Detection of carbohydrate in SDS-polyacrylamide gels by PAS staining

40ml of each of the following solutions were used.

	hours		
A) To remove excess SDS which absorbs light significantly at 550nm.			
Isopropyl alcohol-acetic acid-H_2O	1		Room temperature
Isopropyl alcohol-acetic acid-H_2O	24		
10% (V/V) acetic acid	2x2		
10% (V/V) acetic acid	1x4		
B) Periodic acid (0.5% V/V)	2	*	
C) Distilled water	30sec.		
D) To neutralize unreacted periodic acid that may oxidize the leucofuchsin			
Sodium arsenite (0.5% W/V) in 5% ac. acid	1	*	$4^{\circ}C$
E) To remove excess sodium arsenite			
Distilled water	60sec.		
10% (V/V) acetic acid	3x2		
F) Schiff reagent	12		
Distilled water	60sec.		
5% (V/V) acetic acid	2x1		
5% (V/V) acetic acid	24		
Distilled water	24		
5% (V/V) methanol	24	*	Room temperature

* : with frequent mixing.

2.11 DETERMINATION OF THE MOLECULAR WEIGHT OF S-CARBOXYMETHYLATED SGP

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

2.11.1 SDS-acrylamide gel electrophoresis was performed as described in Section 2.10 using gels of varying porosities (5%, 7.5% and 10% final acrylamide concentration). Bovine serum albumin and ovalbumin were used as molecular weight markers.

2.11.2 Analytical ultracentrifugation

These analyses were performed on purified S-carboxymethylated protein, by the meniscus depletion technique of Yphantis (1964), as described by Chervenka (1970). A Spiro Model E ultracentrifuge was used equipped with interference optics.

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

Results were analysed according to the procedure of Roark and Yphantis (1969) using a computer program kindly supplied by Dr. Roark.

	S-carboxymethylated SGP	S-carboxymethylated collagenase treated SGP
Protein concentration (mg/ml)	0.7	0.5
Temperature (^{o}C)	20	20
Speed (rpm)	25980	42040
$\bar{v}$ (cm^2/g)	0.701	0.701
ρ (g/litre)	1.1260	1.1260
Cell	12 mm with double sector centre piece	
Optical system	Rayleigh interference optics.	

Preparation of SGP for ultracentrifugation runs

Both SGP and collagenase-treated SGP were S-carboxymethylated as described in Section 2.9.

Aliquots of S-carboxymethylated SGP were dissolved in 5M guanidine-HCl (specially purified, see Section 2.2.1) -0.1M Tris (pH 7.4) prior to exhaustive dialysis against several changes of the same buffer using acetylated 8/32 Visking tubing.

The density of GuHCl solutions was determined by picnometry. Partial specific volumes ($\bar{v}$) were obtained from compositional data (Zamyatnin, 1972; Gibbons, 1966).

2.12 CHEMICAL ANALYSES

2.12.1 Amino acid analysis

Hydrolyses were carried out in constant-boiling HCl (5ml/mg protein) containing 0.01M thioglycolic acid at 110°C under N_2.

The samples were taken down to dryness in a rotary film evaporator , the temperature of the bath being maintained at 30°C. Aminoacid analyses were carried out on a single column Locarte amino acid analyser. Norleucine was used as internal standard and corrections were made for hydrolytic losses (Fig. 9). Hydroxyproline was estimated independently on protein hydrolysates by the colorimetric procedure of Serafini-Cessi and Cessi (1964), which is based on the measurement of the absorption at 550nm of the chromophore produced by the steam-distillation of an unsaturated heterocyclic compound (2-methyl pyrrole) formed by the oxidation of hydroxyproline by hydrogen peroxide and successive coupling with p-dimethylamino-benzaldehyde.

Test tubes containing 1ml of the sample, 1ml of 0.05M $CuSO_4$ and 1ml of 2.5N NaOH were placed in a water bath at 40°C. One ml of 6% H_2O_2 was then added to each test tube. These were frequently shaken to remove excess of oxygen developed. After 10min., the tubes were cooled in running water and maintained at that temperature until distilled. Immediately before distillation, 2ml of saturated KCl, 1ml of 3N HCl and one crystal (approx. 10mg) of stannous chloride were added, and the solution distilled in a micro-distillation apparatus. Distillate (3.5ml) was collected into a 10ml volumetric flask containing 6.5ml of Ehrlich reagent (1g of p-dimethylamino-

benzaldehyde in 55ml of glocial acetic acid and 10ml of 10N H_2SO_4). The chromophore which developed immediately, was read at 550nm within 15min. The concentration of hydroxyproline in the hydrolysate was deduced from a standard curve.

Tryptophan was determined colorimetrically according to the method of Spies and Chamber (1949).

Suitable protein samples containing between 10 and 110μ g of tryptophan were treated in a round bottomed flask with 30mg of p-dimethylaminobenzaldehyde (DMAB), 10ml of 19N H_2SO_4 and left at 25°C in the dark for 2hr. After this period, 0.1ml of freshly prepared 0.04% sodium nitrite solution was added to the flasks that were left in the dark, for 30min. at room temperature. The absorbance was measured in a spectrophotometer at 580nm using a blank prepared in the same way. A calibration curve was drawn by treating a series of tryptophan solutions (1ml) of known concentration (concentrations ranging between 12.5 g/ml and 100 g/ml) with 8ml of 23.8 N H_2SO_4 and 1ml of DMAB solution (30mg/ml in 2 N H_2SO_4). The standards were subjected to the same procedure followed for the protein samples.

2.12.2 Hexosamine analysis

Hydrolyses of samples were performed in 4N hydrochloric acid under N_2 at 90°C for 6 and 8hr. Total hexosamine content was determined on the amino acid analyser using D-glucosamine and D-galactosamine as standards. Results were checked by performing a GLC analysis according to the procedure of Bhatti et al. (1970) as described in Section 2.12.3.

2.12.3 Estimation of carbohydrate content by gas-liquid chromatography

Neutral sugars, hexosamines and sialic acid were quantitated by gas-liquid chromatography using the technique of Bhatti et al. (1970). The instrument used was a Pye series 104 Gas Chromatograph equipped with hydrogen flame ionisation detectors coupled to a Philips 8000 recorder. Dual column operation was used, the glass columns (250 x 0.32cm) being packed with 3% SE30 on Diatomite CQ (Pye Unicam). The column temperature was programmed to increase from 110°C to 200°C with a 0.5°C/min. increment, the upper limit being held until the last peak emerged. The carrier gas was oxygen-free nitrogen at a flow rate of 50ml/min. Hydrolysis of samples was performed in methanolic-HCl prepared by adding magnesium turnings (2.5g) and iodine (0.1g) to methanol (500ml) and heating the mixture under reflux for 1hr, after which the dry methanol was distilled into a clean container. Dry HCl gas was then slowly bubbled until the solution was 1.5M.

Conversion of sugars into trimethylsilyl derivatives was performed by treatment with a freshly prepared mixture consisting of pyridine, trimethylchlorosilane and hexamethyldisilazane (5:1:1, by Vol.)

The samples for analysis were prepared by accurately weighing suitable aliquots (1-2mg) of the carboxymethylated SGP into pyrex test-tubes together with internal standard (perseitol, 0.1ml of a 1 μmole /ml solution). The content of the test-tubes was lyophilized and dried overnight in a vacuum desiccator over phosphoric oxide.

Methanolic-HCl (0.5ml) was added to each tube and a steady stream of nitrogen bubbled for 30sec. The test-tubes were immediately

sealed and placed in an oil bath at 90°C for 24hr, after which the acid was neutralized by addition of solid silver carbonate. Acetic anhydride (0.05ml) was added to each tube which was then covered with parafilm and left at room temperature for at least 6hr. After thorough trituration, the content of each tube was centrifuged and the supernatant transferred to a 5ml pear-shaped flask. The trituration and centrifugation steps were repeated with three further additions of methanol and the pooled supernatants evaporated under reduced pressure at 37°C. The samples were then dried in a vacuum desiccator over phosphoric oxide for at least 12hr. Trimethylsilylating agent (0.05ml) was then added to the dried material, and after trituration, the flask was stoppered and allowed to stand at room temperature for 30min. Aliquots (5 to 10µl) of the solution were then injected into the GLC column.

Quantitative results were obtained by the technique of internal standardisation and the molar response factor for each monosaccharide was determined as follows.

Perseitol (0.05µmole) was added to a standard solution containing mannose, galactose, glucose, N-acetylglucosamine and sialic acid in a series of ampoules giving a concentration range of 0.01 to 0.1µmole for each monosaccharide. After lyophilization the samples were subjected to the same procedure described above. Peak areas were calculated from the chromatogram by transferring peaks profiles onto tracing paper of uniform thickness, each peak being cut and accurately weighed. Total peak weight for each monosaccharide was obtained by summing the peak weights of the various isomers. The ratio of the total peak weight to the peak weight of the internal standard (total peak area ratio) was plotted against the mole ratio of the monosaccharide to the internal standard. The molar

relative response factor was then deduced by the slope of the graph. The μmole of each monosaccharide in the sample was calculated from the equation:

$$\frac{\text{Total peak area ratio} \times \mu\text{mole internal standard}}{\text{Molar relative response factor}}$$

2.13.4 Colorimetric determination of sialic acid

Sialic acid was also quantitated by the colorimetric procedure of Jourdian et al. (1971). This method allows the determination of bound sialic acid (no hydrolysis is therefore necessary before the test) by oxidation with periodate to produce a chromogen that reacting with resorcinol gives a chromophore with maximum absorption at 620nm. The reagents used were:

Periodic Acid 0.4M: freshly prepared

Resorcinol reagent: 0.6g resorcinol (from a stock solution of 6% (W/V) resorcinol), 60ml of 28% (V/V) hydrochloric acid, 40ml water and 25 μ moles of copper sulphate.

95% (V/V) tert-butyl alcohol

To measure total sialic acid, an aliquot of the carboxymethylated SGP was suspended in 0.5ml water and added to 0.1ml of periodic acid solution (test carried out in triplicate). After thoroughly mixing and cooling the solutions in an ice-bath for 35min. 1.25ml of resorcinol reagent was added, the solutions mixed and placed in the ice-bath for a further 5min. before being heated at 100°C for 15min. and then cooled under tap water. Tert-butyl alcohol (1.25ml) was finally added, vigorously mixed and the solutions placed at 37°C in a water bath for 3min. to allow the colour to stabilize.

The absorbances were measured at 630nm against blanks using

1cm light-path cuvettes at room temperature. Concentrations of sialic acid in the samples were deduced from a standard curve obtained by similarly treating free standard sialic acid samples except that the oxidation period was 20min. rather than 35min. as for bound sialic acid, as free N-acetyl neuraminic acid produces a chromogen which is unstable after 20min. incubation. The different stability at 37°C of the chromogen produced by N-acetylneuraminic acid and N-glycolylneuraminic acid can be used to identify the sialic acid in the test samples.

In fact while the chromogen formed by N-glycolyl neuraminic acid is stable for up to 100min. at 37°C that formed by the N-acetyl derivative is almost completely destroyed after such a length of time. Another aliquot of protein was therefore subjected to the same treatment as outlined above with the oxidation step performed at 37°C for 100min.

2.13 LYSYL OXIDASE ACTIVITY ASSAY

2.13.1 Preparation of ^{3}H-labelled elastin substrate

The ^{3}H-labelled aortic protein substrate was prepared by organ culture employing the method of Pinnell and Martin (1968).

Twenty five aortae were removed from 15 to 17 day old chick embryos, cut into small pieces, and added to 10ml of Eagle's minimal essential medium lacking lysine and glutamine but supplemented with β-aminopropionitrile fumarate (50μg ml^{-1}), ascorbic acid (50μg ml^{-1}) and kanamycin (50μg/ml).

Culture vessels were gassed with O_2-CO_2 (95:5, V/V) and incubations carried out at 37°C with constant agitation for 48hr.

Prior to the start of each incubation, the medium was further supplemented by addition of 250μCi of L- 4,5-^{3}H lysine monohydrochloride (specific activity 76 Ci $mmol^{-1}$). After incubating for 48hr, the aortae were rinsed in distilled water, lyophilized and stored at 4°C.

The lyophilized ^{3}H-labelled substrate was prepared for enzyme assay as follows: the lyophilized aortae were homogenized in 5ml of 0.15M NaCl using an Ultra Turrax TP 10N homogenizer. The insoluble pellet was collected by centrifugation at 17,500g for 10min. and extracted twice with 0.15M NaCl, once with 1M HCl, in order to inactivate endogenous enzyme (as suggested by Kagan et al, (1974), once with 0.1M NaH_2PO_4 - 0.15M NaCl, pH 7.7, and twice with 0.15M NaCl.

The final residue was resuspended in 0.1M NaH_2PO_4, 0.15M NaCl, pH 7.7 so that portions (0.02ml) of the final substrate suspension contained approximately 280,000 dpm.

2.13.2 Preparation of the 'enzyme'

Aliquots (3mg) of SGP purified by ultrafiltration were dissolved in 1ml of 8M urea-0.1M Tris buffer pH7.4 containing 3% 2-mercaptoethanol, left stirring under N_2 for 6hr at room temperature and dialyzed as follows:

a) exhaustive dialysis against water at 4°C,

b) dialysis against $CuCl_2$ 10^{-3}M solution pH6.2 at 4°C for 48hr,

c) exhaustive dialysis against distilled water.

The protein was then redissolved in urea-mercaptoethanol buffer and dialysed against 0.1M NaH_2PO_4 -0.15M NaCl pH7.7 for 24hr. The protein solution was fairly unstable and began to precipitate after 6hr from the end of dialysis and therefore had to be immediately used for the assay.

2.13.3 Enzyme assay

Each assay was performed at least in duplicate. Appropriate controls containing substrate were carried out to establish the range of background values (2463 ± 391 dpm). All components of the assay system were prepared in 0.1M NaH_2PO_4 - 0.15M NaCl, pH7.7 buffer. Enzymic reactions were carried out in 1ml Reacti-Vials fitted with Teflon coated triangular stirring bars (Pierce Chemical Company).

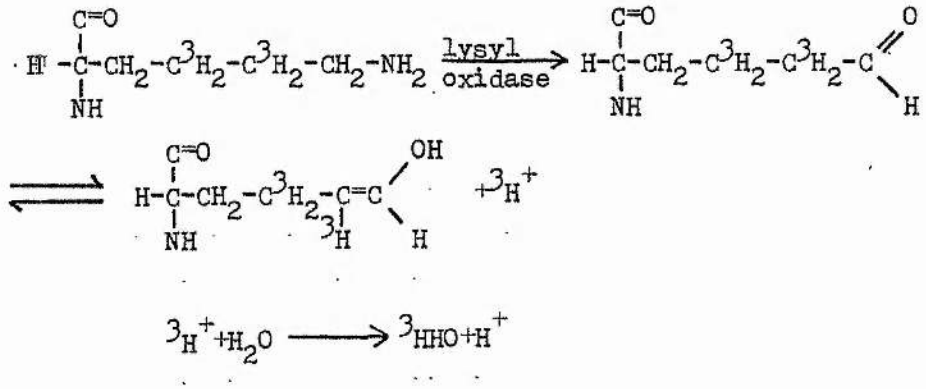
The normal assay system comprised: ^3H-labelled substrate, 0.02ml (containing approximately 280,000 dpm); 'enzyme preparation', 0.02ml (containing 24-41µg protein) and 0.1M NaH_2PO_4 - 0.15M NaCl, pH7.7 buffer, 0.5ml.

β-Amino propionitrile fumarate (final concentration 50-1000µg ml^{-1}) and/or lysine (final concentration 0.01M) were added

to certain tubes before incubation.

Reaction mixtures were incubated at 37°C with continuous stirring for 2hr. In all reactions, the air space above the reaction mixture was exchanged with 100% O_2 for 1min. before capping, since molecular oxygen has been shown to enhance allysine formation (Siegel et al., 1970). Reactions were terminated by cooling on ice. The ^3H released from the protein substrate was monitored as ^3H_2O formed during the assay, and determined by the microdistillation procedure described by Misiorowski et al. (1976). The efficiency of recovery following distillation was assessed using ^3H_2O and found to be between 83-90%. The complete distillate was used in the determination of radioactivity by addition of 5ml of Packard Picofluor-30 scintillation fluid to the frozen distillate, contained in glass pico-scintillation vials.

Determination of ^3H release was performed in a Packard, 'Prias', liquid scintillation spectrometer, utilising a computer programme to calculate dpm from the observed cpm using a calibrated external standard.



Release of tritium and production of tritiated water during aldehyde formation in 4-5 ^3H tritiated lysine.

2.14 PRONASE DIGESTION OF SGP

An aliquot (26.5mg) of SGP purified by ultrafiltration (see section 2.6.1) was suspended in 5ml of 0.1M Tris buffer, pH 7.9 containing 0.002M $CaCl_2$ and incubated at 37°C under toluene with 1% (W/W) pronase in a pH-stat set at 7.9 and loaded with NaOH 0.1M. After 24hr, a further addition of 1% pronase was made and incubation carried out for a total of 70hr until no further enzymic activity was detectable. The suspension was then centrifuged and the supernatant applied to a Sephadex G-50 column (1.4 x 88cm). Elution was carried out with water and glycopeptides identified by analyzing each fraction for carbohydrate by the phenol-H_2SO_4 method described in section 2.15 scaled down to one-fifth volume.

The glycopeptide material, which was eluted as two peaks, was pooled in one single fraction, lyophilized and carboxymethylated (see section 2.8) in order to assess whether the two peaks obtained were due to a difference in the carbohydrate composition or were caused by a different degree of degradation of the protein moiety by pronase due to incomplete digestion around disulphide bonds.

The carboxymethylated material was subjected to a new pronase digestion and Sephadex G-50 chromatography as previously described. The fractions from peak A and B (Fig. 12) were lyophilized and aliquots (0.5mg) from each peak subjected to the treatment described in Section 2.13.3 to be examined by gas-liquid chromatography.

2.15 PHENOL-H_2SO_4 ASSAY FOR CARBOHYDRATE DETECTION

This assay was performed according to the procedure of Hodge et al. (1962) scaled down to one-fifth volume.

To 0.2ml of sugar solution contained in a Pyrex test-tube, 0.2ml of 5% (W/V) phenol solution was added and the solution mixed. Then 1ml of concentrated sulphuric acid was rapidly added, the stream of acid being directed against the liquid surface rather than against the side of the test-tube in order to obtain a good mixing. The tubes were allowed to stand 10min., then they were shaken and placed for 10 to 20 min. in a water bath at 25°-30°C.

The colour that developed was stable for several hours and the optical density was measured at 490nm using 1ml cuvettes.

2.16 ELECTRON MICROSCOPY

An SGP preparation, which had been dialysed against 10mM $CuCl_2$, was dissolved in 8M urea -0.1M Tris (pH 7.4), containing 3% (V/V) 2-mercaptoethanol, and dialysed against distilled water at 4°C. The precipitate that formed after 24hr was suspended in 12mM uranyl formate (pH 2.2) and sprayed on carbon-coated grids. An aliquot of the precipitate in the form of a pellet was fixed in 4% paraformaldehyde -5% glutaraldehyde in 0.8M sodium cacodylate (pH 7.3), dehydrated in ethanol and embedded in Araldite. Ultrathin sections were cut on a Reichardt OMU2 ultramicrotome and stained with 2% uranyl acetate in 50% ethanol for 25min., followed by 0.4% lead citrate in 0.1M NaOH for 5 min. Grids were examined in an AEI-EM6B electron microscope. Micrographs were taken at an instrument magnification of 40,000, calibrated using beef liver catalase crystals (Wrigley, 1968).

SECTION THREE

RESULTS

RESULTS

3.1 ISOLATION AND PURIFICATION OF SGP

SGP was isolated from bovine ligamentum nuchae with a yield of 0.1% relative to the defatted, dry ligamentum weight.

Guanidine-hydrochloride-mercaptoethanol extracts obtained in the presence and absence of protease inhibitors were examined by SDS-polyacrylamide gel electrophoresis, but no differences were detected in the banding pattern of the two preparations (Fig. 2), suggesting that no significant proteolytic activity is present in such extracts. The banding patterns were similar to those obtained under analogous experimental conditions by Bach and Bentley (1980) from bovine thoracic aorta and showed the presence of a complex mixture of proteins. Of these the most prominent component was a 35 000 molecular weight species giving rise to a periodic acid-Schiff-positive band.

The purification procedure was performed in two stages, the ultrafiltration producing a 95% pure preparation of SGP.

Preparative gel electrophoresis proved to be effective in isolating a homogeneous preparation of the periodic acid-Schiff-positive component. The absorbance elution profile of the run on preparative electrophoresis is shown in Fig. 4 and the pooled fractions 27 to 33 yielded a single band on analytical SDS-polyacrylamide gel electrophoresis. (Fig. 3.)

Fig. 2:

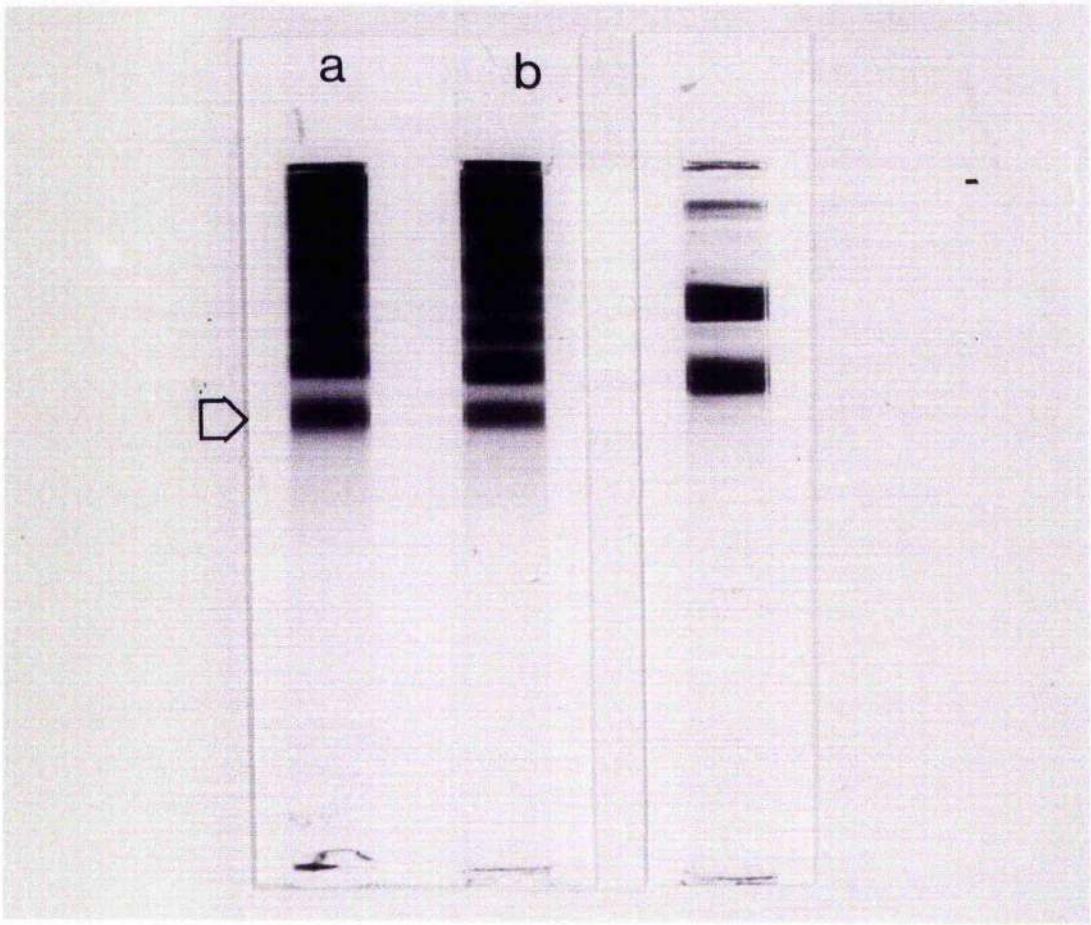
SDS-PAGE of crude 5M guanidine hydrochloride-2-mercaptoethanol extracts of ligamentum nuchae carried out in the presence (a) and absence (b) of protease inhibitors. Gels were stained with Coomassie brilliant blue. 100µg of crude extract were loaded on each gel.

The arrow shows the PAS positive band.

Fig. 3:

(a) SDS-PAGE of pooled fractions (27-33) obtained from the preparative gel electrophoretic separation described in section 2.6.2.

(b) SDS-PAGE of bovine serum albumin and ovalbumin.


a
b

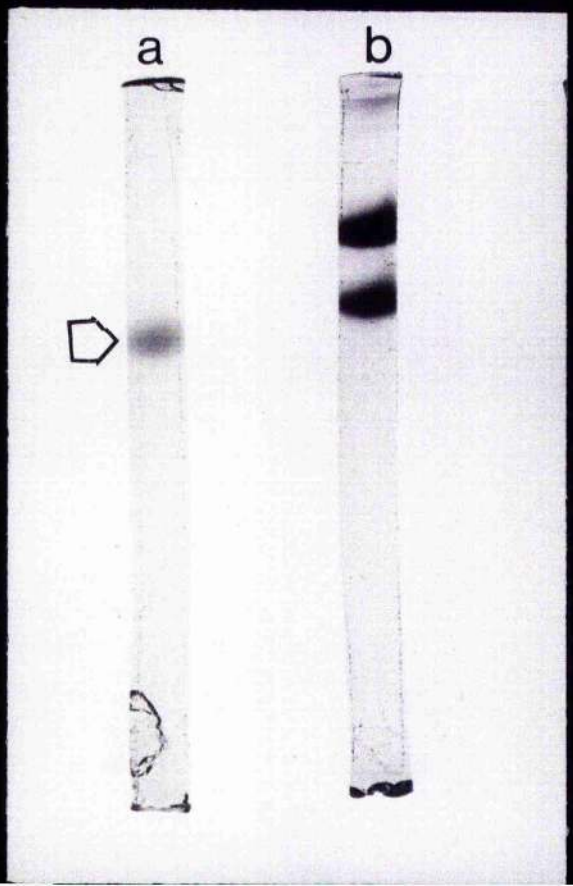
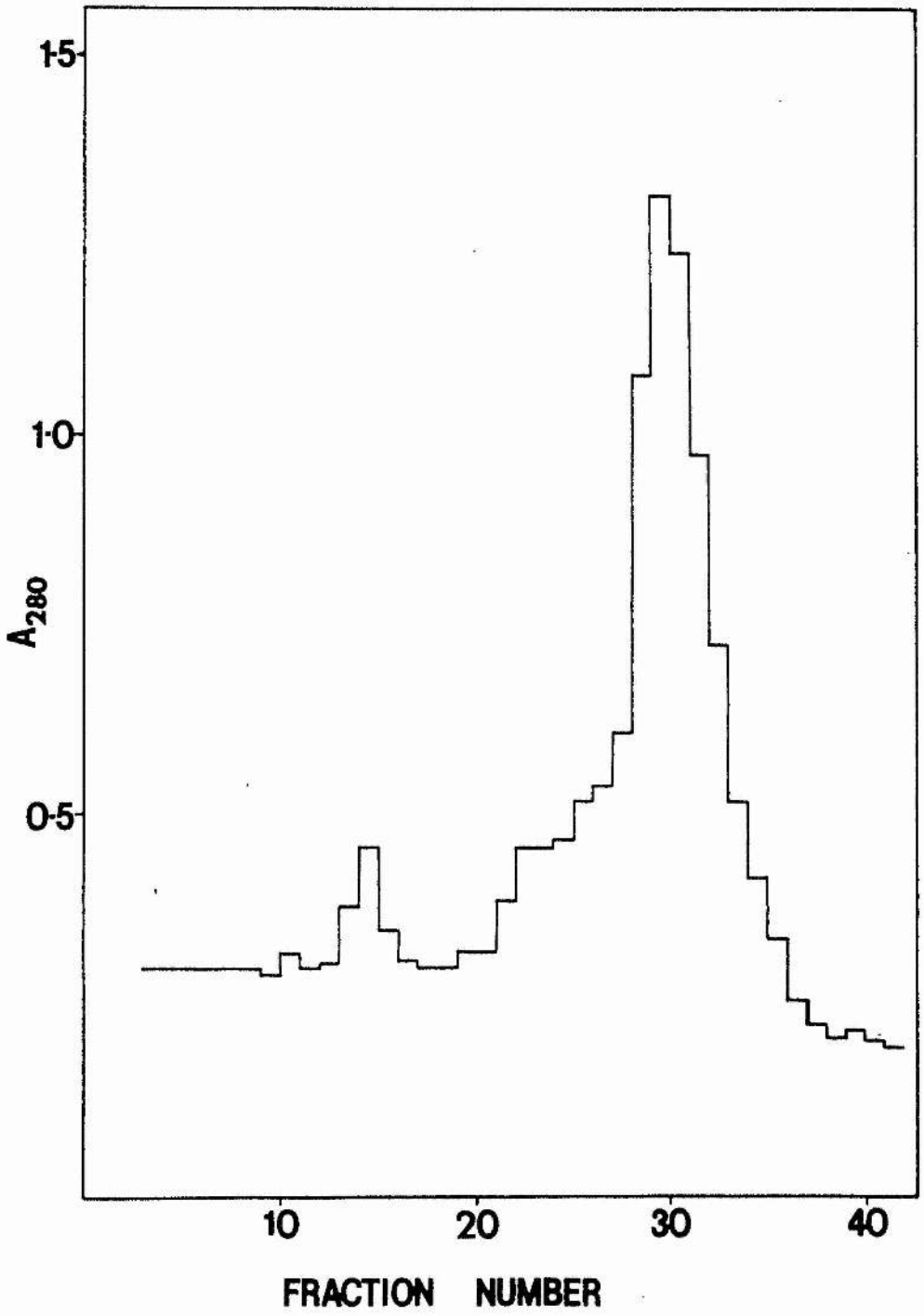

a
b

Fig. 4:

Absorbance profile of the leading fractions from a preparative gel electrophoretic separation of a crude extract of ligamentum nuchae.


1·5
1·0
0·5
A_{280}
10
20
30
40
FRACTION NUMBER

3.2 MOLECULAR WEIGHT DETERMINATION

Molecular weight of this SGP preparation, determined by SDS-PAGE on gels of different porosity (Segrest and Jackson, 1972) ranged between 37000 on 5% gels and 35000 on 10% gels (Fig 5 and 5a).

A molecular weight of 33280 was obtained by analytical ultracentrifugation for the carboxymethylated SGP in 5M guanidine hydrochloride. The partial specific volume ($\bar{V}$) value used was 0.706ml/g and was obtained from compositional data (Zamyatnin, 1972). Molecular weights were calculated from the corresponding reduced molecular weight moments, $\sigma_i(r)$ as defined by Yphantis (1964):

$$\sigma_i(r) = M_i, \text{ app}(r) \frac{(1 - \bar{V}\rho)\omega^2}{RT}$$

In Fig. 6a are plotted the values for the standard apparent point-average molecular weight moments (N, W and Z) and those relative to the ideal moment Y_8 calculated according to Roark and Yphantis (1969) which gives the most realistic estimate of molecular weight, having been corrected for the effects of concentration and non ideality in the system. A value of 11600 daltons was obtained under analogous conditions for the collagenase treated SGP (Fig 7a).

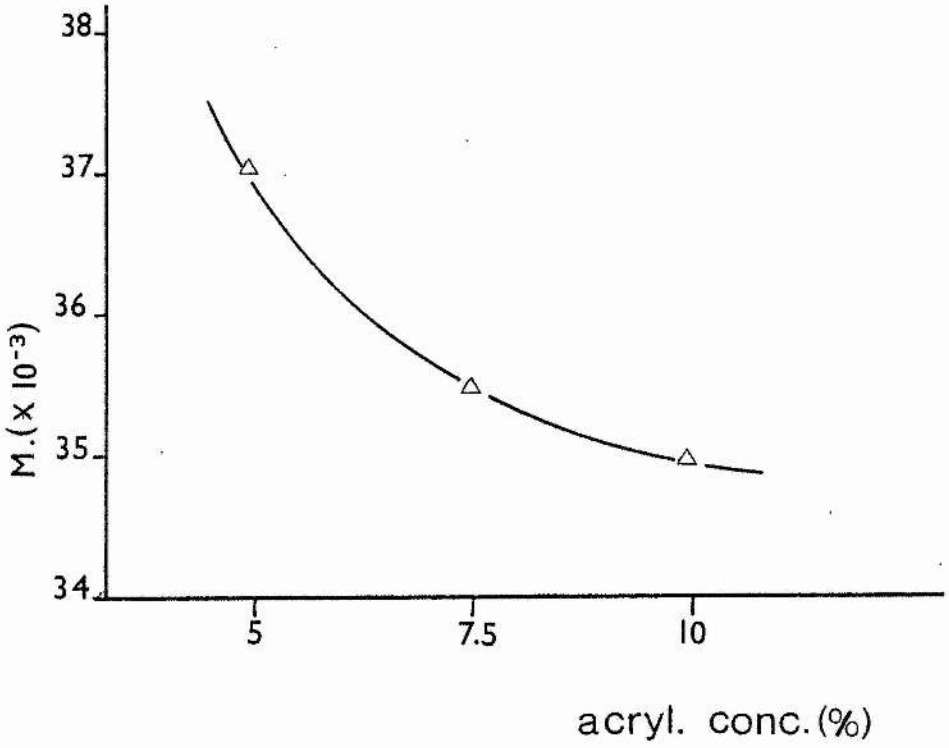
Minimum molecular weight of SGP was also determined by using analytical data according to Black and Hogness (1969), using the computer programme of Bryce (1979) modified to account for sugar residues. In the range between 25000 and 45000 the minimum of the fraction of maximum deviation corresponded to a molecular weight of 34400 (Fig. 8) with a refined integer fit of amino acid and sugar residues reported in Table 1 (column b).

Fig. 5:

Observed molecular weight of SGP calculated by electrophoretic mobility relative to the standard curves in Fig. 5a versus acrylamide gel concentration.

Fig. 5a:

Log of molecular weight versus mobility by SDS-PAGE of three standard proteins run in increasing concentration of acrylamide.
The plots from right to left represent 5, 7.5, 10% gels.


38
37
36
35
34
M.(X 10⁻³)
5
7.5
10
acryl. conc.(%)

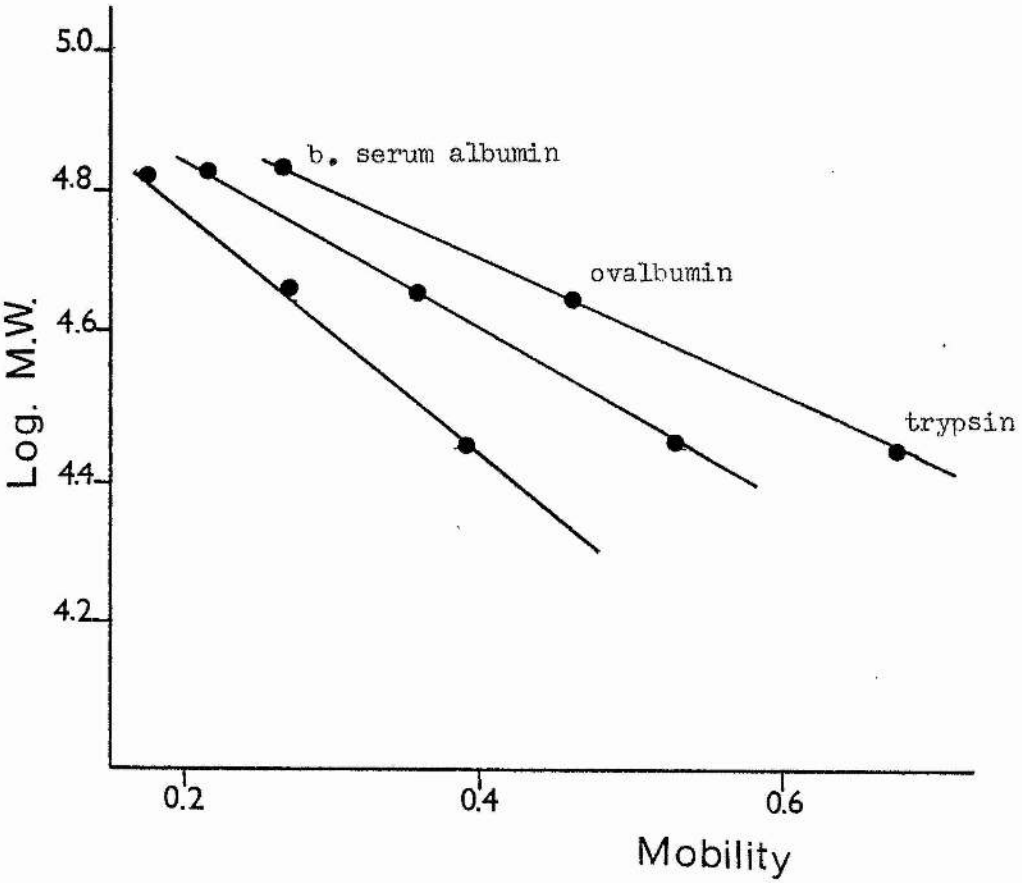

5.0
4.8
4.6
4.4
4.2
Log. M.W.
b. serum albumin
ovalbumin
trypsin
0.2
0.4
0.6
Mobility

Fig. 6a:

Point-average molecular weight moments of SGP, in 5M guanidine hydrochloride -0.1M Tris (pH7.4) are shown as functions of the concentration in the cell.

(▲) σ_Z

(△) σ_W

(■) σ_N

(◆) σ_{Y8}

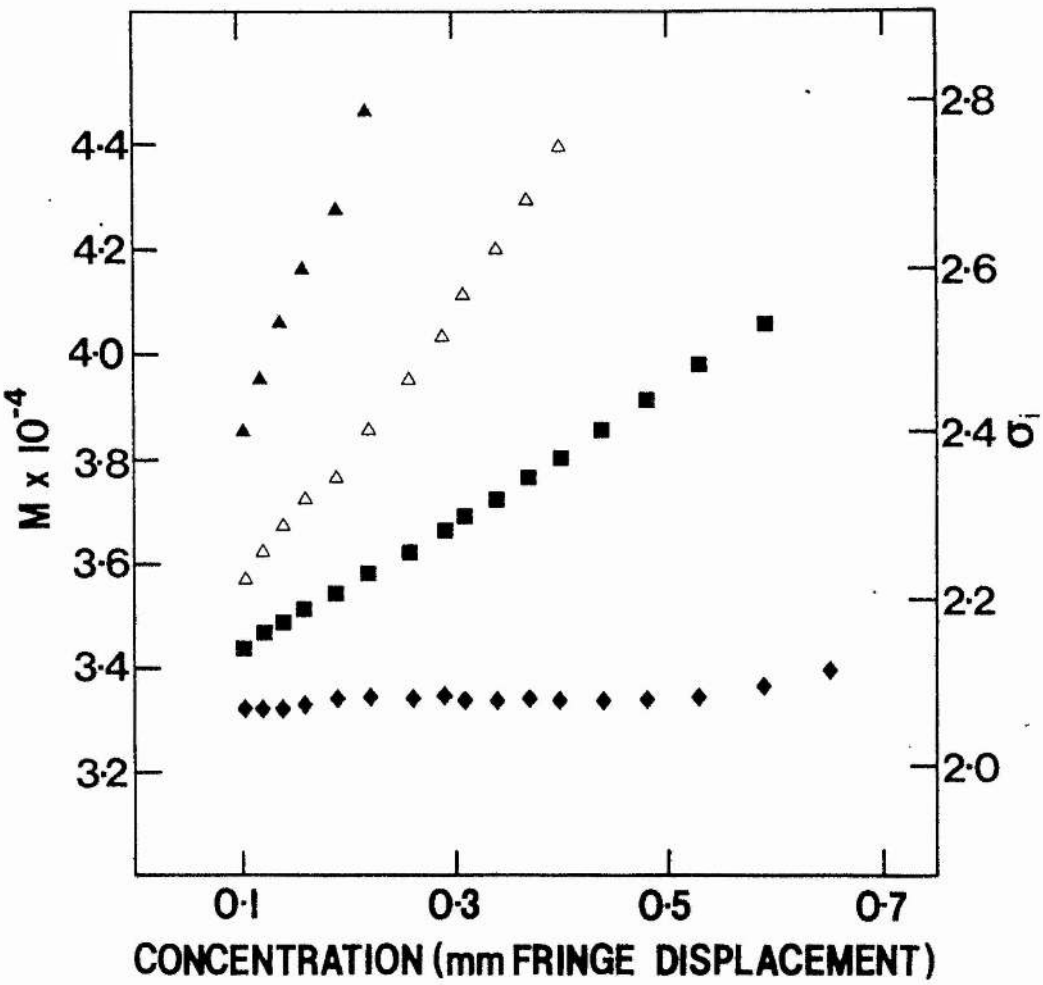

4·4
4·2
4·0
3·8
3·6
3·4
3·2
M x 10⁻⁴
2·8
2·6
2·4
2·2
2·0
σ
0·1
0·3
0·5
0·7
CONCENTRATION (mm FRINGE DISPLACEMENT)

Fig 6b:

Appearance of the interference fringes at equilibrium in the meniscus depletion experiment for the molecular weight determination of SGP.

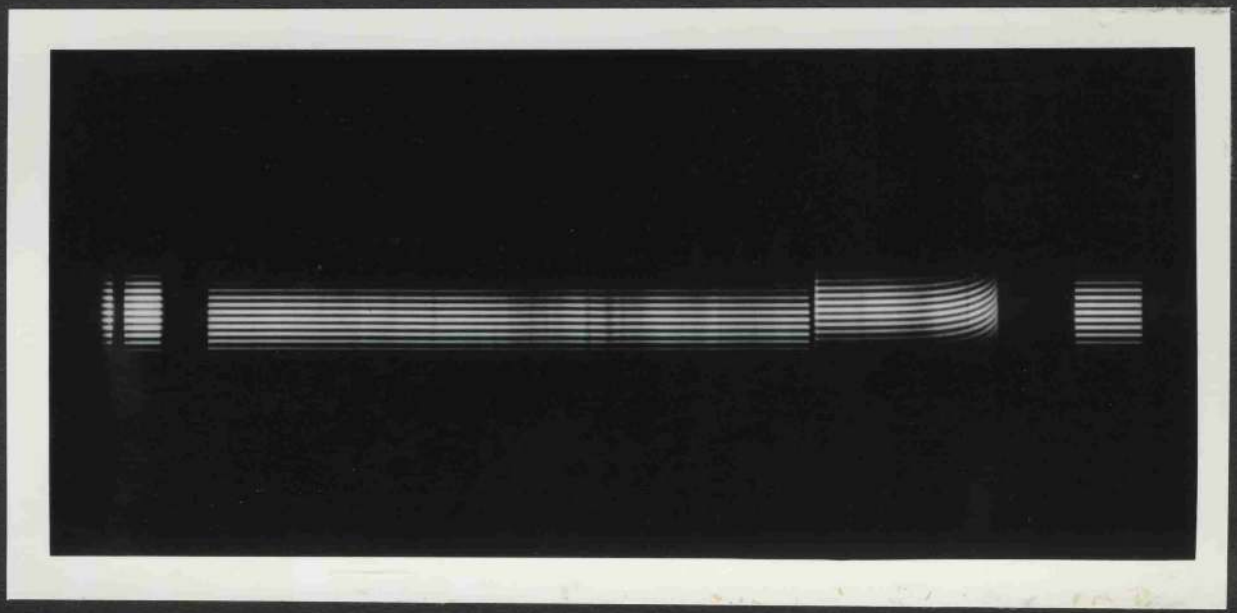


Fig. 7a:

Point-average molecular weight moments of collagenase treated SGP in 5M guanidine-hydrochloride -0.1M Tris (pH7.4), are shown as functions of the concentration in the cell.

(□)σ_Z

(△)σ_W

(◇)σ_N

(○)σ_{Y8}

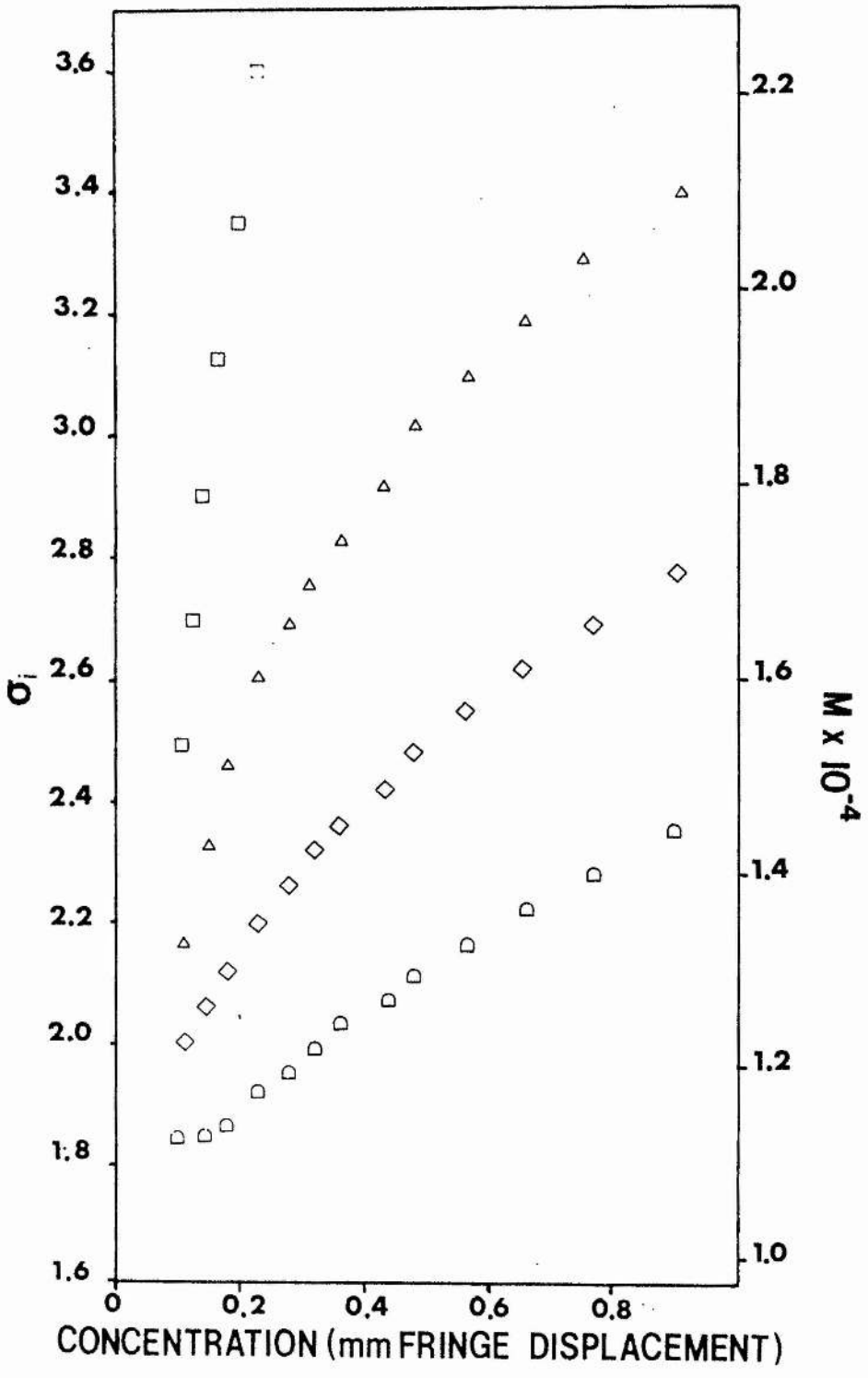

3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
σ_i
2.2
2.0
1.8
1.6
1.4
1.2
1.0
M x 10^{-4}
0
0.2
0.4
0.6
0.8
CONCENTRATION (mm FRINGE DISPLACEMENT)

Fig 7b:

Appearance of the interference fringes at equilibrium in the meniscus depletion experiment for the molecular weight determination of collagenase treated SGP.

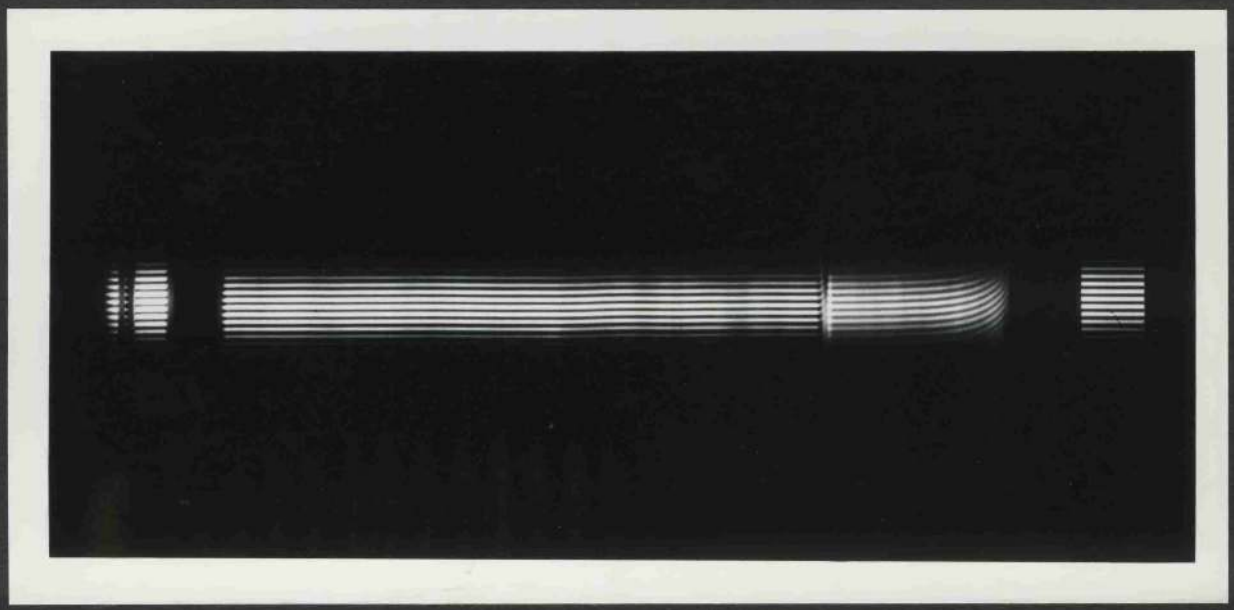
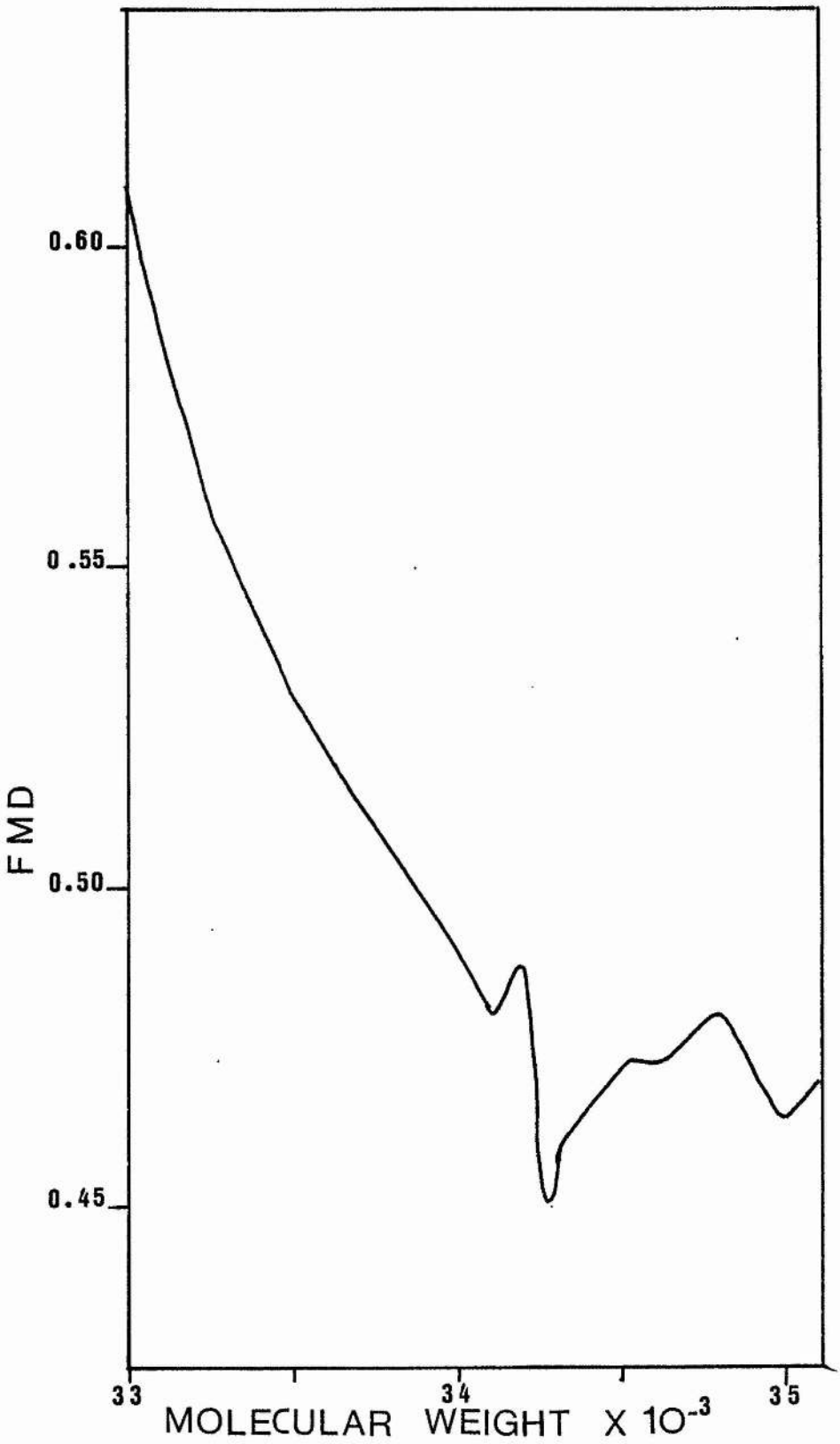


Fig 8:

Relationship between the molecular weight and the fraction of maximum deviation, FMD, of the amino acid frequencies.


0.60
0.55
0.50
0.45
FMD
33
34
35
MOLECULAR WEIGHT X 10-3

3.3 CHEMICAL ANALYSES

3.3.1 Amino acid analysis

Table 1 (column a) reports the composition of SGP isolated from the mature bovine ligamentum nuchae. Serine and threonine values have been corrected for hydrolytic losses (Fig. 9), by performing timed hydrolyses and extrapolating the results at zero time. No decomposition was detectable for all the others amino acids, with the exception of tryptophan that was totally destroyed.

3.3.2 Carbohydrate composition

GLC analysis revealed the presence of mannose, galactose, N-acetylglucosamine and sialic acid in a 6:5:3:3 molar ratio for a total of 10% of the glycoprotein weight. The compositional data reported in Table 1, column a, have been obtained by GLC analysis. Values for N-acetylglucosamine and sialic acid were also determined by reaction with no ninhydrin on the amino acid analyzer (Section 2.12.2) and colorimetrically (Section 2.13.4) and were found to be in close agreement with those derived from GLC studies.

Fig. 10 and Fig. 10a represent the GLC chromatograms of two SGP preparations before and after purification. Since the level of glucose decreased after purification its presence in the crude SGP sample was ascribed to contamination.

In Fig. 11 are reported the calibration curves used for the determination of the molar response factors of mannose, glucose, galactose, N-acetylglucosamine and sialic acid.

The stability for 100min. at 37°C of the chromogen produced at 630nm by periodate oxidation of SGP (Jourdian et al., 1971) suggests that sialic acid is present as N-glycolyl neuraminic acid (see following table).

Compound (μ mole)	Absorbance at 630nm		
	0°C 20min.	0°C 35min.	37°C 100min.
N-acetyl neuraminic acid (0.076)	0.47	-	-
N-glycolyl neuraminic acid (0.154)	0.80	-	-
N-acetyl neuraminic acid (0.038) + N-glycolyl neuraminic acid (0.077)	-	-	0.02
Carboxymethylated SGP (0.037 μmole/mg)	-	0.47	0.50

It was discovered from this experiment, and later confirmed by GLC analysis, that the commercialy (Sigma Ltd.) obtained N-glycolyl neuraminic acid was in fact N-acetyl neuraminic acid.

Colorimetric determination of sialic acid (Jourdian et al., 1971).

Fig. 9:

Recovery of serine and threonine from HCl hydrolysis as a function of time.

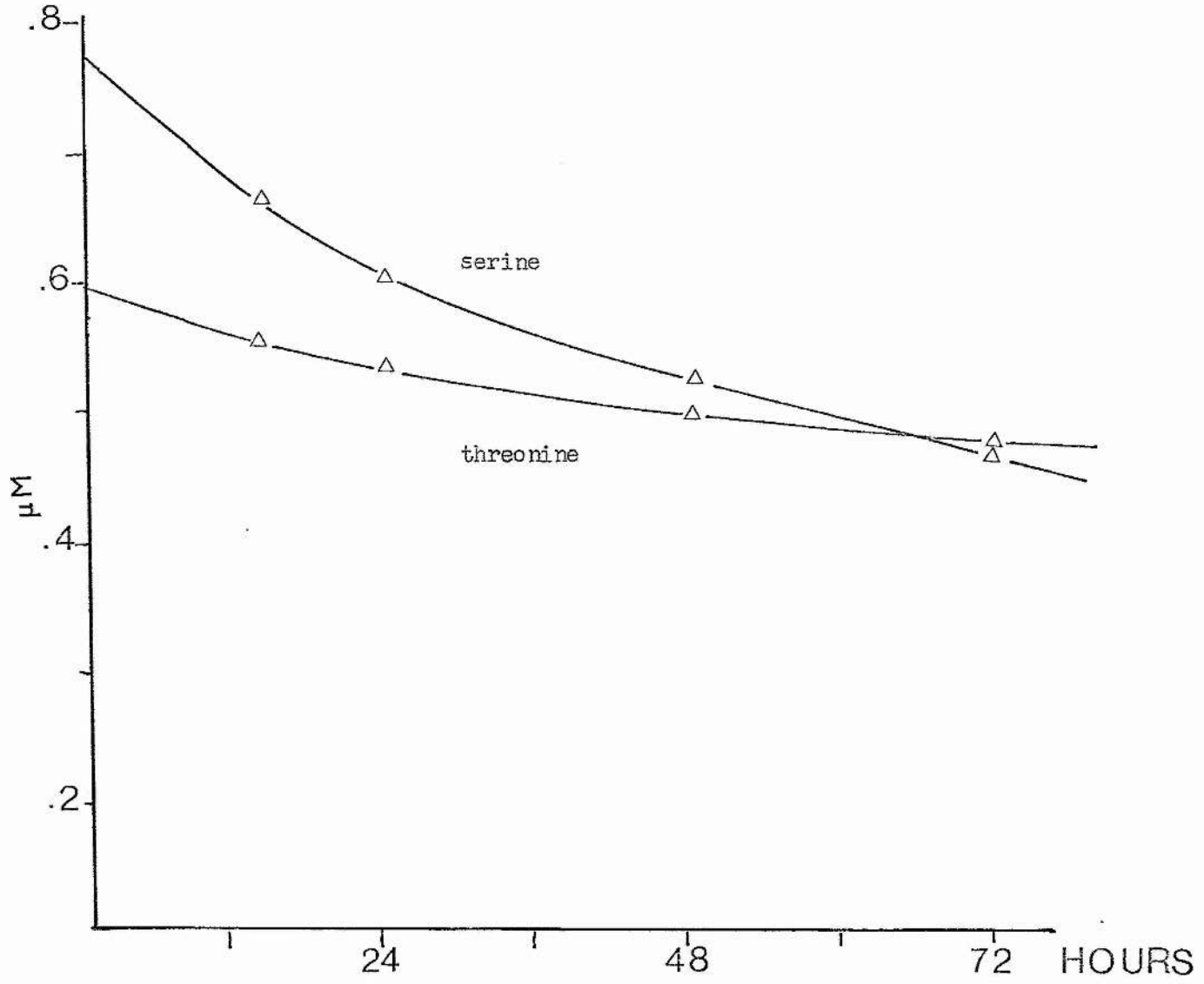

.8
.6
.4
.2
μM
serine
threonine
24
48
72
HOURS

Table I : Composition of Ligamentum Nuchae SGP

	analytical results[a] (a)	refined integer fit from analytical data (mol.wt. 34 400) (b)
Hydroxyproline	0.0	
Aspartic acid	118.8	33
Threonine	44.2	12
Serine	60.8	17
Glutamic acid	103.3	29
Proline	47.0	13
Glycine	111.7	31
Alanine	72.3	20
Cysteine	27.0	8
Valine	56.4	16
Methionine	14.1	4
Isoleucine	33.1	9
Leucine	83.9	23
Tyrosine	43.3	12
Phenylalanine	48.2	13
Tryptophan	16.8	5
Lysine	47.1	13
Histidine	17.5	5
Arginine	54.5	15
Total	1000.0	
N-Acetyl glucosamine	17.6	5
Galactose	9.0	3
Mannose	22.0	6
Sialic acid	10.0	3

[a]Values, corrected for hydrolytic losses, are expressed as residues per 1000 amino acid residues.

Fig. 10:

GLC analysis of TMS glycosides of a crude SGP preparation. Peaks are 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.
Perseitol (internal standard)	12, 13.
Sialic acid	14, 15.

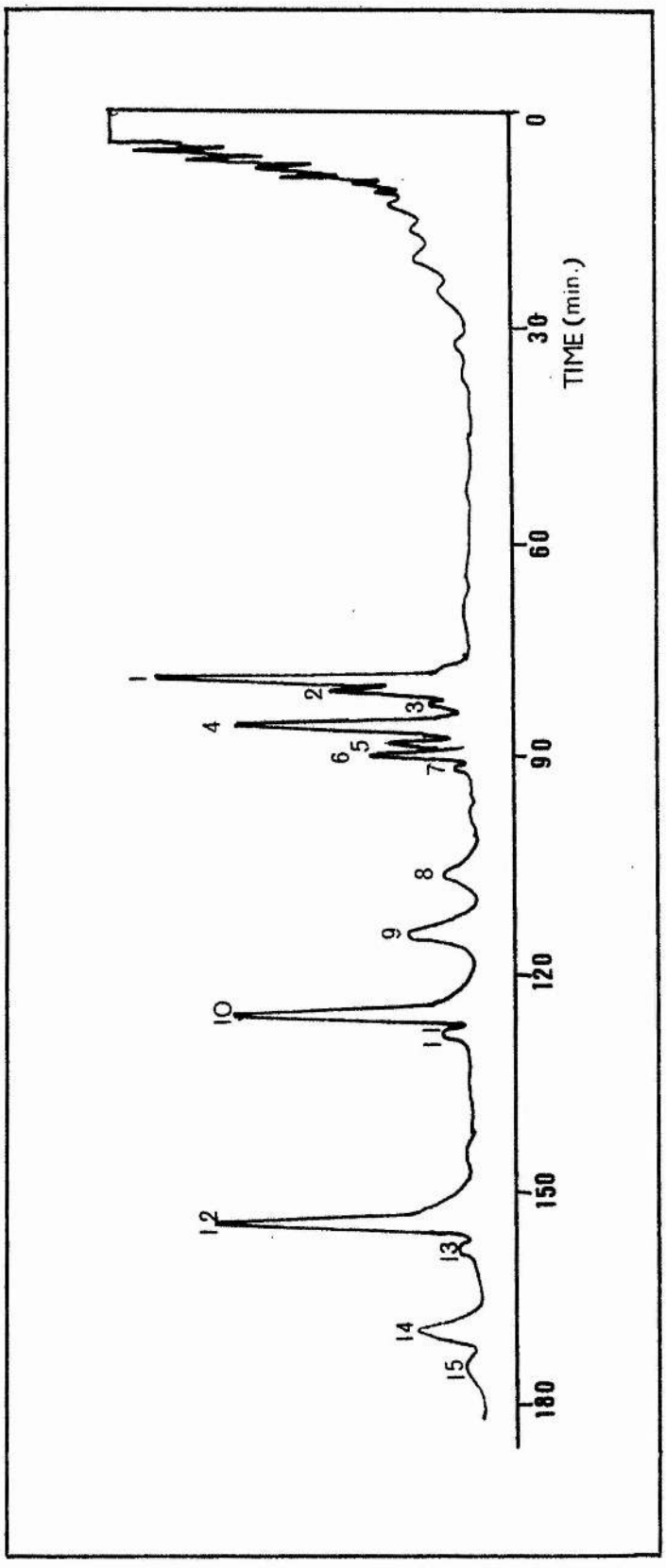

0
30
60
90
120
150
180
TIME (min.)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Fig. 10a:

GLC analysis of TMS glycosides of a pure SGP preparation. Peaks are numbered as in Fig. 10.

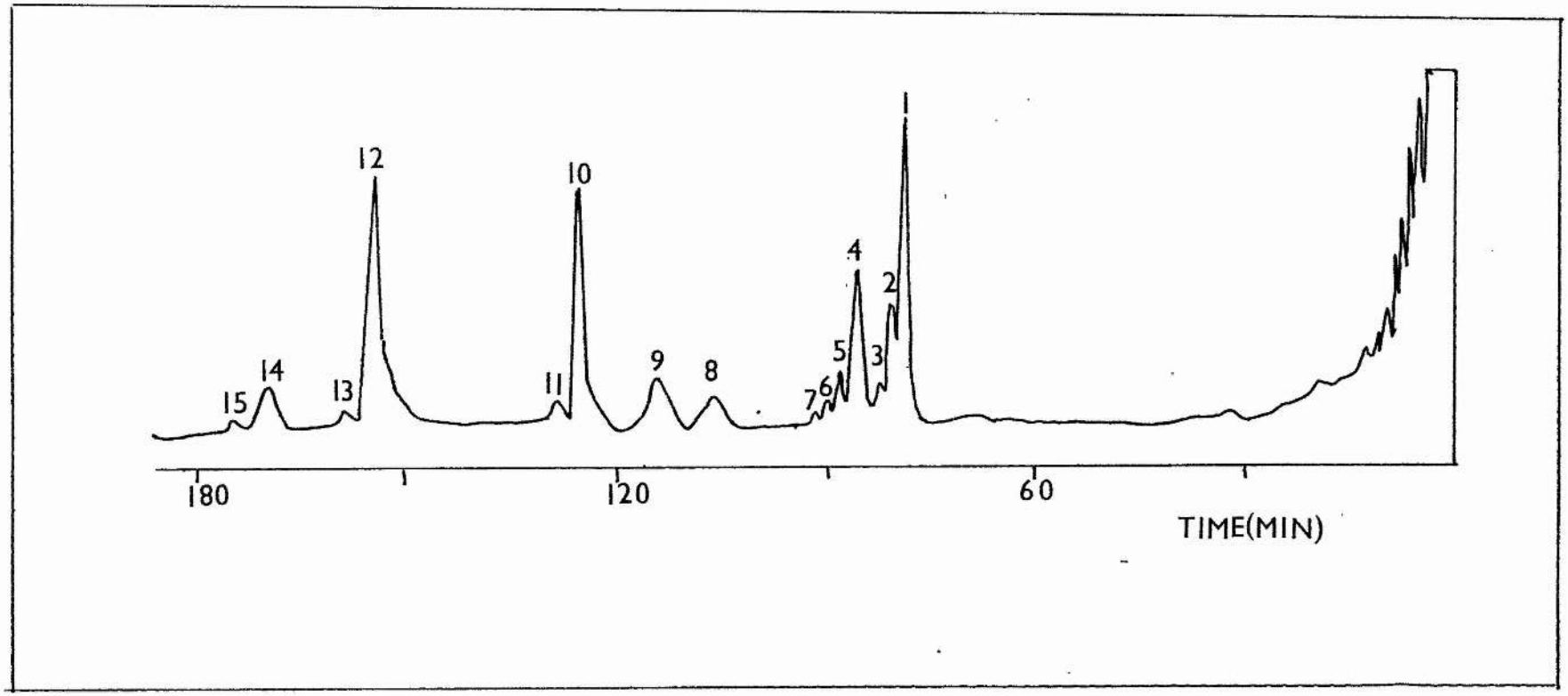

12
10
14
15
13
11
9
8
4
2
5
3
7
6
1
180
120
60
TIME(MIN)

Fig. 10b:

GLC analysis of glycopeptide A.

Peaks are numbered as in Fig. 10.

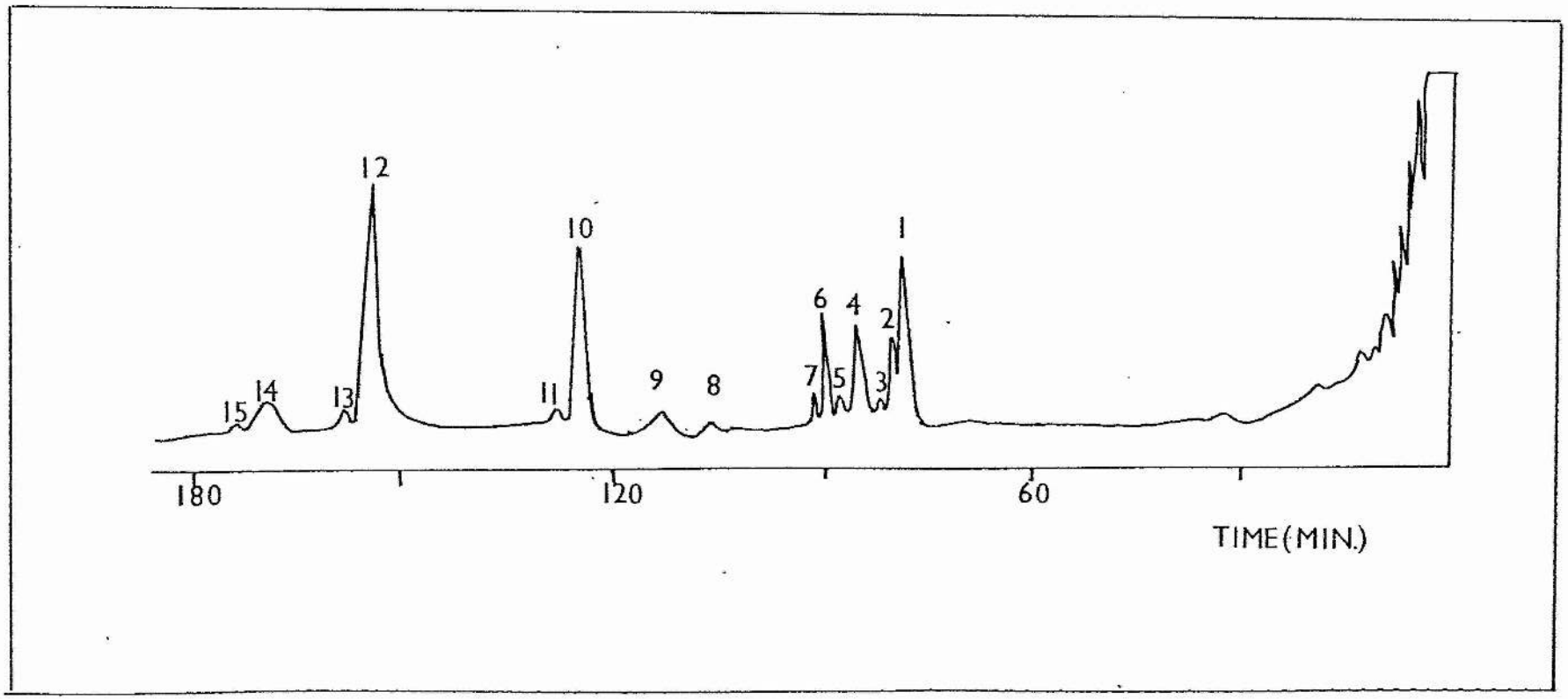

12
10
1
6
4
2
15
14
13
11
9
8
7
5
3
180
120
60
TIME(MIN.)

Fig. 10c:

GLC analysis of glycopeptide B.

Peaks are numbered as in Fig. 10.

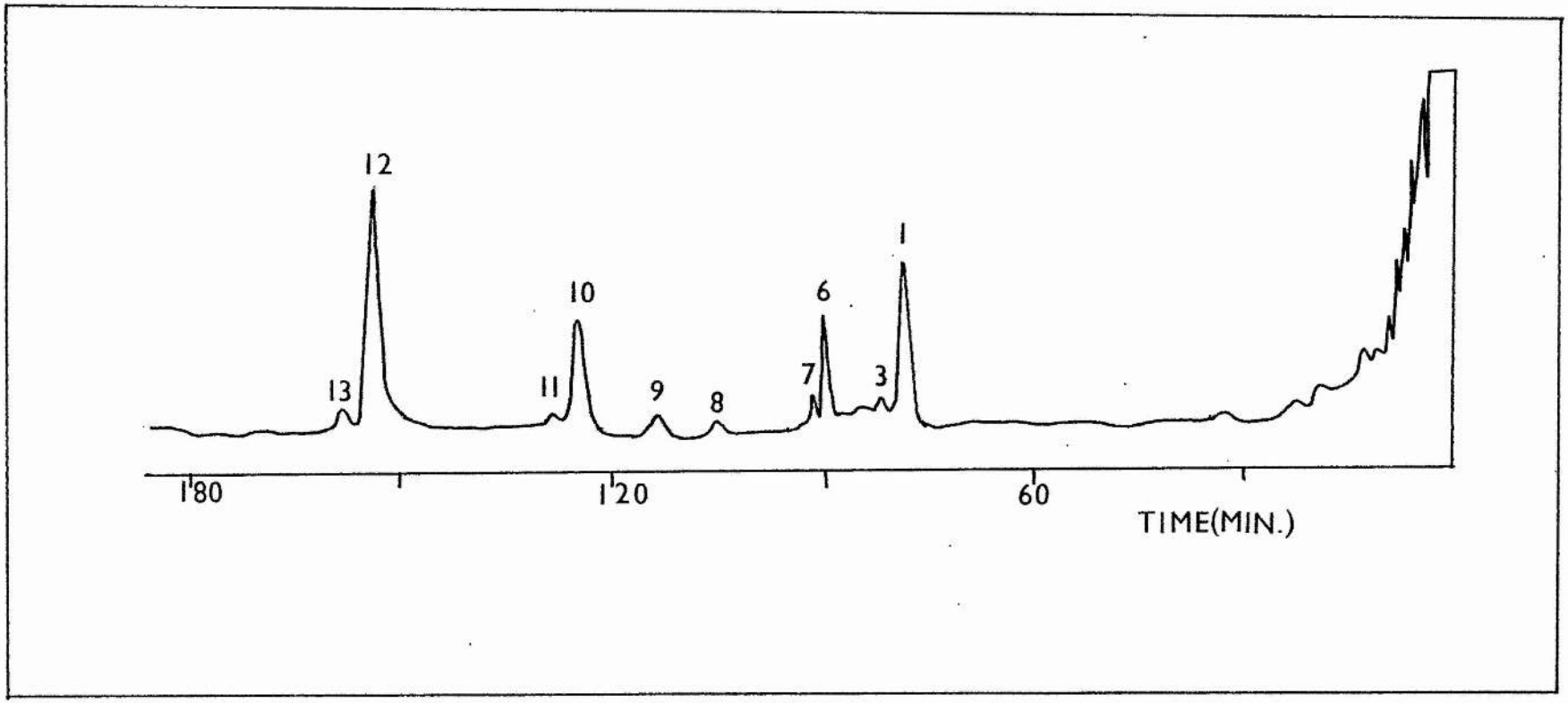

12
13
10
11
9
8
6
7
3
1
180
120
60
TIME(MIN.)

Fig. 11:

Molar Relative Response Factor determination for:

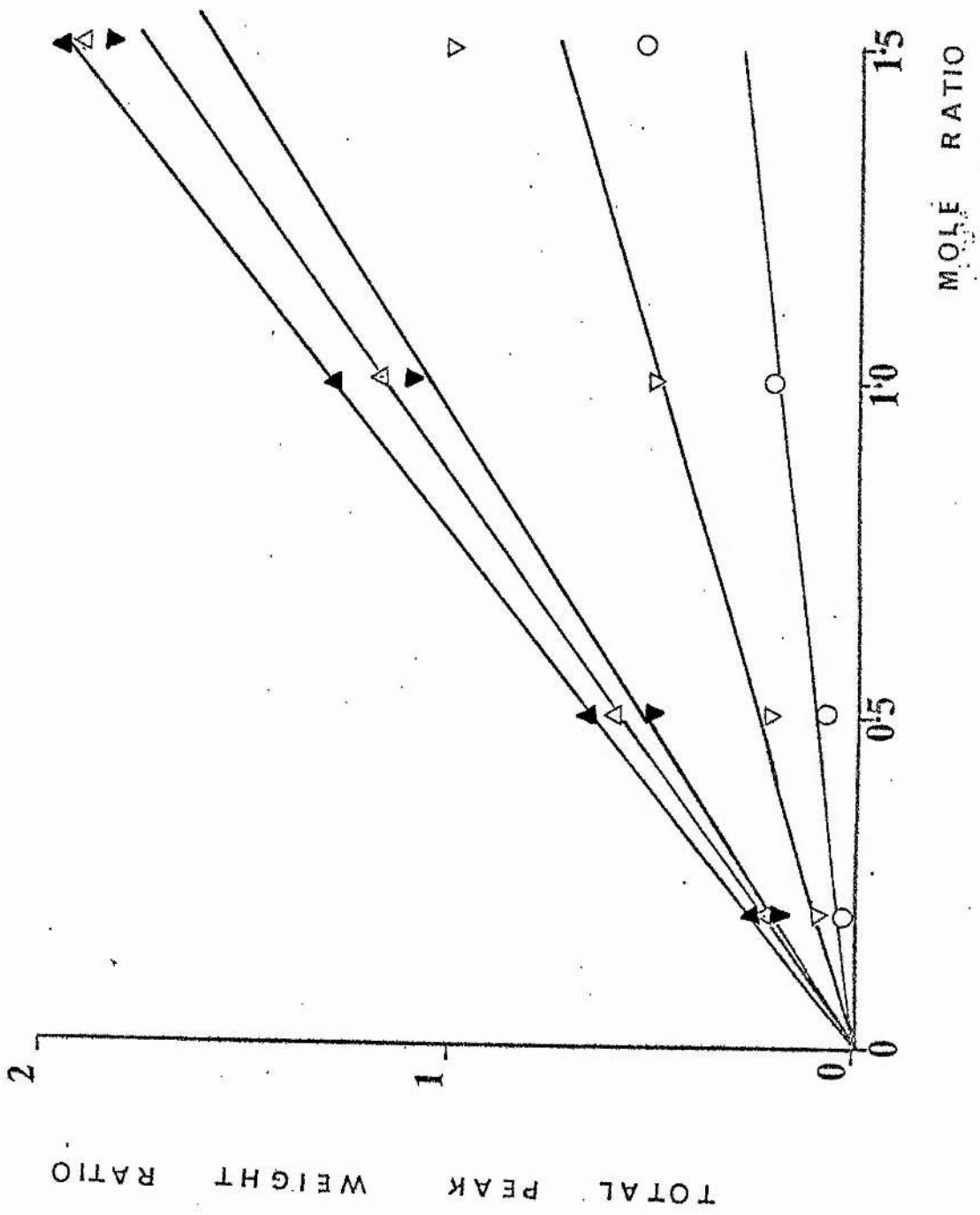
(▲) : Glucose

(△) : Mannose

(▼) : Galactose

(▽) : N-Acetylglucosamine

(○) : Sialic acid


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

3.4 PRONASE DIGESTION OF SGP

The Sephadex-G50 elution profile of the material obtained after the second pronase treatment (section 2.14) was identical with that obtained after the first digestion of the non carboxymethylated SGP yielding two peaks, A and B, (Fig. 12) with a carbohydrate content of approx. 1.4mg and 1.1mg respectively (determined colorimetrically by the phenol-H_2SO_4 assay).

The GLC analysis revealed that glycopeptide A contained mannose, galactose, N-acetylglucosamine and sialic acid in a 3:2:4:2 molar ratio while glycopeptide B contained only mannose and N-acetylglucosamine in a 3:2 molar ratio (Fig. 10b and 10c).

	moles/mg of glycopeptide	
	Peak A	Peak B
Mannose	0.28	0.32
Galactose	0.20	-
N-acetylglucosamine	0.36	0.20
Sialic acid	0.22	-

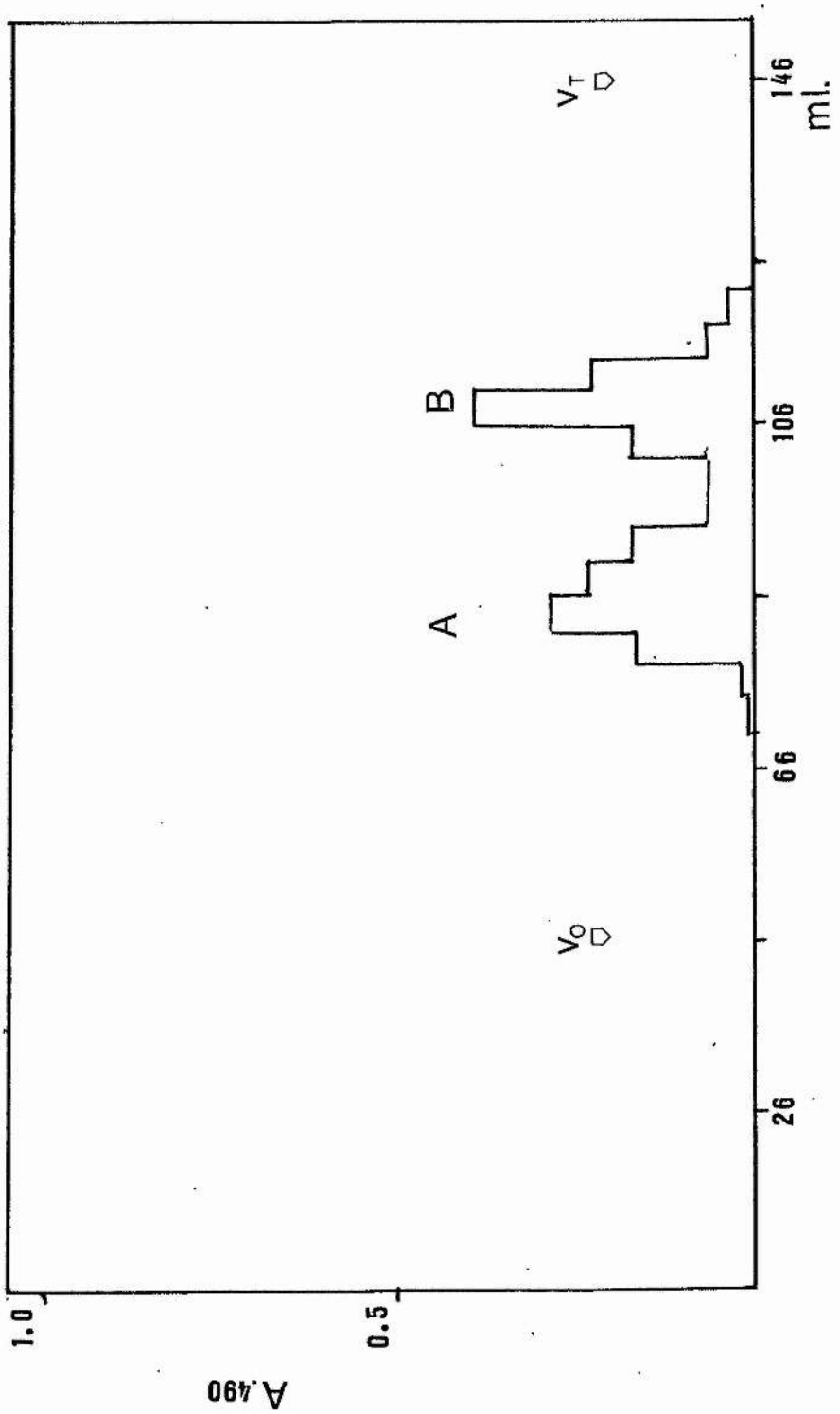
Carbohydrate composition of peak A and B separated on Sephadex-G50 column after Pronase digestion.

The presence of glucose in these two glycopeptides is probably due to contamination arising from the Sephadex column.

Fig. 12:

Sephadex G-50 elution profile of the glycopeptides obtained after pronase digestion of S-carboxymethylated SGP.

Values given as measure of hexose.


A 490
1.0
0.5
26
66
106
146
ml.
V_O
V_T
A
B

3.5 ELECTRON MICROSCOPY

SGP precipitates in the presence of copper ions appeared to have the structure of cylindrical tactoids (Fig. 13) whose diameters measured on thin sections of the embedded precipitate were found to average 11nm.

It must be noted that when guanidine-hydrochloride was removed in the absence of copper ions, SGP aggregates appeared amorphous when examined under the electron microscope.

3.6 LYSYL OXIDASE ACTIVITY ASSAY

Table II summarizes the results of the lysyl oxidase assay performed according to Pinnell and Martin (1968).

Preparation 1 and 2 refer to two different SGP preparations of different activity. A progressive reduction of tritium release was observed (50% in 4 days) as SGP underwent time-dependent aggregation.

Fig. 13:

Precipitate of SGP, obtained after exposure to copper ions, negatively stained with uranyl formate. X 182,000.

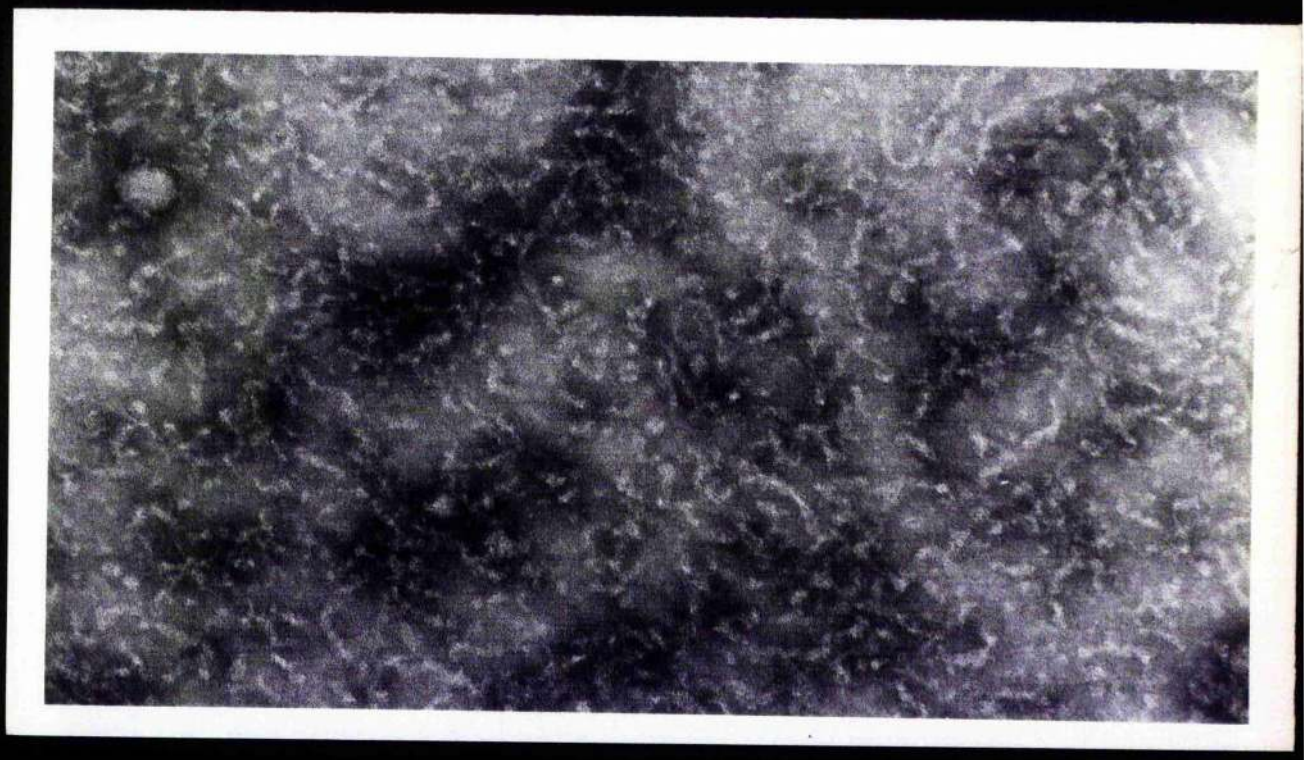


Table II : Enzyme Assay for SGP[a]

SGP preparation	SGP added to assay mixture (µg)	Age of SGP following final dialysis (days)	Substrate (at final concentration)	Net $[^3H]$ release[b] (dpm)	10^{-4} x Specific Activity[c] (dpm/mg protein)
1	24	1	$[^3H]$Aorta	12 469	59.71
	24	1	$[^3H]$Aorta + BAPN(0.27mM)	10 699	51.23
	24	1	$[^3H]$Aorta + L-Lysine(10mM)	6 988	33.47
	24	4	$[^3H]$Aorta	6 187	29.63
2	41	1	$[^3H]$Lysine + L-Lysine(0.1mM)	5 172	14.52
	30	2	$[^3H]$Aorta	4 489	17.20
	30	2	$[^3H]$Aorta + BAPN(0.27mM)	3 879	14.86
	30	2	$[^3H]$Aorta + BAPN(2.7mM)	2 201	8.44
	30	2	$[^3H]$Aorta + BAPN(5.4mM)	1 406	5.39
	30	2	$[^3H]$Aorta + BAPN(27.0mM)	1 500	5.68

[a]Enzyme activity, determined by $[^3H]$ release (isolated as ^3H_2O), was quantified by the microdistillation procedure of Misiorowski et al., (1976). Assays were performed at least in duplicate, mean values are quoted (Max. variations: ± 9%).

[b]Net $[^3H]$ release in dpm was quantified following subtraction of a blank value (2463 ± 391 dpm), the mean of five determinations. Similar blank values were obtained using either substrate alone or substrate + boiled enzyme.

[c]Specific enzyme activity was defined as the dpm of ^3H_2O formed/mg purified SGP protein/2h incubation. Values are corrected for distillation losses.

SECTION FOUR

DISCUSSION

DISCUSSION

The data that have emerged from the present study allow us to draw some interesting considerations about SGP and its correlation with the microfibrillar component extracted from bovine ligamentum nuchae by Ross and Bornstein (1969), the structural glycoprotein isolated from pig aorta by Moczar et al. (1977) and the lysyl oxidases of Jordan et al. (1977) and Kagan et al. (1979).

First of all the difficulties encountered in the isolation and purification of SGP can be explained by the presence of 27 residues of cysteine per 1000 of amino acids residues, probably involved in disulphide bonds, that made SGP virtually insoluble, in spite of the high content of polar amino acids. In fact the protein can be solubilized only in denaturing solvent such as 5M guanidine-HCl containing 2-mercaptoethanol or other reducing agents.

As mentioned in the introduction, electron microscopic studies have shown the presence in bovine ligamentum nuchae of a microfibrillar component in the form of fibrils (11nm diameter) which surround the elastic fibres. The fact that SGP aggregates in vitro, in the presence of copper ions, to form fibrils of 11nm diameter strongly suggests that SGP is the, or one of the constituents of this connective tissue component observed by Ross and Bornstein (1969). The much higher yield reported by these authors (5-10% compaired with about 0.1% of SGP) could be linked to the fact that the percentage of the microfibrillar component in the elastic tissue decreases with ageing as has been reported by several authors (Gotte and Serafini-Fracassini, 1963; Greenlee et al., 1966; Fahrenbach et al. 1966). On the other hand the amino acid composition of the two preparations, although similar, shows some discrepancies (Table III) probably because of the different purification procedure adopted by Ross and Bornstein that involves a treatment with collagenase. In our

TABLE III

	Ross and Bornstein 1969	Moczar 1977
	Residues/1000 Residues	
Hydroxyproline	0.0	0.0
Aspartic Acid	114.0	94.9
Threonine	55.1	54.1
Serine	58.9	66.0
Glutamic Acid	111.0	136.4
Proline	70.4	62.3
Glycine	120.0	104.2
Alanine	58.9	79.1
$\frac{1}{2}$ Cystine	60.3	12.0
Valine	54.1	50.9
Methionine	15.8	3.6
Isoleucine	45.2	54.6
Leucine	57.2	23.2
Phenylalanine	32.1	38.1
Lysine	36.9	57.4
Histidine	14.2	15.1
Tryptophan	n.d.	8.2
Arginine	45.2	47.7

case the use of this enzyme, (purified by affinity chromatography to reduce the non specific activity known to be present in most commercially available preparations) for the purification of crude SGP caused extensive cleavage of the glycoprotein yielding fragments of about 11500 molecular weight.

The correlation between the structural glycoprotein isolated by Moczar from pig aorta and bovine ligamentum nuchae SGP is based essentially on the identity of the molecular weight, since amino acid and carbohydrate compositions are rather different (Table III) maybe because of tissue and species specificity.

To determine the molecular weight of SGP three different approaches were used, since each of them taken on its own did not prove to be conclusive.

On SDS-polyacrylamide gel electrophoresis the apparent molecular weight of SGP varied between 35000 and 37000 according to the cross-linking of the gels used. Glycoproteins with 10% or more carbohydrate are known to behave anomalously on SDS-PAGE (Weber et al., 1972; Pitt-Rivers and Ambesi Impiombato, 1968) showing a reduced mobility that could be due to an interaction between carbohydrate and polyacrylamide and/or to decreased binding of SDS per gram of glycoprotein as compared with standard proteins. The SDS binding results in a decreased charge to mass ratio for glycoproteins versus standard proteins, a decreased mobility during SDS gel electrophoresis and thus a higher apparent molecular weight. (Schubert, 1976; Segrest and Jackson, 1972). These problems are partly overcome by running glycoproteins on gels of different porosities. Under these conditions the apparent molecular weight decreases with the increasing of the porosity approaching asymptoticly values closer to the real molecular weight since, of the two factors (charge and molecular sieving) involved in electrophoresis in SDS gels, molecular sieving

predominates when the cross-linking of polyacrylamide gels is increased (Segrest and Jackson, 1972).

Data obtained by ultracentrifugation (yielding a molecular weight of around 33000) could not be considered decisive either since the presence of guanidine hydrochloride causes a considerable degree of uncertainty in determining the apparent specific volume because of selective bindings. Nevertheless the close agreement shown by the results obtained by ultracentrifugation, electrophoresis and compositional analysis can justify a certain confidence in fixing the molecular weight of SGP at around 34000 daltons.

The amounts of tritium released during the assay for lysyl oxidase activity were significant enough to assume an oxidase activity for SGP toward an aortic substrate.

The presence of free lysine at high concentration in the assay mixture together with the aortic substrate caused a drop of only 44% of the initial activity. This indicates that SGP possesses at least dual substrate specificity as shown by the experiment in which free lysine tagged with carrier ^{3}H lysine (specific activity of tritium in the mixture $\sim$ 2μCi/mol) was used as substrate.

The presence of BAPN caused a 14% inhibition when its concentration was in the order of 0.27mM. The inhibition went up to 70% when higher BAPN concentrations were used. The fact that BAPN, at low concentrations, induces only slight reduction in activity may indicate that it is metabolized by the amino-oxidase activity of SGP. This behaviour of SGP in relation to the inhibitory effect of BAPN and free lysine is markedly different from that exhibited by lysyl oxidases isolated from connective tissues.

However the urea-soluble lysyl oxidases isolated from bovine ligamentum nuchae and aorta by Jordan and Kagan respectively share with SGP the same

tendency to aggregate in the absence of denaturing agents forming multimeric species that are functionally active (Jordan et al., 1977). They also show a similar molecular weight (approx. 32000-33000 daltons) and amino acid composition (Table IV) but differ from SGP as they show only amine oxidase activity toward peptydyl-lysine.

If SGP is, as it seems, a lysyl oxidase it is open to speculation exactly what its biological function might be, particularly in relation to other isoenzymes capable of catalyzing the same type of reaction and to its presence in connective tissue in a highly aggregated form.

Stassen (1976) has reported that in vitro all four species of lysyl oxidase of chick-embryo cartilage oxidize both elastin and collagen substrates. However it is possible that lysines in different environments within each of these connective-tissue proteins may require enzyme species of different specificities. Furthermore, although the enzyme has been isolated as a monomer of about 30 000 molecular weight, it is not clear at this time what the state of aggregation may be when the enzyme oxidatively deaminates peptidyl lysine in its natural environment. It is conceivable, that multimeric forms of lysyl oxidase composed of various mixtures of the individual, subunits might exist in vivo and that these higher molecular weight aggregates may have unique substrate specificities (Kagan et al., 1979).

The state of aggregation of the substrate itself . seems to have a considerable importance on the activity of lysyl oxidases. Experiments carried out with highly purified enzyme preparations have shown that the best collagen substrate is a loosely aggregated collagen fibril rather than collagen monomers and similar conclusions have been reached for elastin (Siegel 1979). This suggests that aldehyde formation occurs extracellularly during biosynthesis in vivo after cleavage of the registration peptides by procollagen peptidase. It seems therefore

TABLE IV

Residues/1000 Res.	I	II	III	IV
Aspartic Acid	123.1	125	121.5	94.3
Threonine	56.7	55	55.7	47.5
Serine	103.7	86.3	103.7	121.6
Glutamic Acid	112.7	135.7	135.8	124.1
Proline	59.8	51.1	49.8	50.6
Glycine	120.3	86.8	107.7	176.8
Alanine	71.1	80.7	82.8	86.4
Valine	39	42.3	34.7	31.7
Cysteine	26.9	24	18.3	14.3
Methionine	15.3	16.1	14.6	16.9
Isoleucine	30	27.2	30.6	28.5
Leucine	64.3	86.1	78.3	64.7
Tyrosine	25	31.4	21.4	17.8
Phenylalanine	27.2	30.3	25.9	24.8
Lysine	30.8	35.8	35.6	28.5
Histidine	38.6	25.4	27.1	22.7
Arginine	55.5	60.5	56.3	49.3

I, II, III, IV: Amino acid composition of the four isoenzymes isolated by Kagan et al., 1979.

probable that lysyl oxidase acts on the substrate, when this has acquired its definite quaternary structure to allow the maximum number of couplings between lysine residues to be established prior the formation of the covalent cross-links.

Two kinds of cross-links are formed in the collagen fiber: intramolecular (between the α chains of tropocollagen molecules) and intermolecular (between different tropocollagen units).

The tropocollagen molecule is stabilized by a single intramolecular covalent cross-link. This involves lysyl residues which are located in the N-terminal non-helical region of the α chains. These lysyl residues are oxidatively deaminated and the resulting allysyl residues condense with one another to form an acid-stable aldol condensation product.

From a chemical point of view, four types of intermolecular cross-links have been clearly defined so far:

1. An acid-stable aldol condensation product, formed by the reaction of one molecule of allysine and one of hydroxyallysine. This is similar in nature to the intramolecular cross-link (Bailey et al., 1969).
2. Schiff bases which are acid-labile and which can consequently be isolated and identified only after stabilization by reduction with sodium borohydride.

   (a) Dehydrolysinonorleucine formed by condensation of allysine with the ε-amino group of a peptide-bound lysine.

   (b) Dehydrohydroxylysinonorleucine resulting from the condensation of either allysine with the ε-amino group of hydroxylysine or hydroxyallysine with the ε-amino group of lysine.

   (c) Dehydrohydroxylysinohydroxynorleucine, formed by the condensation of hydroxyallysine with the ε-amino group of hydroxylysine.

3. Stable, naturally reduced cross-links. Hydroxylysinohydroxynorleucine is the only cross-link of this type identified so far in appreciable amount in mature collagen.

4. Cross-links involving histidine, formed by condensation of an aldol condensation product with histidine.

Aldol condensation products and lysinonorleucine are also found in elastin. Peculiar to elastin are merodesmosine, desmosine and isodesmosine. Merodesmosine can be formed from the reaction of the aldehyde function of an aldol condensation product with the amino group of a lysine side chain, on the other hand desmosine is formed by the interaction of three allysine residues with one residue of lysine to form a pyridinium ring. Isodesmosine is similarly formed, the difference being a different disposition of the allysine residues.

All these cross-links are found in regions of elastin polypeptide chain which are rich in alanine and lysine with two lysyl residues being separated by two or three alanines.

The importance of these cross-links in the maturation of both collagen and elastin is demonstrated by the pathological states in which lysyl oxidase is lacking.

The administration of BAPN or other lathyrogenic substance to experimental animals inhibits cross-linking of collagen molecules and considerably increases the amount of soluble collagen.

In the X-linked Ehlers-Danlos Syndrome (Type V) lysyl oxidase deficiency causes atrial septal defect, musculoskeletal weakness, stretchy skin, and skeletal deformities.

Similarly patients affected by X-linked Cutis Laxa although not exhibiting cardiovascular abnormalities, show long, thin faces with redundant upper eyelids, mild join laxity and loose skin over the

dorsum of the hands and feet. Both types of patients show marked increases in the solubility of collagen synthesized by fibroblasts.

Copper is essential for lysyl oxidase activity and copper-deficient animals are characterized by an extreme weakness of their connective tissues. Patients with Menke's syndrome have low intestinal copper absorption and show connective tissue defects such as spinal curvature and cardiovascular abnormalities. Their skin collagen is characterized by an abnormally high degree of solubility.

Finally lysyl oxidase activity in extracts of skin of aneurysm-prone mice is markedly lower than the activity in control mice resulting in weakness of skin and of blood vessels.

Because of the difference in substrate specificity between lysyl oxidases and SGP and the uncertainties still surrounding the classification of amine oxidases it is difficult to give an exact enzyme classification of SGP.

Lysyl oxidase is defined by its strict substrate specificity and the type of enzymic reaction, cofactors and inhibitors involved suggest that the enzyme is similar to other amine oxidases (EC 1.4.3.6). However lysyl oxidase seems to be immunologically different from Cu-amine oxidases of the same species and could therefore be the first of a separate class of enzymes.

On the other hand SGP could be more closely related to human placental diamine oxidase (Crabbe et al., 1976) which is copper dependent and has both amine and lysyl oxidase activity.

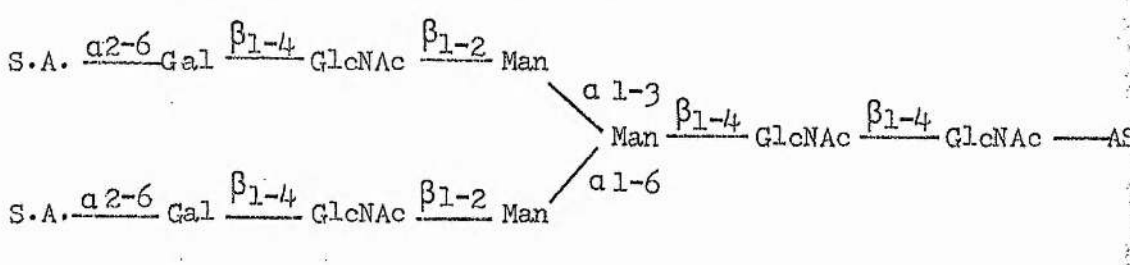
The isolation of two glycopeptides from SGP after pronase digestion clearly showed the presence of two carbohydrate chains in SGP. One of these (A) contains only mannose and N-acetylglucosamine (molar ratio 3:2)

while the other (B), probably branched, contains mannose, N-acetylglucosamine, galactose and sialic acid (molar ratios 3:4:2:2). It should be noted that the presence of a single carbohydrate chain at different stages of completion cannot be totally excluded.

A further point worth mentioning is the identification of glucose peaks in the GLC chromatograms of the two glycopeptides. As mentioned before it seems reasonable to ascribe the presence of glucose to contamination arising from the Sephadex column since virtually no glucose was detectable in the purified SGP.

However the presence of glucose in the glycoprotein cannot be totally excluded since Spiro et al. (1967) have demonstrated the existence of a glucosylated lipid-oligosaccharide which acts as an intermediate in the biosynthesis of some glycoproteins and from which glucose is removed in the final stages of completion of the macromolecule. It could be possible therefore to have oligosaccharide chains in which the glucose has not been removed. However no definite evidence has yet been obtained to justify this hypothesis.

The information so far available does not allow the final structure of these glycopeptides to be established but a first guess based on the molar ratios and on the available literature about glycopeptide structures could be that glycopeptide B has a structure similar to that proposed by Toyoshima et al. (1973) for human transferrin glycopeptide:



(S.A.: sialic acid; Gal: galactose; GlcNAc: N-acetylglucosamine; Man: mannose; ASN: asparagine)

Glycopeptide A probably has a simpler structure containing the same GlcNAc-GlcNAc-Man core of glycopeptide A with two additional mannose residues.

Phosphorylated sugar residues have been found to be the key structure for the pynocytosis of lysosomal enzymes by cultured human fibroblasts (Hasilik and Neufeld, 1980; Distler et al., 1979).

For instance, bovine testicular β-galactosidase is rapidly and strongly inhibited by mannose 6-phosphate and by a glycoprotein fraction isolated from bovine testes (glycoprotein inhibitors) thus suggesting that β-galactosidase and the glycoprotein inhibitors have a common recognition marker that contains mannose 6-phosphate (Distler et al., 1979).

The possibility that a similar recognition marker may be present on SGP too is suggested by the observation that SGP precipitated under the conditions described in Section 2.16 for electron microscopy and spread onto plastic Petri dishes induces the adhesion of fibroblasts on its surface and causes their rapid proliferation. In this respect SGP seems to behave like fibronectin. (P. Wells, unpublished results.)

Furthermore electron microscopic observation on foetal ligamentum nuchae at the stage of maximum elastic synthesis (7 months-old) showed that active fibroblasts adhere to nascent elastic fibres. These fibroblasts are characterized by increased content of ribosomal material and cysternal spaces.

At the interface between elastin and the fibroblast there is a layer of microfibrillar material in a precipitate state. If an identity between this microfibrillar material and SGP can be assumed, it could be postulated that the initial stages of cross-link formation of the elastin precursor are performed on the cell surface by the action of SGP that subsequently precipitates and acts as a signal (through its carbohydrate

moiety) for another fibroblast to adhere on the new surface for further synthesis of elastin.

SECTION FIVE

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