# STRUCTURE AND METABOLISM OF BOVINE AORTIC PROTEOGLYCANS

by

EILEEN MURRAY

Submitted in fulfilment of the requirement

for the degree of

DOCTOR OF PHILOSOPHY

in the

FACULTY OF MEDICINE (MEDICAL BIOCHEMISTRY)

UNIVERSITY OF CAPE TOWN

August 1983

## CERTIFICATE OF SUPERVISOR

In terms of Paragraph 8 of "Regulations for the degree of Ph.D.", I, as supervisor of the candidate, Eileen Murray, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

Signed by candidate

Dr Timothy Scott-Burden

The following papers relating to the work described in this thesis have been accepted for publication:

1. Scott-Burden, T., Murray, E., Diehl, T. and Gevers, W. "Glycosaminoglycan synthesis by smooth muscle cells cultured in absence and presence of ascorbic acid". Hoppe-Seyler's Z. Physiol. Chem. 364, 61-70, 1983.

2. Murray, E., Scott-Burden, T., Ferguson, P. and Gevers, W. "Increased sulphation level and altered composition of glycosaminoglycans synthesized by cultured smooth muscle cells in the presence of β-D-xylosides". Biochim. Biophys. Acta. Accepted for publication 10.8.83.

## ABSTRACT

Although a number of studies of arterial glycosaminoglycans have been carried out, there is comparatively little information available regarding the native state in which these glycosaminoglycans occur, namely covalently bound to protein to form proteoglycans. There are numerous factors that make arterial proteoglycans difficult to study, such as their relatively low concentration in the tissue as compared to cartilaginous tissue, and their association with the fibrous elements of the matrix which make quantitative extraction difficult. In recent years reports of characterizations of arterial proteoglycans have appeared in the literature but they differ markedly in several respects. In order to minimize such variables, proteoglycans isolated from pure medial tissue of bovine aortas from different age groups have been studied. Isolation of these proteoglycans was carried out essentially as described for their counterparts from cartilage. Furthermore, proteoglycans have been isolated from a cultured clonal line of foetal bovine aortic medial smooth muscle cells and comparisons made between the two sources of material in terms of their proteoglycan monomer size and glycosaminoglycan composition.

Material isolated from the aortas of all ages studied contained two main sizes of proteoglycan monomer with $K_{av}$'s of 0,31 and 0,56 when chromatographed on Sepharose CL-2B columns. The larger species was the predominant one in foetal tissue but in the adult tissue the smaller species

predominated. The differences in size could be attributed partially to differences in glycosaminoglycan chain length as this was found to decrease with age. However, the glycosaminoglycan chains of all ages were very long (with a molecular weight of greater than 40000) and thus were at least twice as long as those found in cartilage proteoglycans. Proteoglycan monomers from young tissue appeared unable to interact with both endogenous and exogenous hyaluronic acid, whereas those from adult tissue could do so to some degree. Chondroitin-4- and -6-sulphate made up the bulk of the glycosaminoglycans from the proteoglycans of all ages. No dermatan sulphate was found in proteoglycans from foetal tissue but its concentration increased with age. Small amounts of heparan sulphate/heparin were found in all ages and its concentration also increased with age. The presence of keratan sulphate-like oligosaccharide chains was suggested by the presence of sialic acid-containing material.

When $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine labelled proteoglycans were isolated from the culture medium and cell layer of cultured foetal bovine aortic smooth muscle cells, the sizes, as assessed by Sepharose CL-2B chromatography, were similar to those found for the proteoglycans extracted from foetal tissue. Two sizes of proteoglycan monomer were found in both the medium and cell-layer, with $K_{av}$'s of 0,31 and 0,60 for the medium proteoglycans and 0,27 and 0,76 for proteoglycans from the cell layer. However, the bulk of the sulphated material was found in the culture medium and the sizes of the medium proteoglycans were the same as those from foetal tissue. The

glycosaminoglycan chains were all found to be the same size and the same as those from foetal tissue (MW approx. 50000). About 16% of the proteoglycans from the medium could interact with exogenous hyaluronic acid, whereas those isolated from the cell layer appeared unable to form aggregates. Chondroitin sulphate was the major glycosaminoglycan found in the cultures, but significant amounts of dermatan sulphate were found which had been shown to be absent from proteoglycans derived from the foetal tissue. No hyaluronic acid or keratanase-sensitive material was synthesized by the cultured cells. The glycosaminoglycan composition of the different culture compartments was shown to be quite distinct, with chondroitin sulphate making up the bulk in the extracellular compartment (medium); heparan sulphate predominated in the pericellular-matrix compartment and dermatan sulphate was found in highest concentration in the intracellular compartment.

The effects of ascorbic acid and p-nitrophenyl-β-D-xyloside on the biosynthesis of glycosaminoglycans by the smooth muscle cells was studied. Ascorbic acid supplementation resulted in the cells producing a more copious extracellular matrix which consisted largely of collagen, whereas in its absence the matrix contained less than 2% collagen. Ascorbic acid addition was also shown to increase the incorporation of $|^{35}S|$ sulphate into glycosaminoglycans, particularly in the pericellular-matrix culture compartment. This increased incorporation was shown to be partly as a result of increased synthesis and partly due to increased sulphation of the

glycosaminoglycan chains. In addition, the effects of ascorbic acid were shown to be mediated, at least in part, by the presence of the vitamin itself and not via its effects on collagen production in the matrix. β-D-xylosides have been shown to act as artificial acceptors for glycosaminoglycan chains, thus circumventing the requirement for core protein and the initial xylosyltransferase enzyme involved in glycosaminoglycan synthesis. The addition of p-nitrophenyl-β-D-xylopyranoside to the cells in culture caused a marked increase in $|^{35}S|$ sulphate incorporation into glycosaminoglycans which were readily secreted into the medium. The chains were shorter than normal and the increased incorporation was again shown to result both from increased synthesis and increased sulphation of the glycosaminoglycans. In addition, β-D-xyloside had the most marked effect on incorporation of labelled precursor into the dermatan sulphate fraction so that its relative concentration increased in the presence of the drug. β-D-xyloside was also shown to decrease the amount of material synthesized on core protein and the glycosaminoglycan chains formed on the core protein in the presence of the drug were also shown to be shorter than normal and to have increased levels of sulphation.

The turnover of proteoglycans was also studied using the tissue culture system. When added to cells in culture radioactively labelled proteoglycans were internalized via receptor-mediated endocytosis, and unlabelled proteoglycans competed with their radiolabelled counterparts for these binding sites. Degradation of the proteoglycans, once inside

the cell, was inhibited by lysomotrophic agents which indicated that they were degraded by the lysosomal enzyme system. Degradation of the whole molecule occurred without prior partial desulphation as has been suggested by some workers.

Thus the proteoglycans from the medial layer of bovine aortas of different ages have been characterized with respect to monomer size, glycosaminoglycan chain length, glycosaminoglycan composition and ability to interact with hyaluronic acid. Furthermore, it was shown that the smooth muscle cell line derived from such medial tissue largely retained its differentiated function with respect to the synthesis of proteoglycans. The effects of various exogenous agents on the biosynthesis and degradation of these molecules by the cells in culture have been studied.

## ACKNOWLEDGEMENTS

I wish to express my appreciation and thanks to the following:

Dr Timothy Scott-Burden for his excellent supervision, enthusiasm and unending encouragement throughout the accomplishment of this thesis.

Prof. W. Gevers for the many hours of stimulating discussion and for providing invaluable assistance, without which this thesis would not have been possible.

Dr M.B.E. Sweet for providing laboratory facilities over a three-month period during the course of this work.

Rose Marie Estment for all her help, particularly in regard to the typing of this manuscript.

Karen Judge and Patricia Ferguson for the efficient running of the tissue culture unit.

Patricia Ferguson for technical assistance, for preparing the photographs for this thesis and for much more.

Dr E. Merrifield for performing detailed sugar analysis on samples.

Messrs S. Groeneveldt and A. Adonis for assistance in the laboratory.

Many other friends and colleagues for providing a pleasant working atmosphere.

Mr McSweeney and staff at the Cape Town Municipal Abattoir for their willingness to assist in the not-so-pleasant task of collecting tissue.

Mr D. Sanan from the Medical Research Council Electron Microscopy Unit for his assistance.

My parents for their support, both financial and emotional, without which my studies would never have progressed thus far.

My husband, Glen, whose faith in the ultimate outcome of this project never faltered. Thank you.

The S.A. Medical Research Council, Harry Crossley Foundation and University of Cape Town for financial support.

## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ADP | = adenosine diphosphate |
| APS | = adenosine-5'-phosphosulphate |
| ATP | = adenosine triphosphate |
| BSA | = bovine serum albumin |
| C | = Celsius |
| Ci | = Curie ($3,7 \times 10^{10}$ disintegrations per second) |
| cm | = centimeter |
| $cm^3$ | = cubic centimeter |
| CDP | = cytosine diphosphate |
| CMP | = cytosine monophosphate |
| DEAE-cellulose | = diethylaminoethyl cellulose |
| ΔDi-4S | = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose |
| ΔDi-6S | = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose |
| ΔDi-0S | = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose |
| ΔDi-diS | = disulphated unsaturated disaccharide obtained by digestion of dermatan sulphate by chondroitinase ABC |
| DMSO | = dimethylsulphoxide |
| DNA | = deoxyribonucleic acid |
| DPM | = disintegrations per minute |
| EDTA | = ethylenediamine tetraacetic acid |
| g | = acceleration due to gravity |
| GDP | = guanidine diphosphate |
| h | = hours |
| $K_{av}$ | = $\frac{V_e - V_o}{V_t - V_o}$ |
| $K_d$ | = dissociation constant |

| | |
|---|---|
| l | = litre |
| LiCl | = lithium chloride |
| M | = molar |
| mg | = milligram |
| min | = minutes |
| ml | = millilitre |
| mm | = millimeter |
| mM | = millimolar |
| MW | = molecular weight |
| nm | = nanometer |
| PAGE | = polyacrylamide gel electrophoresis |
| PAPS | = phosphoadenosine-5'-phosphosulphate |
| PBS | = phosphate buffered saline |
| PGM | = proteoglycan monomer |
| pH | = negative logarithm of the hydrogen ion concentration |
| PMSF | = phenylmethylsulphonyl fluoride |
| rpm | = revolutions per minute |
| RNA | = ribonucleic acid |
| S.D. | = standard deviation $\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}$ |
| SDS | = sodium dodecyl sulphate |
| TCA | = trichloroacetic acid |
| Tris | = tris (hydroxymethyl) amino methane |
| UDP | = uridine diphosphate |
| μ (prefix) | = micro ($10^{-6}$x) |
| μg | = microgram |
| μl | = microlitre |
| μM | = micromolar |

$V_e$ = elution volume

$V_o$ = void volume

$V_t$ = total volume

v/v = volume per volume

w/v = weight per volume

% = percent

|| = concentration

CONTENTS

# CHAPTER 1

# INTRODUCTION

## 1.1. THE MOLECULAR STRUCTURE OF PROTEOGLYCANS

The name proteoglycans was introduced in 1967 to describe a large group of complex macromolecules which are made up of a protein core to which polysaccharide side chains(s) are attached (1). Within this class of molecules, there is enormous variability; the name encompasses a range of structures which are presumed to have different core proteins, and carbohydrate chains which have been shown to differ in length, composition and number (2-5). These molecules are found in almost all parts of the body and serve many diverse functions related to their characteristic physico-chemical properties. Many of these functions result from their ability to interact specifically with other biological macromolecules (see Section 1.4).

The polysaccharide side chains, which usually make up the bulk of the proteoglycan molecule are called glycosaminoglycans. To date seven different types of glycosaminoglycans have been identified on the basis of their structural units (Tables 1.1 and 1.2) (6). They all consist of repeating disaccharide units linked together to yield unbranched macromolecules. The repeating disaccharide units are usually composed of an N-acetylated hexosamine linked to a hexuronic acid residue. The unit also often contains O- or N-linked

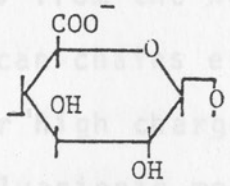
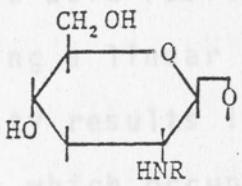
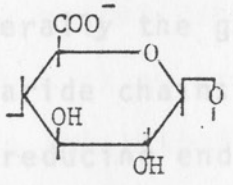
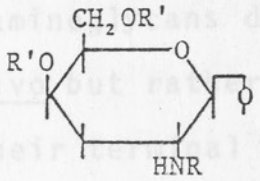
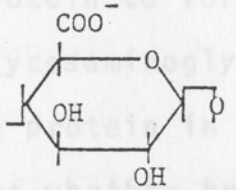
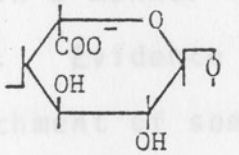
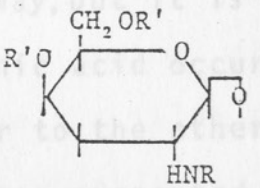
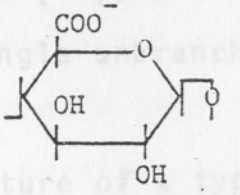
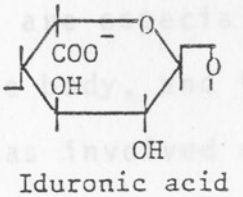
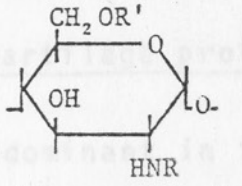
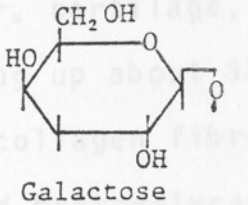
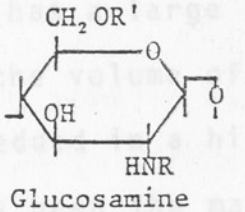
TABLE 1.1.

MOLECULAR FEATURES AND DISTRIBUTION OF GLYCOSAMINOGLYCANS OF VERTEBRATE CONNECTIVE TISSUE

| Polysaccharide | Molecular Weight Range ($x10^{-3}$) (ref. 18) | Disaccharide Repeating Unit | Type of Sulphation | Charge/ Disaccharide Unit | Examples of Tissue Distribution |
|---|---|---|---|---|---|
| Hyaluronate | 4000 - 8000 | D-glucuronic acid<br>D-glucosamine | - | 1,0 | Synovial fluid, vitreous humor, umbilical cord, cartilage, skin |
| Chondroitin-4-sulphate | 5 - 50 | D-glucuronic acid<br>D-galactosamine | $O\text{-}SO_3^-$ | 1,1-2,0 | Cartilage, bone, cornea, skin, aorta |
| Chondroitin-6-sulphate | 5 - 50 | D-glucuronic acid<br>D-galactosamine | $O\text{-}SO_3^-$ | 1,2-2,3 | Cartilage, bone, umbilical cord, skin, intervertebral disc, aorta |
| Dermatan sulphate | 15 - 40 | D-glucuronic acid<br>L-iduronic acid<br>D-galactosamine | $O\text{-}SO_3^-$ | 2,0-2,2 | Cartilage, bone, skin, tendon, aorta. |
| Heparan sulphate | 50 | D-glucuronic acid<br>L-iduronic acid<br>D-glucosamine | $O\text{-}SO_3^-$, $N\text{-}SO_3^-$ | 1,1-2,8 | Lung, liver, skin |
| Heparin | 4-16 | D-glucuronic acid<br>L-iduronic acid<br>D-glucosamine<br>D-glucosamine-$SO_4$ | $O\text{-}SO_3^-$<br>$N\text{-}SO_3^-$ | 3-4 | Lung, liver, skin, intestinal mucosa |
| Keratan sulphate | 4-19 | D-galactose<br>D-glucosamine-6-$SO_4$ | $O\text{-}SO_3^-$ | 0,9-1,8 | Cornea, cartilage, bone intervertebral disc |

TABLE 1.2.

STRUCTURE OF GLYCOSAMINOGLYCANS

| Polysaccharide | Monosaccharide Units | | Substituents |
|---|---|---|---|
| Hyaluronate |  Glucuronic acid |  Glucosamine | R = $-C(=O)CH_3$ |
| Chondroitin sulphates |  Glucuronic acid |  Galactosamine | R = $-C(=O)CH_3$<br>R' = -H or $-SO_3^-$ |
| Dermatan sulphate |  Glucuronic acid<br>  Iduronic acid |  Galactosamine | R = $-C(=O)CH_3$<br>R' = -H or $-SO_3^-$ |
| Heparin, heparan sulphate |  Glucuronic acid<br>  Iduronic acid |  Glucosamine | R = $-SO_3^-$ or $-C(=O)CH_3$<br>R' = -H or $-SO_3^-$ |
| Keratan sulphate |  Galactose |  Glucosamine | R = $-C(=O)CH_3$<br>R' = -H or $-SO_3^-$ |

sulphate residues and these, in conjunction with the negative carboxyl groups from the hexuronic acid residue, result in glycosaminoglycan chains exhibiting a linear array of anionic groups. Their high charge density results in proteoglycans behaving as polyanionic molecules which occupy large molecular domains. Generally the glycosaminoglycans do not occur as free polysaccharide chains *in vivo* but rather are covalently linked by the reducing end of their terminal sugar residue to the core protein to form proteoglycans (7). Six of the seven known glycosaminoglycans have been shown to be covalently linked to core protein in this way, but it is not established for certain yet whether hyaluronic acid occurs free or linked to a protein in a manner similar to the other glycosaminoglycans (8,9). Evidence has been presented which suggests covalent attachment of some protein to hyaluronic acid in synovial fluid (10), but most information points to its existence as a single unbranched carbohydrate polymer.

### 1.1.1. Structure of a typical cartilage proteoglycan monomer

Proteoglycans are especially predominant in the connective tissues of the body, and therefore a great deal of the research carried out has involved connective tissue-derived polymers. In particular, cartilage, which has a large extracellular matrix, making up about 90% of the volume of the tissue, and composed of collagen fibres embedded in a high concentration of aggregated proteoglycans, has been the main source of material used for research on these glycoconjugates; apparently 10 to 30% of the tissue dry weight is glycosaminoglycan (11).

A typical cartilage proteoglycan monomer is depicted in Fig. 1.1. It consists of a protein backbone of about 3000 amino acid residues resulting in a molecular weight of between 270 to 350000, and has been shown by electron microscopy to have a length of some 300 nm (12,13). In spite of having such a large molecular weight compared to that of other connective tissue proteins such as collagen α-chains (MW approx. 100000) and fibronectin (MW 220000), the core protein makes up only 5 to 12% of the mass of the large proteoglycan molecule, which has an overall molecular weight of $0.5 \times 10^6$ to $4 \times 10^6$ (14). The remainder is made up of the carbohydrate side chains. It has been shown that a typical cartilage proteoglycan contains about 100 chondroitin sulphate chains (MW approx. 20000) and about 60 keratan sulphate chains (MW approx. 5500) and a number of short N-linked and O-linked oligosaccharides (MW 1200 to 2000) attached to the core protein. The glycosaminoglycans are concentrated in specific regions of the core protein to give a C-terminal chondroitin-rich domain (15,16) and a keratan-rich area towards the N-terminal portion of the core protein (15). The molecule thus consists of approximately 80 to 84% chondroitin sulphate, 7 to 12% keratan sulphate, and 1 to 3% oligosaccharides, the remainder being protein (17).

The primary structure of the core protein has not yet been entirely resolved and remains open to further revision. The problems that have arisen in the elucidation of this structure are primarily due to the general features of proteoglycans

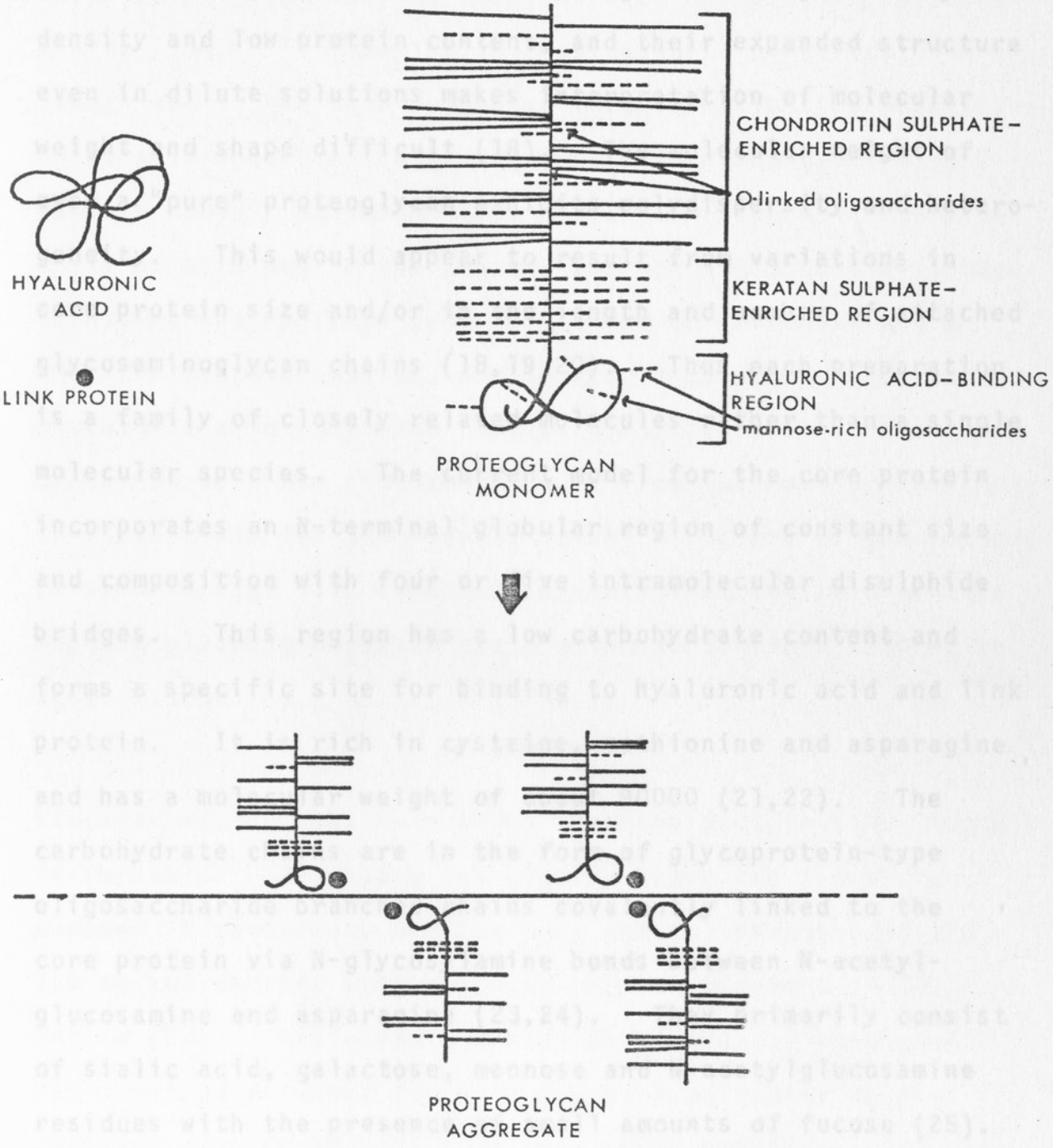



Fig. 1.1. Schematic representation of cartilage proteoglycan monomer and aggregation of cartilage proteoglycans.

that make them difficult to study e.g. their high carbohydrate density and low protein content, and their expanded structure even in dilute solutions makes interpretation of molecular weight and shape difficult (18). The molecular weight of even a "pure" proteoglycan exhibits polydispersity and heterogeneity. This would appear to result from variations in core protein size and/or in the length and number of attached glycosaminoglycan chains (18,19,20). Thus each preparation is a family of closely related molecules rather than a single molecular species. The current model for the core protein incorporates an N-terminal globular region of constant size and composition with four or five intramolecular disulphide bridges. This region has a low carbohydrate content and forms a specific site for binding to hyaluronic acid and link protein. It is rich in cysteine, methionine and asparagine and has a molecular weight of about 90000 (21,22). The carbohydrate chains are in the form of glycoprotein-type oligosaccharide branched chains covalently linked to the core protein via N-glycosylamine bonds between N-acetylglucosamine and asparagine (23,24). They primarily consist of sialic acid, galactose, mannose and N-acetylglucosamine residues with the presence of small amounts of fucose (25). Adjacent to the hyaluronic acid-binding region is an extended polypeptide region with many keratan sulphate chains attached (15). These keratan sulphate chains are bound to the core protein by O-glycosidic bonds between N-acetylgalactosamine and the hydroxyl groups of serine and/or threonine (23,24). However, the greater part of the core protein exists as an

extended polypeptide with many O-linked chondroitin sulphate chains (15). This region is considered to be variable in length and it has been suggested that it exhibits a repetitive amino acid sequence containing many serine and glycine residues in roughly equimolar amounts (22). Each O-linked side chain is attached to the protein through a glycosidic linkage involving the hydroxyl group of serine or threonine and a specific tetrasaccharide, namely xylose-galactose-galactose-glucuronic acid (26) (see Section 1.2.3.1). It is the extension of these side chains away from the core protein that is responsible for the "bottle brush" structure of the monomer as seen in Fig. 1.1. Evidence suggests that the core protein has one glycosaminoglycan chain attached to every 12th amino acid residue (6).

Electron micrographs have shown that the protein core length is variable. Rosenberg has clearly shown two types of monomer in cartilage, one having a central filament of 170 - 190 nm and another with a longer central filament of 320 - 340 nm (27). Cell-free translation of proteoglycan mRNA has provided evidence for a single high molecular weight protein core (MW 300000) from both chick cartilage (28) and calf articular cartilage (29). It thus remains to be seen to what extent the heterogeneity within different proteoglycans is related to variations in the length of the chondroitin sulphate attachment region and whether this is due to a biosynthetic control mechanism or results from normal non-pathological proteolytic degradation of core protein

taking place in the extracellular matrix.

### 1.1.2. Formation of proteoglycan aggregates

In their native state in cartilage, proteoglycan monomers exist as large aggregates composed of many monomers bound to hyaluronic acid chains (Fig. 1.1). This aggregation has been studied in great detail using the techniques of associative and dissociative caesium chloride (CsCl) buoyant density centrifugation. In 1969 Hascall and Sajdera showed that when material from bovine nasal septum cartilage was analysed in the ultracentrifuge under associative solvent conditions (0,4 M guanidine HCl) the proteoglycans remained aggregated and had a high sedimentation coefficient (50 to 60 S). In dissociative solvents (4 M guanidine HCl) the aggregate dissociated into its individual components; the individual monomers had higher buoyant densities than free hyaluronic acid and thus could be separated from the latter (30). As mentioned previously, the typical monomer has a globular elongated foot at one end of the molecule which interacts with hyaluronic acid. Reduction and alkylation of the core protein prevents subsequent aggregate formation and interaction with hyaluronate (31,32). This suggests that the conformation of the binding region is critical for the binding process. Selective chemical modification of the core protein has implicated the amino acid residues arginine, tryptophan and lysine as being involved in this binding process. These amino acids may be involved directly in the interaction of core protein with hyaluronic acid or indirectly

by merely maintaining the stability of the binding site (32, 33). It has been shown that a minimum of 5 hyaluronate disaccharide units are required for binding of monomer to occur (34,35) and calculations have shown that up to 200 proteoglycan monomers can bind to a single hyaluronic acid chain of molecular weight $1.6 \times 10^6$. This would give rise to an aggregate 4 μm in length (36). The distance between monomers has been suggested to be about 40 disaccharide units (22). Kimura *et al.* showed that the addition of hyaluronic acid oligosaccharides (averaging about 50 monosaccharides) to the culture medium of chondrocytes inhibited aggregate formation by as much as 94% (37,38).

The interaction of core protein with hyaluronic acid is specific and has a dissociation constant of about $10^{-6}$ - $10^{-7}$ M (39); no other anionic biopolymer has been found that will compete with hyaluronic acid for binding (35,40). Modification of the glucuronyl carboxyl groups with diazomethane prevented aggregation with protein monomer and it appeared that four consecutive unmodified carboxyls were the minimum requirement for interaction (41). Furthermore, an absolute specificity for N-acetylglucosamine was observed since polymers such as chondroitin sulphate, which contain N-acetylgalactosamine, were unable to interact with monomers even at high concentrations (22,39).

The interaction between proteoglycan core protein and hyaluronic acid does not involve covalent bonding but is stabilized by specific link proteins (22). Once they are

present, the proteoglycan monomers are essentially locked into position on the hyaluronic acid and the dissociation constant becomes too low to measure accurately (35,42). Two types of link protein have been shown to exist in cartilage and they are structurally related to each other (31). They have molecular weights of about 42000 and 500000, respectively (43,44). They have the same amino acid composition, and exhibit the same electrophoretic behaviour, but it has been suggested that the larger form is made up of a longer polypeptide chain, and contains more N-linked oligosaccharide residues (44). The link protein binds to a length of hyaluronate adjacent to the hyaluronate-binding region of the proteoglycan (45), and it also interacts with portions of the core protein in this region (46). It has been calculated that a minimum of 15 hyaluronate disaccharide units are required for the formation of stable hyaluronate-link protein-monomer aggregates (12). The molar ratio of proteoglycan to link protein has been shown to be 1:1 (47). The tertiary complex of hyaluronic acid, link protein and proteoglycan monomer has been found to be stable (22) and absence of link protein results in instability of the hyaluronic acid-proteoglycan monomer complex (42). In cultures of chondrosarcoma-derived chondrocytes it has been found that there is no exchange of link protein between monomer-link complexes and added exogenous monomers (47). This suggested that the formation of stable tertiary complexes was a sequential process, involving link-monomer complex first and subsequent binding of this to hyaluronic acid. This however does not account for the observation that hyaluronate

can combine with a proteoglycan monomer to form a complex that is only stabilized on subsequent addition of link protein.

Aggregation of proteoglycan monomers with hyaluronic acid only occurs after secretion and takes some finite time to be completed (37). The mechanisms for the regulation of aggregate assembly and for their organised deposition within the collagen network of the extracellular matrix are not yet understood. It has been proposed that in cartilage aggregation is largely responsible for the"fixation" of the proteoglycan monomer in the matrix.

### 1.1.3. Functional aspects of proteoglycans

As a consequence of their chemical composition, proteoglycans have an overall hydrophilic character and exhibit extended structures in solution, thus enabling them to occupy very large molecular domains (14,48). As a consequence of their structure, these expanded molecules behave as molecular exclusion media and are able to "sieve out" large molecules, thus preventing their passage through connective tissue (49, 50). Proteoglycan molecules are reversibly compressible when subjected to compressive loads; as solvent is displaced from their domains, interactions between the glycosaminoglycan chains increase. Once the load is removed the glycosaminoglycan chains "reclaim" their molecular domains. This is an important property essential for the normal physiological functioning of cartilage, which must be able to act as a cushion for variable, compressive loads. The collagen fibrils

of the cartilage matrix define shape and the interspersed proteoglycan aggregates provide a hydrated, viscous medium that absorbs compressive loads.

A variety of proteoglycans are found in different tissues. Some tissues have proteoglycans with features very similar to those found in cartilage, whereas others have entirely different structures and clearly serve different functions. For example, ovarian follicular fluid contains a proteoglycan which has smaller amounts of chondroitin sulphate chains compared with cartilage proteoglycan chains (about 10 to 20 per core protein), but these chains are much longer (MW approx. 55000) (2,51). The ovarian follicle proteoglycan core protein is substituted with a very large number of mucin-type O-linked oligosaccharides (about 300 to 400), and the monomer does not aggregate with hyaluronic acid. A small proteoglycan containing about one to three keratan sulphate chains (MW 5000 to 7000) has been isolated from bovine corneal stroma (4,52) and both rat liver (3) and bovine aortic tissue (53) contain a heparan sulphate proteoglycan. Recently human aortas have been shown to contain proteoglycans which have either chondroitin sulphate or dermatan sulphate glycosaminoglycan chains (54).

The properties of the different tissue proteoglycans result from their diverse biochemical structures and interactions with tissue proteins. These aspects of proteoglycans, which will be discussed fully later (Section 1.4), are all related to their widespread functional roles in the extracellular

matrix. These include the regulation of tissue water content (55) and consequently the resistance of tissues to compressional forces, the control of diffusion rates for nutrient and degraded molecules through tissue (49.50), and most likely the control of collagen polymerization to form fibrils in the extracellular matrix (56,57).

## 1.2. BIOSYNTHESIS AND SECRETION OF PROTEOGLYCANS

Recently considerable progress has been made in the elucidation of the proteoglycan biosynthetic pathways and this may, in turn, contribute to a better knowledge of both the structure and function of these molecules. Earlier studies were concerned almost exclusively with the biosynthesis of the carbohydrate side chains but in order to understand the physiology of the extracellular matrix, it is essential that the mechanisms leading to the coordination of all the reactions necessary for the formation of the whole molecule be elucidated.

Many of these early biosynthetic studies were carried out on pathological specimens or on cultured cell lines. In recent times we have become increasingly aware of the artifactual results that may result from using such systems. However, cautious interpretation of these results plus the use of cell-free systems have enabled us to obtain a good overall view of the biosynthetic processes involved in the synthesis of proteoglycan monomers in a variety of forms.

Essentially, the process involves the synthesis of the core

protein, the initiation of the carbohydrate side chain on this molecule, glycosaminoglycan chain elongation, sulphation and chain termination.

### 1.2.1. Synthesis of core protein

There is no evidence to contest the assumption that core protein biosynthesis takes place via the normally accepted Paladel pathway for secretory proteins (58). The control mechanisms for this process are not yet elucidated and neither is it known if there is some discrete compartmentalization of the synthesis of different protein cores that allows for the heterogeneity of proteoglycan species. It is almost certain that different proteoglycans are the products of closely related genes for core protein that have originated by gene duplication, since it is known that core proteins of different proteoglycans differ in structure and represent different gene products, e.g. the keratan sulphate proteoglycan in the corneal stroma has been shown to have a different core protein to the corneal chondroitin sulphate proteoglycan (4,59). All preparations of cartilage specific proteoglycans studied so far have been found to be polydisperse, with variability in the chondroitin sulphate binding region of the core protein (60). It seems unreasonable that such a variability occurs during biosynthesis unless there is a variation in processing or more than one gene product is involved.

It is deducible that certain amino acid sequences code for the attachment of the glycosaminoglycan chain and this code

must of necessity contain L-seryl residues for the majority of glycosaminoglycans or L-asparaginyl residues for some keratan sulphate chains, since these residues are part of the glycopeptide linkages.

Cultured chondrocytes have been shown to contain a large intracellular precursor pool of core protein having a molecular weight of 370000 by analysis on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) after immunoprecipitation with highly purified antiserum to the hyaluronic acid-binding region of core protein (61). However, it may already contain N-linked and partially completed O-linked oligosaccharides and thus the actual molecular weight should be less. Other workers have succeeded in obtaining putative core protein mRNA from embryonic chick sternal cartilage differentiated limb bud cultures (28) and calf articular chondrocytes (29) which, when translated in cell-free systems, gave rise to a product with a molecular weight of 340000 on SDS PAGE. These cell-free generated polypeptides may still have their hydrophobic "signal" peptides attached. However, the general agreement on the size of isolated core protein as compared to the cell-free translation product suggests that in the tissues studied the proteoglycan core protein is a large (MW $\pm$ 350000) single chain polypeptide.

### 1.2.2. Formation of carbohydrate precursors

The carbohydrate components of glycosaminoglycans are derived from D-glucose without alteration in the carbon

backbone. These sugars are activated using primarily UTP to form UDP-sugars. However, in the case of fucose and sialic acid, GTP and CTP, respectively, are required for activation. In Fig. 1.2 a detailed description of the major sugars involved in glycosaminoglycan synthesis and their activated derivatives is shown.

1.2.3. Synthesis of carbohydrate side chain (Fig. 1.3)

Whether or not initiation of carbohydrate chain synthesis occurs on nascent core peptide or after peptide completion remains to be clearly established. In the synthesis of glycoproteins containing N-glycosidically linked carbohydrate chains, lipid intermediates have been shown to be involved in the transfer of activated sugars to the nascent protein core whilst it is still present in the rough-surfaced endoplasmic reticulum. A similar process may occur in the biosynthesis of certain glycosaminoglycans, namely N-linked keratan sulphate and oligosaccharide chains. Studies using the antibiotic tunicamycin, which inhibits N-glycosylation up to 95%, have shown that biosynthesis of glycosaminoglycans by cultured chick embryo fibroblasts (62, 63) and of corneal keratan sulphate (64) was inhibited. However, it has also been found that the antibiotic does not inhibit the biosynthesis of sulphated glycosaminoglycans by chondrocytes (62). It is thus possible that the N-linked oligosaccharides and glycosaminoglycans are synthesized on the nascent core protein chains in a manner analogous to glycoprotein biosynthesis.

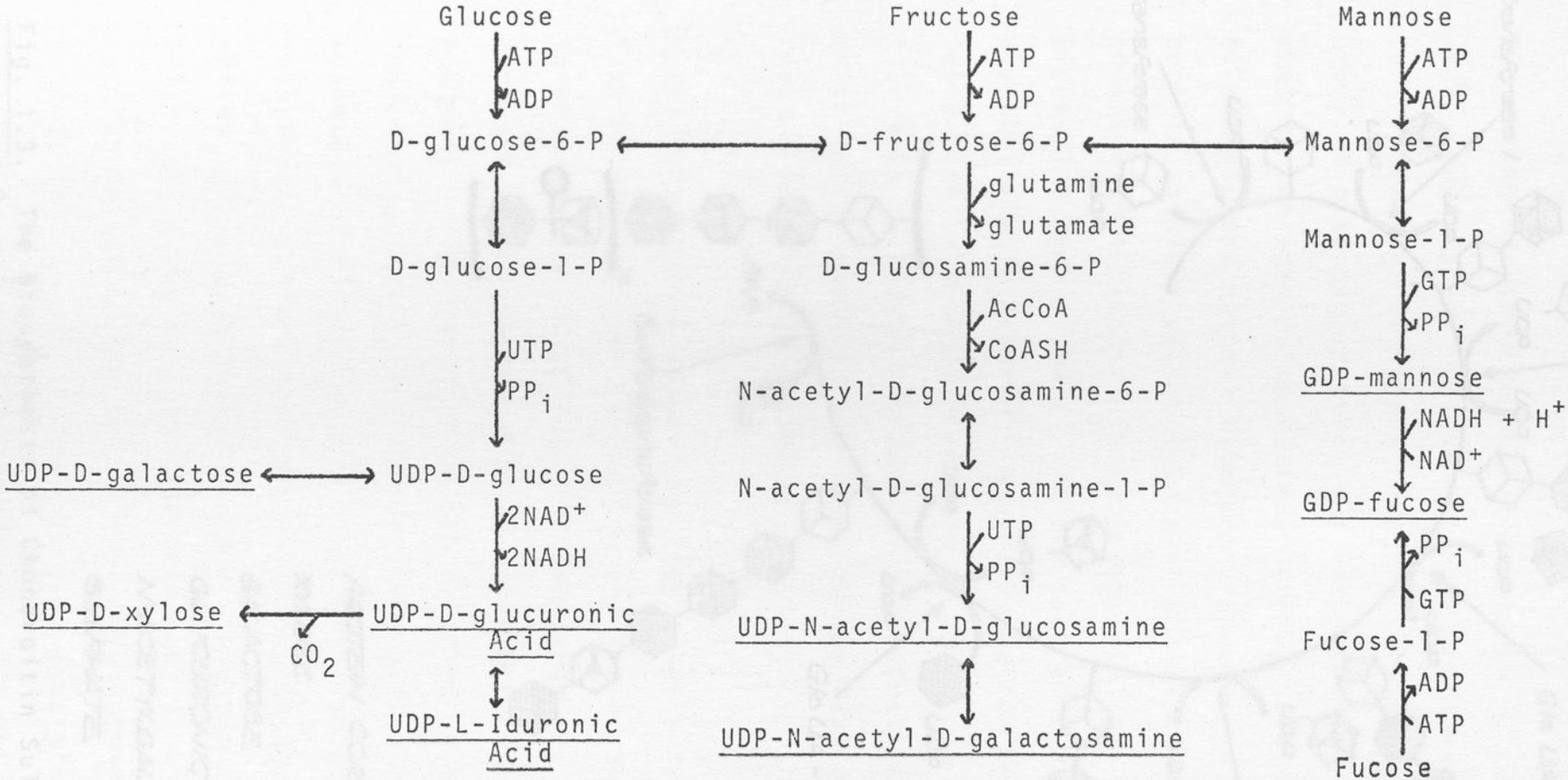



Fig. 1.2. The formation of activated sugars involved in the synthesis of glycosaminoglycans.

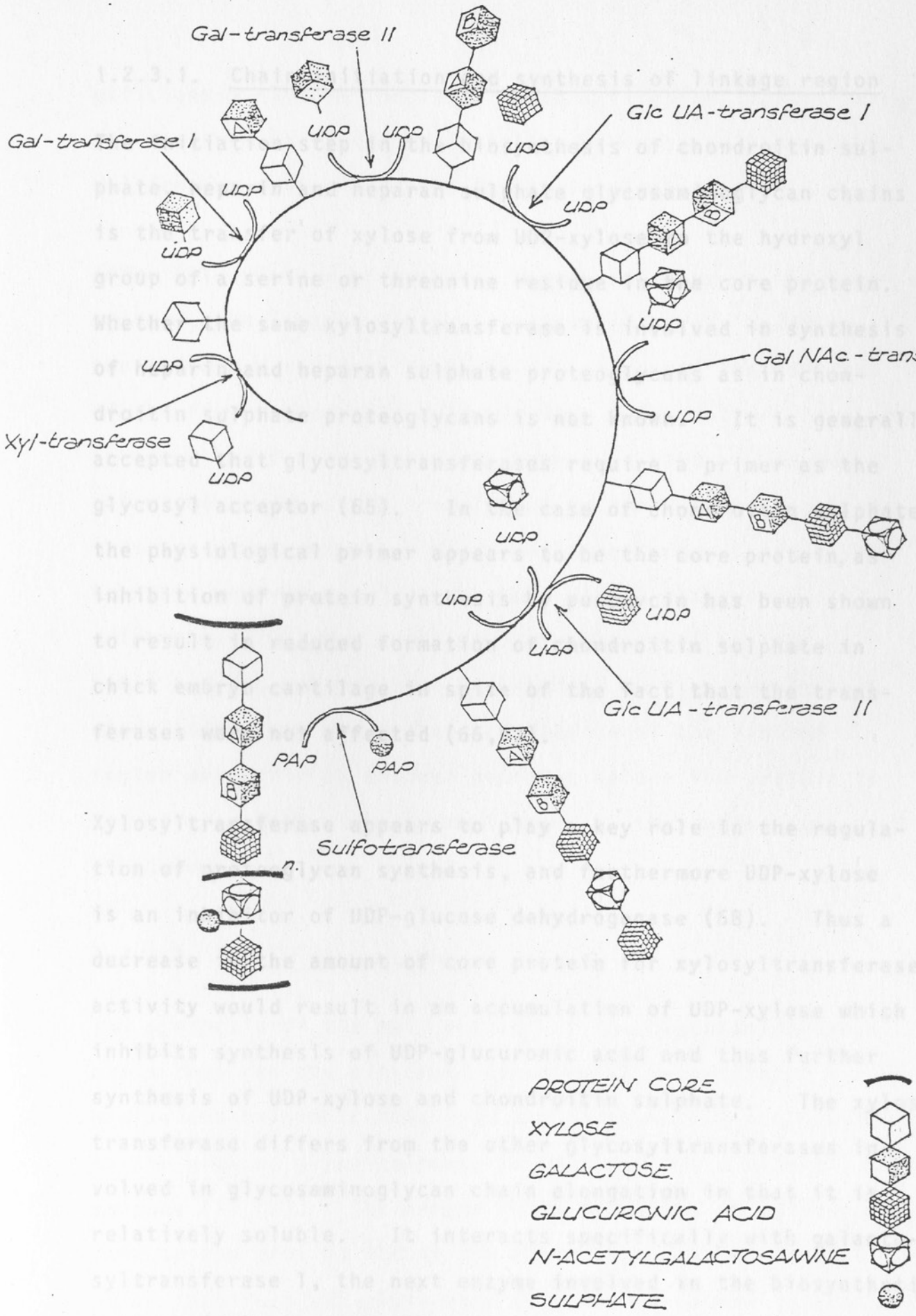



Fig. 1.3. The Biosynthesis of Chondroitin Sulphate - a schematic representation.

1.2.3.1. Chain initiation and synthesis of linkage region

The initiation step in the biosynthesis of chondroitin sulphate, heparin and heparan sulphate glycosaminoglycan chains is the transfer of xylose from UDP-xylose to the hydroxyl group of a serine or threonine residue in the core protein. Whether the same xylosyltransferase is involved in synthesis of heparin and heparan sulphate proteoglycans as in chondroitin sulphate proteoglycans is not known. It is generally accepted that glycosyltransferases require a primer as the glycosyl acceptor (65). In the case of chondroitin sulphate the physiological primer appears to be the core protein, as inhibition of protein synthesis by puromycin has been shown to result in reduced formation of chondroitin sulphate in chick embryo cartilage in spite of the fact that the transferases were not affected (66,67).

Xylosyltransferase appears to play a key role in the regulation of proteoglycan synthesis, and furthermore UDP-xylose is an inhibitor of UDP-glucose dehydrogenase (68). Thus a decrease in the amount of core protein for xylosyltransferase activity would result in an accumulation of UDP-xylose which inhibits synthesis of UDP-glucuronic acid and thus further synthesis of UDP-xylose and chondroitin sulphate. The xylosyltransferase differs from the other glycosyltransferases involved in glycosaminoglycan chain elongation in that it is relatively soluble. It interacts specifically with galactosyltransferase 1, the next enzyme involved in the biosynthetic pathway (69). Its interaction with core protein, substrate UDP-xylose, and galactotransferase may serve to coordinate

efficiently glycosaminoglycan chain initiation, and to bring the core protein into juxtaposition with the membrane-bound multienzyme system required for chain elongation.

After xylosylation of specific serine residues in the core protein, the linkage region is completed by the addition of two galactose residues from UDP-galactose and the addition of the first glucuronic acid residue. The enzymes involved in these and further steps are membrane-bound. The two galactose residues are transferred by two different galactosyltransferase enzymes. Galactosyltransferase I is loosely bound to membranes and can be solubilized with nonionic detergents (70), but galactosyltransferase II is tightly bound and has not yet been isolated. It is probable that chain initiation and perhaps completion of the linkage region may occur on nascent peptides before the protein is sequestered into the cisternal lumen of the endoplasmic reticulum.

1.2.3.2. Chain elongation

The synthesis of the main portion of the glycosaminoglycan chain requires the alternate transfer of N-acetylated hexosamine and hexuronic acid from their respective nucleotides to the growing glycosaminoglycan chain by two distinct enzymes (71,72). The enzyme which transfers UDP-glucuronic acid to the growing end of the chain is different to that which transfers UDP-glucuronic acid to the second galactose residue of the linkage region (73). Studies using electro-

microscopic autoradiography (74,75) and purified Golgi preparations from mouse mastocytoma cells (76) have demonstrated that the site of elongation of glycosaminoglycan chains is the Golgi. Extensive evidence indicates that in cartilage keratan sulphate chains are linked to the same core protein as the chondroitin sulphate chains (77). No information is available regarding the site and time of addition of O-glycosidically linked keratan sulphate or oligosaccharide chains but their synthesis is presumed to follow a similar pathway of synthesis to that of chondroitin sulphate. The mechanism of hyaluronic acid synthesis is poorly understood, but it has been shown to be insensitive to inhibition of protein synthesis (78,79), and is also unaffected by tunicamycin (80), thus excluding the possibility that it is synthesised on a core protein and that it requires dolicholpyrophosphate derivatives for its polymerisation. However, some coordination of hyaluronic acid and proteoglycan biosynthesis must exist, especially in tissues that contain predominantly aggregated proteoglycans. The conversion of glucuronic acid to L-iduronic acid via the action of C5-epimerase, to give rise to dermatan and heparan sulphates, takes place during elongation and not after completion of the glycosaminoglycan chain (81).

1.2.3.3. Sulphation

Incorporation of sulphate into glycosaminoglycan chains has been shown to follow a pattern similar to that described for the incorporation of sugar moieties in that the sulphate

is first activated and then transferred to the acceptor. The activated sulphate is formed from inorganic sulphate in a two-step process:

1) $SO_4^{2-}$ + ATP $\xrightarrow{A}$ adenosine-5'-phosphosulphate (APS) + PPi

2) APS + ATP $\xrightarrow{B}$ 3' phosphoadenosine-5'-phosphosulphate (PAPS) + ADP (82-84)

where A = sulphate adenylyltransferase and B = adenylyl-sulphate kinase.

Specific 4- and 6- sulphotransferases transfer sulphate from PAPS to the 4- or 6- position of the hexosamine residue in the glycosaminoglycan chain at some stage after their polymerisation to the growing molecule, and electron microscopic autoradiography has shown that the vesicular component of the Golgi apparatus is the actual site of sulphation (85,86). On examination of various chondroitin-4-sulphate fragments obtained from rabbit ear cartilage, Derge and Davidson showed that they contained stoichiometric amounts of sulphate hemi-ester groups which suggests that a close relationship exists between extension and O-sulphation of the chondroitin chains (87). Such stoichiometry is less likely to be achieved when sulphation occurs after polysaccharide completion. Other experiments are consistent with the theory that sulphation occurs concurrently with polysaccharide elongation (88,89). Hybrid molecules with both 4- and 6- sulphates have been shown (90,91), and specificity for the position of sulphation (4- or 6- position) is unknown. It is a reasonable assumption that the site of sulphate incorporation

on the disaccharide unit may depend on the activity of a specific sulphotransferase rather than on the acceptor, since in the case of dermatan sulphate, sulphation appears to occur before epimerization of glucuronic acid to L-iduronic acid (92).

Heparin and Heparan sulphate also possess N-sulphate groups. There is some evidence that sulphation of these residues takes place after the completion of the polymer. However, it has been suggested from work using aortic microsomal fractions that rapid N-sulphation precedes O-sulphation of heparan sulphate chains and that O-sulphation only occurs on previously N-sulphated sugars (93).

1.2.3.4. Chain termination

The termination of the glycosaminoglycan chain synthesis is not understood. Chain lengths of all chondroitin sulphate preparations examined so far appear to be polydisperse, thus there may be different termination signals for different glycosaminoglycan populations. One possible termination mechanism in the biosynthesis of chondroitin sulphate chains was suggested by Telser's finding that oligosaccharides containing non-reducing terminal N-acetylgalactosamine-4-sulphate residues do not serve as acceptors for further glucuronic acid addition, but that those containing N-acetylgalactosamine-6-sulphate are excellent acceptors (66). The addition of sialic acid residues may also function as a termination signal for the carbohydrate chains or as a trigger for the secretion

of proteoglycans from the cell in the same manner as has been suggested for glycoproteins. Chain termination may also perhaps partially depend on a decreased affinity of the elongation enzymes for acceptors above a certain size.

### 1.2.4. Secretion of proteoglycans

Little is known with certainty of the mechanism of proteoglycan secretion. It appears that not all of the newly synthesized proteoglycans are destined to be secreted. Studies on the fibroblasts derived from the skin of patients affected by metabolic disorders in which abnormal intracellular accumulation of glycosaminoglycans takes place (Hurler's and Hunter's syndromes) have shown there to be two different pools of glycosaminoglycans inside the cell: those that are secreted into the extracellular matrix and those that remain inside the cell and are slowly degraded (94). The completed molecules destined for export are collected in secretory vacuoles which also appear to contain procollagen (95,96). Electron microscopy has revealed that the secretory product is transported to the Golgi zone in smooth surfaced vesicles which fuse with one another to give rise to vacuoles of increasing size. Under electron microscopy the material in the large vacuoles is similar to the extracellular matrix in appearance and probably represents a combination of collagen in fibrillar and amorphous form, together with proteoglycans which are visualized as dense granules. These vacuoles can be seen to fuse with the surface of the cell and the contents are secreted into the matrix. Kimura *et al*

have shown that in chondrocyte cultures the contents of the vacuoles are secreted to the matrix over a period of 10 to 30 min following a 5 min pulse with $|^{35}S|$sulphate (47). One hour after addition of radioactive label most of the radioactivity is in the matrix. Once outside the cell, the proteoglycan interacts with link protein, hyaluronic acid and the matrix proteins to become an integral part of the matrix (47).

### 1.2.5. Effects of exogenous agents on proteoglycan biosynthesis

#### 1.2.5.1. Effects of β-D-xylosides

As described above, the majority of the sulphated glycosaminoglycans in animal tissues are covalently linked to serine residues of the core protein via a specific tetrasaccharide:

$$\text{Glycosaminoglycan - Glucuronic Acid} \overset{\beta 1,3}{---} \text{Galactose} \overset{\beta 1,3}{---} \text{Galactose} \overset{\beta 1,4}{---} \text{Xylose} \overset{\beta 1,3}{---} \text{Serine}$$

(26,97).

The biosynthesis of the completed proteoglycan monomer requires the initial formation of the protein core via the normal protein synthetic machinery, followed by the stepwise addition of the specific monosaccharides from their respective uridine diphosphosugars by distinct transferases.

The addition of β-D-xylosides to intact cells has been shown to provide an artificial primer for new glycosaminoglycan synthesis and thus eliminates the need for core protein as an acceptor for xylose (98-100). The levels of core protein,

however, remain the same in control cells and in cells treated with xyloside (101). Thus use of β-D-xylosides as initiators of new glycosaminoglycan chains has made available a potent tool for the dissection of the pathways of proteoglycan synthesis.

It has been shown that the β- conformation of the xyloside is essential, since α-xylosides are unable to initiate glycosaminoglycan synthesis (102,103). The aglycone moiety of the molecule is also important; non-polar residues (e.g. n-butyl, octyl, benzyl, phenyl xylosides) have been shown to maximally promote glycosaminoglycan chain initiation (102). This may reflect differences in the rate of transport through membranes or differences in the affinity of these xylosides for the enzymes involved in the initiation of glycosaminoglycan synthesis. Other sugar derivatives further along the glycosaminoglycan chain sequence, such as β-galactosides, do not initiate synthesis (102,103). Thus it seems that only the first galactosyltransferase enzyme (galactotransferase 1) is able to make use of a monosaccharide as an acceptor for the activated galactose moiety.

Most workers find that addition of β-xylosides to culture systems causes a significant increase in the amount of $|^{35}S|$ sulphate incorporated into glycosaminoglycans (100,104,105). Most of this radioactivity is recovered in the medium, indicating that the glycosaminoglycans formed on the acceptor are probably more soluble (100). The possibility exists

that they may be secreted via a different pathway, or more likely, that the protein core plays a vital role in "positioning" the proteoglycan molecules in the extracellular matrix; in the absence of core protein the glycosaminoglycans move rapidly into the medium.

Previous studies have also shown that there is a decrease in the average glycosaminoglycan chain length in the presence of these "false" acceptors, pointing to a role for the core protein in the normal regulation of chain length (102,103). It is possible that the length of the chains can be inversely correlated with the rate of synthesis. This is supported by the observation that a decrease in temperature leads to a slower rate of synthesis of glycosaminoglycans and these chains are longer than those of controls at normal temperature (106). In the presence of xylosides many more chains are being synthesized and premature termination may be due to the large amount of acceptor (xyloside) available for the initiation of new glycosaminoglycan chains. Furthermore, the free glycosaminoglycans may be more readily secreted by the cell and thus may reside for a shorter period of time at the sites for glycosyl transfer. It has also been observed that in the presence of β-D-xylosides, chondroitin sulphate is the predominant type of glycosaminoglycan synthesized, even in cell types that normally synthesize only small amounts of this glycosaminoglycan (103,107).

1.2.5.2. Effects of ascorbic acid

The importance of ascorbic acid for the biosynthesis of connective tissue proteins has been well documented by numerous laboratories (108-111). From time to time further roles for the vitamin have been suggested (112-114). One of these involves the participation of ascorbic acid in the process of biological sulphate transfer. Several workers have suggested that the sulphated derivative of ascorbic acid (ascorbic acid-2-sulphate) plays a direct role in biological sulphate transfer, including the sulphation process which occurs in proteoglycan synthesis (115,116). Hatanaka and Egami studied sulphate incorporation from ascorbic acid-2-sulphate into chondroitin sulphate synthesized by embryonic chick cartilage epiphyses (117). They postulated that ascorbic acid-2-sulphate is synthesized *in vivo* from 3' phosphoadenylyl sulphate and they proposed that it acted as a long-term storage pool of ascorbic acid in the body. However, Shapiro and Poon postulated that sulphation of glycosaminoglycans did not result from direct transfer of $|^{35}S|$ sulphate from ascorbic acid-2-sulphate but rather from a decomposition product thereof (118). They found negligible direct sulphation of glycosaminoglycans by chondrocytes using freshly prepared ascorbic acid-2-sulphate but some sulphation with ascorbic acid-2-sulphate that had been stored at -20°C for several days. Evidence was presented to show that the sulphation pathway with the decomposition product involved exchange with inorganic sulphate and they suggested that sulphation proceeded via the normal PAPS pathway (see Section 1.2.3.3).

However, Hatanaka and Egami disagreed with the above findings. They did not detect such time-dependent decomposition during storage at -20°C for at least three months and in addition the extent of incorporation of radioactivity from ascorbic acid-2-|$^{35}$S|sulphate remained constant for at least three months. Furthermore, they showed that the rate of incorporation of radioactive sulphate into glycosaminoglycan chains from ascorbic acid-2-|$^{35}$S|sulphate or inorganic |$^{35}$S| sulphate were different (117).

Thus the question as to whether ascorbic acid plays a direct role in the sulphation of proteoglycan molecules remains unresolved. Transfer of sulphate from ascorbic acid-2-sulphate may or may not be physiologically significant. Ascorbic acid has many other functions, particularly involving the formation of the extracellular matrix and the maintenance of its integrity (108,119,120). Its influence on proteoglycan biosynthesis may thus be secondary and may result from its effects on the synthesis of extracellular matrix generally. Ascorbic acid has also been shown to have a role in the promotion of microtubule assembly (114) and may thus facilitate secretion of proteoglycans from the cell.

1.2.5.3. Effects of the ionophore, monensin

Monensin is a monovalent, carboxylic ionophore which has been shown to reversibly block secretion by several cell types. It has a common and specific effect on intracellular transport at the level of the Golgi complex (121-124). Non-regulated

cells, whose rate of secretion is free from short term controls (as opposed to regulated cells which secrete only when appropriately stimulated) lack a conspicuous storage compartment for secretory protein. All the data accumulated so far have indicated that the ongoing "basal" secretory rate in such cells is maximal. For secretion, vesicles originating from the rough endoplasmic reticulum obligatorily fuse with Golgi cisternae, and a secondary population of smooth-surfaced organelles derived from this fusion pass towards the cell surface (58). In non-regulated cells the second population of organelles corresponds to small Golgi-derived vesicles endowed with a specificity which allows them to fuse with the plasma membrane. Monensin causes a partial equilibration of cellular Na/K levels, which leads to the arrest of vesicular traffic in the region of the Golgi, thus creating a secondary organelle population. This appearance of many intracellular membranous vacuoles on exposure to monensin has been recorded consistently (122,124,125). Monensin has been shown to have no deleterious effects on protein synthesis (123) and minimal effects upon energy production in cells (126). Ledger et al. have shown inhibition of secretion of both procollagen and fibronectin from human fibroblasts in culture (124). Immunofluorescence microscopy showed these proteins to be concentrated in the Golgi zone which became filled with a new population of smooth-membraned vacuoles; normal Golgi complexes were not found. They also noticed dilated rough endoplasmic reticulum, and this may have indicated an additional blockage at the stage where secretory proteins exited from the endoplasmic reticulum, or it may have been due to the build-up

of protein in the Golgi compartment which exceeded the latter's capacity and thus extended back into the endoplasmic reticulum.

Several workers have noted that the presence of monensin led to a general undersulphation of all the newly synthesized glycosaminoglycan chains (125,127,128). This implies that all proteoglycan molecules and all glycosaminoglycan chains of a given proteoglycan have equal access to membrane-bound sulphotransferases. Undersulphation cannot be explained by decreased access of selected glycosaminoglycans or proteoglycans. Since sulphation of glycosaminoglycans occurs at a late stage in their biosynthesis and takes place in the Golgi elements (83,84), it is reasonable to expect that a Golgi-disruptive ionophore will impair sulphation. At low doses of monensin, proteoglycans, though undersulphated, continued to be secreted; thus it appears that sulphation is not essential for their exit from the cell (128).

The reason for the interference of sulphation of glycosaminoglycan chains by monensin is not yet clear. Inhibition of sulphation even in the presence of β-D-xyloside (127) showed that monensin exerts its effects after core protein synthesis and such synthesis has no bearing on the inhibition of sulphation by monensin. Experiments showed that $|^{35}S|$ sulphate incorporation into $|^{35}S|$ PAPS in the presence of monensin was no different from controls (127). Also, measurements of sulphotransferase activity, sulphatase activity, free sulphate

and glucosamine uptake into cells showed no change in the presence of monensin (127). Studies on cartilage of brachymorphic mice which contain undersulphated glycosaminoglycan chains have shown that the defect is at the level of conversion of APS to PAPS (see Section 1.2.3.3) (129,130). Whether monensin also interferes here or at some other stage in the sulphation pathway remains to be determined.

Monensin is thus a useful probe for studying the relationship between intracellular translocation of macromolecules destined for secretion, and the structural modifications of such molecules while they are en route to the cell surface as it disrupts intracellular flow without permanently damaging cells, and it has minimal effects on energy production and protein synthesis.

1.2.5.4. Effects of inhibitors of protein synthesis

Inhibitors of protein synthesis such as puromycin or cycloheximide have been shown to inhibit synthesis of chondroitin sulphate in cartilage, presumably by preventing the formation of suitable core protein acceptors for glycosaminoglycan chain initiation (66). When protein synthesis inhibitors have been added to cells, in particular rat chondrosarcoma cells, an immediate inhibition of $|^{3}H|$ serine incorporation into core protein was noted, but $|^{35}S|$ sulphate incorporation into glycosaminoglycans was only inhibited gradually (104). This has been taken to imply that certainly in the case of chondrosarcoma cells there is a large intracellular pool of core

protein. Using these cells it has been calculated that the newly synthesized core protein passes through an intracellular pool for 70 to 90 min, before the glycosaminoglycan chains are synthesized on it, and it is then rapidly secreted from the cell with a half time of less than 10 min (104). It remains to be seen, however, whether this lengthy time between core protein synthesis and glycosylation is a general feature of all cell types or whether it is a reflection of abnormal behaviour of this cell type.

In rat chondrosarcoma cells cycloheximide has been shown to interfere with the flow of core protein along the route of intracellular synthesis leading to secretion as well as inhibiting synthesis of further core protein. In treated cultures the proportion of $|^{3}H|$ serine labelled proteoglycan secreted was less than 50% of that in control cultures, and the rate of incorporation into secreted proteoglycan was decreased to 80 min as compared to 120 min for controls (104). Thus some interference of intracellular transport of the core protein has been proposed.

Puromycin has been shown not to inhibit incorporation of $|^{14}C|$ acetate into UDP-N-acetylgalactosamine or of $|^{14}C|$ galactose into UDP-glucuronic acid so it does not appear to have any direct effect on glycosaminoglycan synthesis (66). The addition of β-D-xylosides has also been shown to relieve the inhibition of $|^{14}C|$ or $|^{3}H|$ acetate incorporation into chondroitin sulphate by puromycin and cycloheximide when

studied with intact cartilage (98,102). This confirms that inhibition of protein synthesis has no direct effect on glycosaminoglycan chain elongation. The stimulation of labelled acetate incorporation into chondroitin sulphate, observed in the presence of β-D-xyloside and cycloheximide (102), suggested that the synthesis of core protein may be a rate-controlling step in proteoglycan synthesis. As expected, however, the xyloside has no effect on the incorporation of $|^{3}H|$ serine into core protein (102).

Several workers have also observed an increase in the size of the proteoglycan monomer, due to an increase in glycosaminoglycan chain length in the presence of inhibitors of protein synthesis (104,131). This may be due to a decrease in the overall rate of proteoglycan synthesis as a result of less core protein being available and this slower rate results in an increase in chain length. Previous observations have shown that the length of chondroitin-sulphate chains can be inversely correlated with their rate of synthesis (106). When β-D-xylosides were added together with the inhibitor the length of the glycosaminoglycan chains was found to be the same as those synthesised in the presence of xyloside alone (102,131). This may be explained by the fact that the requirement for the core protein has been obviated and glycosaminoglycan synthesis can proceed at an increased rate.

1.2.5.5. Effects of antimicrotubular and microfilament-modifying agents

In 1968 it was first proposed that microtubules are involved in cell secretion (132). The antimicrotubular agent, colchicine, has been shown to inhibit secretion inter alia of matrix components from osteoblasts and chondrocytes (133-136). Thus it has been suggested that microtubules play an active role in the secretion of collagen, elastin and proteoglycans in these cell types. Morphological and chemical data have suggested that the effects of microtubule depolymerizing agents, such as colchicine, on secretion result from functional disturbances in the microtubular system which cause secondary alterations in the Golgi complex (137,138). These changes in the Golgi complex coupled with a retarded translocation of secretory vacuoles result in inhibition of secretion.

In spite of the observed effects of colchicine on secretion, chondrocytes treated with the drug were shown to retain a large part of their biosynthetic and secretory activities (138). Secretion of proteoglycans from these cells was retarded but the effect was not dramatic. This suggested that either alternative pathways for secretion of proteoglycan existed that were independent of functional microtubules or that the Golgi apparatus, in spite of structural alterations, retained most of its synthetic and secretory capacities. Thus the microtubules may play a facilitatory rather than an obligatory role in intracellular translocation and secretion of

proteoglycans by chondrocytes.

Colchicine has also been shown to inhibit synthesis of proteoglycans in chondrocytes, but here again the effect is not very dramatic, with 30% inhibition at the most, and the molecules synthesised in the presence of the drug have only minor alterations when compared to their counterparts from untreated cultures (139). Treatment of xyloside-stimulated cultures with colchicine has been shown to give comparable inhibition to treatment of non-stimulated cells with colchicine and indicates that its inhibitory effects probably occur after core protein synthesis and xylosyl transfer (139).

Microfilaments are also thought to play a role in intracellular transport phenomena as well as in cell surface events such as pinocytosis (132,140-143). They may also act as a cytoskeleton and thereby maintain cell shape and intracellular organization (144,145). Cytochalasin B, a microfilament modifier, has been shown to have no effect on the secretion of proteoglycans by chondrocytes but it inhibits synthesis of proteoglycans by about 30% (139). The role of microfilaments remains unclear - perhaps the effects of cytochalasin B on microfilaments results in the disruption of uptake and transport of precursors for synthesis. When cells were treated simultaneously with colchicine and cytochalasin B the effect on synthesis was additive, suggesting that the sites of action of the drugs were different (139).

## 1.3. TURNOVER OF PROTEOGLYCANS

In their natural environment proteoglycans are subject to turnover and are constantly being degraded and replaced by new ones. Many experiments have been performed utilizing radioactive precursors, both *in vivo* and *in vitro*, in attempts to establish the pathways of proteoglycan metabolism (146-151). These experiments have established that the pathways of metabolism of the various types of proteoglycans are by no means uniform; metabolic compartments exist and within these compartments there are pools which exhibit metabolic heterogeneity.

In general there are at least four different metabolic compartments: an intracellular compartment and three extracellular compartments made up of aggregating, non-aggregating and cell surface proteoglycans. The intracellular compartment contains molecules which are being degraded and newly synthesized molecules, of which about two-thirds are destined for secretion to the extracellular environment and one-third is diverted to an intracellular storage pool and is ultimately degraded (94). These different intracellular pools could account for the metabolic heterogeneities which have been observed by several workers. Olssen *et al*. have established that there is more than one intracellular pool of proteoglycans that have different metabolic activities (152). Using cultured bovine aortic and human skin fibroblasts, Kresse *et al*. have presented evidence for the existence of topographically distinct glycosaminoglycan pools with varying metabolic

characteristics (153). The different pools may occur as a result of different activities of metabolizing enzymes or as a result of the existence of distinct pools which differ in their accessibility to degradative processes or as a combination of both.

Using *in vivo* studies on cartilage, Lohmander *et al*. have shown that the total fractions of chondroitin sulphate and keratan sulphate contain at least three metabolic pools (150). One has a half-life in the region of a few hours and was tentatively interpreted as representing intracellular synthesis, storage and degradation. The other two pools had components with slow and faster turnover rates: in the order of about 3 days and 30 to 80 days for chondroitin sulphate, and 4 days and 90 days for keratan sulphate. These were assumed to reflect degradation of different pools of matrix proteoglycans.

No simple relationship exists between chemical nature and turnover of proteoglycans. Metabolic heterogeneity exists even at proteoglycan level. Hardingham and Muir have suggested that smaller proteoglycan molecules have a faster rate of turnover than larger molecules even though they may have similar glycosaminoglycan chains (147). They found that proteoglycans with a low chondroitin sulphate content were degraded more rapidly than those with a high chondroitin sulphate content. These results have been supported by other workers (154). It is possible that molecules with fewer side chains may be degraded more rapidly by proteolytic enzymes than those protected by a high density of attached chains.

The turnover rates of various glycosaminoglycan chains appear to be different. Studies using rat epiphyseal cartilages have shown that chondroitin sulphate has a turnover rate of 70 h as compared to hyaluronic acid which has a turnover time of 120 h (148). *In vitro* labelling of bovine corneas showed that chondroitin sulphate turned over every 251 h and keratan sulphate every 723 h (149). The data obtained by Lohmander *et al.*, as mentioned above, do not however support the notion of a considerably slower metabolism of keratan sulphate compared to chondroitin sulphate (150).

In *in vitro* studies of incorporation of $|^{35}S|$ sulphate into pig laryngeal cartilage proteoglycans, radioactivity appeared in chondroitin sulphate after 2 min, but there was a delay of 35 min in the appearance of extractable proteoglycans (147). No precursor-product relationship was found between fractions of different molecular size which appeared to be labelled all at the same time. It has been suggested that molecules of all sizes are synthesized at the same time but this does not exclude the possibility that there are different biosynthetic pools (147). Contrary to these findings, however, are the results of *in vivo* studies on rabbit auricular cartilage which strongly suggested a precursor-product relationship between high molecular weight and low molecular weight proteoglycan molecules (155). However, some doubt exists regarding the validity of these results since it cannot be exclusively proved that the low molecular weight species, which are synthesized at a rate comparable to that

of some of the fractions isolated from epiphyseal cartilage by Handley and Phelps (148), are not themselves distinct terminal biosynthetic products.

### 1.3.1. Degradation of proteoglycans

As most of the enzymes necessary for complete degradation of the proteoglycan molecule are found intracellularly, it is a prerequisite that the initial step in the degradation of those proteoglycans which are found in the extracellular matrix involves "releasing" the molecule from the matrix. In the cartilage matrix most of the proteoglycan molecules occur in aggregate form complexed with hyaluronic acid but in other tissues interactions between the glycosaminoglycans and matrix proteins are thought to be important for the retention of proteoglycans within the extracellular matrix. The proteoglycans can thus be released either by degradation of hyaluronic acid and thus aggregate disruption, or by proteolytic cleavage of the core protein in such a way that the monomer is released. Glycosaminoglycans do not appear to be depolymerized before leaving the tissue (156,157) and hyaluronidase activity has not as yet been demonstrated in epiphyseal cartilage (158), thus the first possibility seems unlikely. Most glycosaminoglycans released during autolysis of cartilage slices were units of several intact chains attached to a polypeptide (159), which indicated that it was indeed the core protein that underwent cleavage. A considerable body of evidence has been built up to support the contention that degradation in adult articular cartilage is initiated by

limited proteolysis of the core protein.

Sandy *et al*. labelled rabbit articular cartilage *in vivo* with $|^{35}S|$ sulphate and then incubated it in organ culture at pH 7.2 (160). During the first 48 h, 65% of the tissue radioactivity appeared in the medium. A significant result was that this released material was only slightly smaller than proteoglycan molecules isolated from similar tissue as a control ($K_{av}$ of 0,67 on Sepharose CL-2B as compared with 0,4), but it had lost its ability to interact with hyaluronic acid and thus diffused readily from the tissue. It thus seems likely that proteolytic cleavage occurs at or near the globular hyaluronic acid-binding region of the core protein. The proteolysis may be limited as a result of a higher rate of release of the degradative product from the tissue, thus preventing its further degradation by other enzymes. These results were supported by Wasteson *et al*. using rat costal cartilage for *in vitro* studies (161). They confirmed that the first phase of the turnover process involved the partial proteolysis of the core protein whilst the polysaccharide chains remained intact.

Several workers have produced evidence for the existence of proteases which cleave the subunit core protein, both at acid and neutral pH (156,160-164). *In vitro* studies of cartilage autolysis showed that the degradative activity was very pronounced at pH 5.0 and relatively insignificant at neutral pH (156,163,164). This led to the conclusion that it is the

lysosomal cathepsins that must be the major degradative proteases. However, there have been a few reports on neutral proteases which are capable of degrading the core protein (162, 165).

In 1964 Ali described an enzyme which attacks peptide bonds involving arginine in the core protein with optimum activity at pH 5,0 (156). Its activation by cysteine and sensitivity to inhibition by iodoacetamide led him to conclude that it was probably cathepsin $B_1$. Dingle et al. raised antiserum to purified cathepsin D and showed that it inhibited degradation of purified bovine nasal proteoglycan by this enzyme (163). They excluded a major role for cathepsin $B_1$ as proposed by Ali. Later Morrison et al. showed that both cathepsins $B_1$ and D degrade proteoglycans from adult human articular cartilage and they suggested that both these enzymes are involved in core protein degradation (164). Sapolsky et al. isolated three distinct metalloproteases from human articular cartilage (162). One was active at neutral pH - 7,25 - and the other two at acid pH's 4,5 and 5,5. These they have shown to be distinct from cathepsins B, D and F.

The question has not been resolved whether it is the lysosomal enzymes or the neutral proteases which are responsible for the initial proteolysis in proteoglycan degradation. It seems probable that in some tissues, particularly connective tissues, the primary lysosomes migrate to the cell surface where they discharge their contents into the extracellular space.

The importance of these acid proteases in normal turnover has been questioned since the pH of the extracellular fluid is about 7,3 (166), and these enzymes have very limited activity at this pH. This limited activity at neutral pH, however, might be sufficient to cause limited proteolysis of the core protein and thus release it from the extracellular matrix. Other possibilities are that lysosomes may transport their environment with them and as a result may generate a local acid environment suitable for their activity, or an acid pH might be generated at the cell surface at the expense of metabolic energy. Although reports on neutral proteases are limited, their involvement in the initial stages of proteoglycan degradation cannot be ruled out; although not quantitatively important they may be important physiologically.

After the initial cleavage of the core protein and the release of the proteoglycan from the extracellular matrix, the molecule must either diffuse into the circulatory system or be taken up by the cells for further intracellular degradation. Kresse et al. have shown that proteoglycans are taken up into cells by a process that exhibits kinetics characteristic for receptor-mediated adsorptive endocytosis, i.e. binding to a receptor on the cell surface followed by internalization via pinocytic membrane movements (167,168). They have shown that endocytosis is dose and temperature dependent and is specific and saturable (167,168). Unlabelled proteoglycans compete for uptake with labelled counterparts (168). Binding to the cell surface for integration into the pericellular pool and

binding for uptake into the cell are separate processes as the proteoglycan concentrations required for half maximal saturation have been found to be quite different (167).

Different glycosaminoglycan components are endocytosed at different rates; dermatan sulphate-rich proteoglycans have the highest rate of uptake (167). This appears to result from the existence of different cell surface receptors, since the uptake of a heparan sulphate-rich proteoglycan was not reduced by the addition of a 10-fold excess of unlabelled dermatan sulphate-rich proteoglycan (167). Prinz et al. showed that the kinetics of binding to the cell surface suggested two types of binding sites: low affinity binding sites which were non-saturable and high affinity saturable binding sites (169). The simplest explanation for the low affinity non-saturable sites would be that the proteoglycans associate with the surface as multilayers owing to self-aggregation rather than as a monolayer. The timing involved does not allow for synthesis of new cell surface receptors (169) and suggested that receptors were recycled in a manner similar to that for low density lipoproteins (170). Calculations showed that the half-life of a cell surface receptor would be less than 3 min at the maximal rate of endocytosis.

It has also been shown that an intact protein moiety was necessary for efficient uptake, as the uptake of glycosaminoglycans initiated on β-D-xylosides was decreased by about 75% (168). This would account for the initial step in degradation

being limited to only slight proteolysis of the core protein. Whether the protein moiety is specifically recognized by the receptor or whether it just organizes the carbohydrate side chains in a configuration necessary for efficient binding is not yet known. When hybrid chondroitin sulphate - dermatan sulphate oligosaccharides were used as competitors for the uptake of xyloside-initiated glycosaminoglycan chains it was shown that decasaccharides successfully competed for uptake but smaller saccharide fragments were ineffective (168). It remains to be tested whether the minimal length of five disaccharide units is required for interaction with only one receptor or with two adjoining binding sites.

It has not been clearly established whether the endocytosed proteoglycans are partially desulphated. There are some suggestions that sulphated proteoglycans may inhibit endocytosis (171). Wasteson et al. showed that proteoglycan molecules released from cartilage by limited proteolysis were partially desulphated (161). Studies on rat smooth muscle cells in culture have shown that degradation appears to take the form of desulphation of sulphated molecules with the desulphation process occurring in the pericellular-matrix compartment (172). Thus partial desulphation may be the first stage in the process of receptor-mediated endocytosis of the proteoglycan molecule.

Once inside the cell the proteoglycan molecules are fully degraded by the lysosomes which contain a full complement of

enzymes necessary for destruction of the core protein and carbohydrate moieties: proteases, β-glucuronidases, β-glycosaminidases and sulphatases. Those proteoglycans which have been endocytosed are degraded more rapidly than those which are already inside the cell. Hickman and Neufeld have suggested that in fibroblasts lysosomal enzymes may first be secreted before they reach the lysosomes by adsorptive pinocytosis (173). In view of this the more rapid catabolism of pinocytosed proteoglycans could be explained by a proximity of receptors for lysosomal enzymes and proteoglycans which would result in the formation of pinosomes with proteoglycans (substrate) and a set of enzymes for adequate and rapid degradation. Catabolism would start when the approximate pH was generated and would no longer depend on random fusion processes.

Addition of glycosaminoglycans such as chondroitin sulphate, dermatan sulphate and heparin to various acid hydrolases resulted in neither reduction nor apparent increase in enzyme activity (174-176). This effect was considered to be due to a protective action of the polysaccharide against enzyme degradation by lysosomal proteinases (20). Studies involving lysosomal enzymes from polymorphonuclear leucocytes have shown that most of the enzymes interacted with glycosaminoglycans and this interaction involved pH-dependent, reversible electrostatic binding (174,177). It was suggested that the enzymes in acidic primary lysosomes occur in a complex with glycosaminoglycans and are hence in a protected and relatively inactive form. Fusion of a primary lysosome with a phagosome

will result in dilution of the intralysosomal acidic fluid and thus an increase in pH. This in turn leads to a dissociation of the complexes and a release of intact active enzyme molecules.

It is generally thought that the endocytosed proteoglycans are at least partially desulphated before total degradation. Buerman et al. have shown that in polymorphonuclear leucocytes the complete degradation of glycosaminoglycans into inorganic sulphate and monosaccharides occurs by a concerted synergistic mechanism involving aryl sulphatases, followed by β-glucuronidases, 4- and 6-sulphatases and β-N-acetyl-galactosaminidases and -glucosaminidases (178). An enzyme isolated from bovine aortic tissue has been shown to release inorganic sulphate from chondroitin-4-sulphate with a pH optimum of 4.4 (179).

## 1.4. EXTRACELLULAR ASSOCIATIONS OF PROTEOGLYCANS

### 1.4.1. Binding to matrix components

Proteoglycans make up a major portion of extracellular matrix material along with collagen, elastin and glycoproteins. Thus the relationships of these molecules to each other and to the water and ions of the extracellular milieu are physiologically extremely important and contribute largely towards the physical properties displayed by the tissue. The deposition of insoluble proteoglycans into the extracellular matrix

arises both from their aggregation with hyaluronic acid which has been discussed in detail in Section 1.1.2, and as a result of interactions between themselves and the other components of the matrix.

Because of their polyanionic nature, the binding of glycosaminoglycans is generally thought to be of an electrostatic nature although other types of interactions may occur. In addition, specific stereochemical factors seem to operate as in general glycosaminoglycans containing L-iduronic acid, such as dermatan sulphate and heparan sulphate, bind to proteins with higher affinity than do those containing D-glucuronic acid such as chondroitin-sulphates, in spite of having a similar charge density. This may be most likely explained by the fact that in the former the carboxyl and hydroxyl groups are in an axial orientation, giving rise to a more favourable conformation of the molecule for binding. The degree of binding of glycosaminoglycans is directly proportional to their charge density and chain length.

### 1.4.1.1. Binding to collagen

The interaction between collagen and glycosaminoglycans has been studied by a variety of techniques (180-185). The results indicate that all glycosaminoglycans bind to collagen by electrostatic interaction at physiological pH and ionic strength except keratan sulphate which lacks carboxyl groups, and hyaluronic acid which lacks sulphate groups. However, some association between collagen and hyaluronic acid by

mutual steric interaction has been postulated (186). As mentioned earlier, higher charge density and larger molecular size as well as the presence of L-iduronic acid residues promotes binding with higher affinity. Dermatan sulphate has been shown to have the strongest interaction with collagen followed by heparan sulphate, heparin and chondroitin sulphate (186). Radhakrishnamurthy has shown that a dermatan sulphate proteoglycan was closely associated with collagen in bovine aorta and could only be released by collagenase digestion (187). Eisenstein et al. have shown three distinct morphological patterns of proteoglycan containing material in epiphyseal growth plate, one of which was intimately associated with collagen and not extracted with guanidinium HCl (188). They have suggested that these proteoglycan molecules may be covalently linked to collagen. The number of polysaccharide chains bound to each collagen monomer usually varies from between two to five chains; however, it has been shown that a dermatan sulphate of high molecular weight (approx. 41000) could bind as many as five molecules of collagen per chain (184,189,190).

Experiments with isolated chondroitin sulphate proteoglycan core protein point to a complex binding situation. Core protein bound strongly to collagen (191,192), yet did not appear to inhibit binding of intact proteoglycan (192,193). Studies by Toole using a sensitive radioactive assay showed that it was likely that the primary specific binding site for collagen lay in the core protein, and this reaction gave rise to a spatial arrangement which maximizes secondary inter-

action between the glycosaminoglycan chains and the basic residues in the collagen molecule (193).

Proteoglycans have been shown to bind to all types of collagen (193,194). Interaction of cartilage proteoglycans with isolated $\alpha$-chains from type I collagen have shown them to have a preference for $\alpha_2$ and $\beta_{12}$ components, but by increasing the concentrations of proteoglycan, $\alpha_1$ and $\beta_{11}$ also bound (192,195). Also *in vitro* competitive-interaction experiments showed that in the presence of equal amounts of collagen type I and II, the proteoglycan bound type I preferentially (195).

The exact binding sites on the collagen molecule have not been established but the positively charged guanido group of arginine and the $\varepsilon$-amino group of lysine are expected to play a major role (196,197). Öbrink *et al*. found at least three binding sites for bovine nasal chondroitin-4-sulphate on each collagen molecule (189,196). Scatchard analysis of the binding gave non-linear results indicating that the binding sites each have different binding strengths.

Proteoglycan-collagen interactions should influence the deposition of collagen fibres *in vivo*. Heparin, heparan sulphate and cartilage proteoglycans have been shown to retard the assembly of collagen molecules during fibrillogenesis if present early enough during the process. Chondroitin sulphate and hyaluronic acid were less effective (192). The molecules that delayed fibrillogenesis also altered the final organization of

the fibril by producing an increase in the degree of aggregation. Thus the proteoglycan-collagen interaction could be important in the organization and functioning of the connective tissue matrices at all stages of development.

1.4.1.2. Binding to elastin

Little information is available concerning the interaction between proteoglycans and elastin. Podrazký and Adams have shown that an insoluble elastic product is formed from α-elastin and sulphated proteoglycans from connective tissue (198). They have also shown an electrostatic interaction between the native precursor of elastin, tropoelastin, and the proteoglycan (199). It is generally accepted that insoluble elastic fibres are formed as a result of cross-linking of the soluble tropoelastin. Prior to cross-linking, the tropoelastin molecules must be arranged in such a way that the correct apposition is achieved for the lysine side chains which are responsible for the cross-link formation (200,201). Hydrophobic associations have been suggested as the ordering force but it is possible that the tropoelastin-proteoglycan interaction is important. However, this binding occurs at very low pH (2,5 - 4,0) with no binding at pH 7,0 (198,199), thus its physiological significance appears uncertain. The binding of proteoglycan seems to induce conformational changes in the elastin molecule, leading to an increased content of helical structure (202).

Radhakrishnamurthy et al. showed that elastase digestion of

bovine aorta solubilized most of the heparan sulphate proteoglycans and some of the dermatan sulphate - chondroitin sulphate species as well (187). The preferential solubilization of heparan sulphate proteoglycan was taken as evidence for a specific binding between this glycosaminoglycan and elastin.

1.4.1.3. Binding to fibronectin

Fibronectin is an important glycoprotein component of cell surfaces and extracellular spaces. It has been shown that fibronectin binds both hyaluronic acid and heparin and to a lesser extent heparan sulphate, but there is no evidence of binding to chondroitin sulphate (203-207). Yamada et al. have shown that binding of hyaluronic acid and heparin was saturable and reversible and occurred at separate sites (203). Binding of one molecule did not affect binding of the other. Scatchard analyses revealed non-linear high-affinity binding with $K_D$ $10^{-7}$ to $10^{-8}$ M for both glycosaminoglycans (203). Results suggested that the binding was specific and not merely due to simple non-specific electrostatic binding of charged polymers. The fibronectin binding site for heparin is localized in a specific 50000 dalton fragment. It is relatively globular in shape and is nearly devoid of cysteine residues and of carbohydrate (208). Its resistance to degradation by moderate concentrations of Pronase is strong evidence for the existence of a tightly folded polypeptide domain containing no regions of exposed peptide. A structural domain for binding hyaluronic acid on fibronectin has not been identified as yet

but competitive inhibition studies show that it recognizes between eight and ten monosaccharide units (203). Hyaluronic acid plays a role in cellular adhesiveness and may have important regulatory effects on cells during embryonic development and thus the binding with cell surface fibronectin may be one of the ways by which hyaluronic acid interacts with the cell.

The binding of glycosaminoglycan to fibronectin-collagen complexes results in the formation of a more stable complex than that of fibronectin and collagen alone (203-207). This observation gave rise to the proposal that all three components are necessary for the formation of a stable, insoluble extracellular matrix.

1.4.2. Binding to plasma proteins

Several examples of interactions between glycosaminoglycans and plasma proteins are known but will only be briefly discussed. Whether these all have physiological roles depends, among other factors, on availability of glycosaminoglycans within the blood vessel wall. Their presence on the endothelial cell surface may provide a means for the selective binding of plasma components to the vessel wall (209,210).

Heparin is synthesized and stored inside mast cells in the close vicinity of blood vessels. The physiological function of endogenous heparin is uncertain in spite of its obvious biological effects. It binds to several plasma components - coagulation factors and antithrombin - but little is known of

the mechanisms and specificity of these interactions (211, 212). The binding is thought to be electrostatic, perhaps inducing conformational changes.

Barber et al. have shown that platelet factor 4 (PF4) is released from human blood platelets by thrombin in the form of a high molecular weight proteoglycan-PF4 complex (213). The composition of this complex shows remarkable stoichiometry: it occurs as a dimer, each monomer consisting of four molecules of PF4 and one molecule of proteoglycan which consists of four chondroitin-4-sulphate chains bound to a core protein. Heparin, heparan sulphate, chondroitin-6-sulphate and dermatan sulphate were also shown to combine stoichiometrically with PF4 but hyaluronic acid did not (213,214). Binding was shown to be electrostatic and was promoted by high charge density and the presence of L-iduronic acid residues. Lysine residues of PF4 have been implicated in binding whilst modification of arginine residues had no effect on binding (214).

The triglyceride-degrading enzymes lipoprotein lipase and hepatic lipase may be released by heparin into the circulation from tissue sites that are presumably located at the luminal surface of capillary endothelial cells and in the liver respectively (215-217). Heparin binds to both enzymes (218), and experiments with chemically modified heparin have shown that they bound to sites in the heparin molecule different to the antithrombin sites (219-221). Both heparan sulphate and dermatan sulphate also bound the enzymes but chondroitin sulphate showed no appreciable affinity for them (222). The

binding of the glycosaminoglycan to the enzyme might cause lipase release as a result of a conformational change in the enzyme or as a result of interaction with the cell surface carrier structure (223). The glycosaminoglycan may, in fact, form part of such structure.

One of the most important interactions between glycosaminoglycans and plasma components must be that between sulphated glycosaminoglycans and serum low and very low density lipoproteins (LDL and VLDL). This interaction has been implicated in the retention of serum lipoprotein in the arterial wall and in the subsequent development of atherosclerosis, and has been studied using a variety of techniques (224-227). Again binding depends on electrostatic forces, increasing with charge density of the glycosaminoglycans, and it can be abolished by N-acetylation of the lipoproteins. Preferential binding of L-iduronic acid-containing species was again noted (224). Vijayogopal et al. showed that removal of the core protein or the glycosaminoglycan chains or desulphation of the proteoglycan molecule prevented interaction with LDL (228). Divalent cations were found to stabilize the complexes and this was thought to be as a result of these cations bridging the anionic groups of the glycosaminoglycan and/or the lipoprotein, thereby producing the cross-links necessary for the formation of insoluble complexes (226,228).

LDL, the major cholesterol-carrying lipoprotein in human plasma, delivers its cholesterol to cells in tissue by a receptor-

mediated process (229). Binding of LDL to its receptor can be reversed by adding heparin or dermatan sulphate at a higher concentration, whereas chondroitin sulphate is ineffectual (230). These observations have suggested that extracellular glycosaminoglycans may influence the binding of lipoproteins to cells and, furthermore, raise the possibility that cell surface glycosaminoglycans may in fact form part of the receptor structure.

Interactions between serum lipoproteins and arterial wall proteoglycans are believed to play a role in the development of the atherosclerotic lesion, involving deposition of lipids in arterial walls (231,232). Dermatan sulphate binds to lipoproteins at physiological ionic strength (224). Kumar et al. analysed glycosaminoglycan composition in human arterial intima and externa in different stages of atherosclerosis and showed that dermatan sulphate was almost exclusively confined to the fatty streaks and pre-stages of this lesion (233). Thus lipoproteins may accumulate and form fatty streaks where dermatan sulphate is abundant.

### 1.4.3. Interchain binding of glycosaminoglycans

A number of natural glycans form gels by cooperative interactions between individual chains e.g. such glycans constitute extraneous coats in bacteria or extracellular matrices in algae and higher plants (234,235). As a result of an initial observation of an ordered conformation for hyaluronic acid, interchain binding for glycosaminoglycans was proposed but

subsequently refuted (236-238). Recently, however, Fransson claimed that copolymeric galactosaminoglycan chains (that contain both D-glucuronosyl- and L-iduronosyl-N-acetyl-D-galactosamine sulphate) bind to one another (239). The interaction was demonstrated by affinity chromatography using polysaccharide-substituted gels. Binding occurred at physiological ionic strength and did not require the presence of divalent cations. Both hydrogen bonding and hydrophobic interactions appeared to play a role as guanidine, urea and trichloroacetate prevented interaction. The presence of both D-glucuronic and L-iduronic residues in the same chain was essential for interactions to occur and the highest degree of association occurred in copolymeric chains containing nearly equimolar proportions of the epimeric uronic acids (239). Chondroitin-6-sulphate showed no binding to copolymeric chains but chondroitin-4-sulphate did (239). Thus it was the 4-sulphated regions of the chains that were proposed to contain the binding zones. It was not established whether the D-glucuronic and L-iduronic regions were arranged in clusters or in an alternating fashion (240). In a recent study on the formation of copolymers by fibroblasts in culture it was observed that alternating sequences were particularly prominent in copolymers isolated from the cell layer after trypsin digestion (241). The study by Fransson also demonstrated binding of heparan sulphate and heparin to copolymeric galatosaminoglycans. Since heparan sulphate is associated with the cell surface (242), this phenomenon may be a factor in concentrating certain proteoglycans containing chondroitin-dermatan copolymers in the immediate vicinity of the cell.

The binding of the copolymeric chains to each other may also explain the anomalous behaviour of such chains upon gel chromatography observed by some workers (243). Copolymers from fibrous cartilage were eluted earlier than both L-iduronic acid-rich and D-glucuronic acid-rich glycans, despite the fact that all fractions appeared to have the same molecular weight. This was presumed to be as a result of the copolymeric chains having a different conformation than the homopolymeric chains but it now seems highly likely that chain-chain interactions leading to aggregation are the cause of such copolymeric chains eluting earlier.

The concept of inter-chain binding of glycosaminoglycans under physiological conditions thus offers new prospects for the functional role of copolymeric chains in the intercellular space and particularly on the cell surface.

### 1.4.4. Cell surface glycosaminoglycans/proteoglycans and the interaction of glycosaminoglycans with cell surface components

#### 1.4.4.1. Cell surface glycosaminoglycans/proteoglycans

Cultured mammalian cells contain a variety of glycosaminoglycans associated with their cell surface (210,244). The predominant proteoglycan/glycosaminoglycan found associated with cell membranes is heparan sulphate (210,242), although both chondroitin sulphate proteoglycan and hyaluronic acid have also been identified (245,246). Diverse functions have been proposed for these cell surface proteoglycans e.g. regulation of cell growth,

cell-cell communication, and service as receptors (247,248). The nature of association between these molecules and the cell surface is largely unknown but is probably variable since they may be loosely or tightly bound. Interactions probably involve recognition of core protein or polysaccharide side chains or both. Attempts to release total membrane-associated proteoglycans from cell surfaces by repeated washing with moderate to high salt buffers have been unsuccessful, suggesting that they may be held in association with the surface by more than mere ionic or electrostatic interactions (249). Kraemer isolated a cell surface heparan sulphate component from the surface of cultured Chinese hamster cells with mild trypsin treatment under conditions which prevented irreversible cell damage (242).

The occurrence of membrane-associated binding sites for heparin-like polysaccharides was conclusively demonstrated in studies on the uptake of radiolabelled heparin by rat liver cells in primary culture (250). The uptake process was time- and temperature-dependent and showed saturation kinetics. It was also reversible since heparin, once bound, could be displaced by addition of excess amounts of unlabelled heparin. Heparin could also displace bound heparan sulphate but the reverse was not true (251). The binding was confirmed to be specific as attempts to displace bound heparin or heparan sulphate using hyaluronate, chondroitin sulphate, dermatan sulphate and some preparations of low-sulphated heparan sulphate were unsuccessful

(250). The latter suggests that a certain high level of sulphation may also play a role in the interaction of heparin and heparan sulphate to cell membrane components.

Kjellen et al. demonstrated that heparan sulphate proteoglycans were associated with hepatocyte plasma membranes by two separate mechanisms (252):

i) a portion was bound to surface receptors that recognize the polysaccharide chains and these could be displaced by the addition of heparin;

ii) some cell surface heparan sulphate proteoglycans resisted heparin treatment but were released after trypsin treatment. The data suggested that in this case the core protein was embedded in the bilayer of the plasma membrane and thus disruption of the membrane by detergent was required to solubilize the proteoglycan in an intact form. The presence of a hydrophobic domain in the proteoglycan molecule was also shown. The detergent-extracted molecule was shown to have a higher molecular weight than the heparin-displaced species and its core protein exhibited marked hydrophobic properties. It was concluded that the larger molecule was directly attached to the hepatocyte plasmalemma and that the smaller molecule may be derived from it by proteolytic cleavage. Some cells lack receptor bound heparan sulphate at their cell surfaces, but have heparan sulphate associatedwith their surfaces, thus a membrane-intercalated proteoglycan may occur generally (6).

It has been suggested that cell-associated proteoglycans may

have their own biosynthetic pathways. Johnston and Keller noticed that the synthesis of surface-associated heparan sulphate was not affected by the addition of exogenous xyloside while that of medium-associated heparan sulphate was enhanced (253). As a result of tandem label experiments Kraemer proposed that the heparan sulphate of the cell surface is derived directly from a small intracellular membrane-associated compartment rather than from the normal intracellular compartmentated pathway (242).

As constituents of membranes, proteoglycans could serve as receptors e.g. for lipoprotein lipase (see Section 1.4.2). They may also be biologically active in cell-cell contacts or in the interaction of the cell with its substratum e.g. via binding to matrix proteins.

1.4.4.2. Interactions of glycosaminoglycans with cell surface components

Many specific cellular functions or behaviours have been attributed to interactions between glycosaminoglycans and structures on the cell surface. The endocytosis of proteoglycans from their extracellular environment for degradation has been shown to occur as a result of glycosaminoglycan-cell surface receptor binding (167).

The interactions of hyaluronic acid with the cell surface are very relevant during morphogenesis. In many systems it has been demonstrated that the cessation of migration and the onset

of differentiation are associated with a dramatic decrease in the extracellular hyaluronic acid concentration (254,255). When added to cultures of embryonic chick somite cells, hyaluronic acid inhibited differentiation (256). This effect could not be reproduced with other biological polyanions e.g. chondroitin sulphates. Hyaluronic acid was also shown to inhibit chondroitin sulphate biosynthesis in chondrocytes (38,257,258). This effect was not noticed in fibroblasts and thus may be one of the factors controlling the essential properties of cartilage matrices. The hyaluronic acid was shown to bind to specific sites on the surface of the chondrocytes and could be removed with brief trypsin treatment (245,259). Scatchard analysis shows that binding is of high affinity and it has been proposed that because of its repetitive sequence, a single hyaluronic acid molecule can bind to many receptors, and this would lead to an overall strong interaction. The binding between protein receptors and hyaluronic acid was proposed to be of a noncovalent nature, but there may be secondary covalent binding of hyaluronic acid to the membrane. Hyaluronic acid did not affect chondroitin sulphate synthesis in the presence of β-D-xylosides (258) and thus acts by interfering with glycosaminoglycan synthesis, either by specifically inhibiting the formation of normal core protein or by inhibiting the initial enzyme involved in the synthesis of the glycosaminoglycan chain, xylosyltransferase.

Hyaluronic acid has also been implicated in the adhesion of cells to each other and to various natural or artificial

substrates. These adhesive properties are considered essential to normal morphogenesis and differentiation, and manifest themselves in markedly selective cell aggregation phenomena *in vitro* (260,261). Using certain lymphoma cells it was shown that hyaluronic acid lost its aggregating properties on partial depolymerization and would not be substituted for by any other glycosaminoglycan (267,263). However, oligosaccharides derived from either hyaluronic acid or chondroitin sulphate were able to inhibit aggregation induced by polymeric hyaluronic acid (263). A possible interpretation is that both hyaluronic acid and chondroitin sulphate bind to the same cell-surface component but due to the differences in chain length, only hyaluronic acid is able to span the intercellular space and thus induce aggregation. Not all cell lines, however, possess the putative hyaluronic acid-binding receptor and are thus non-aggregatable by hyaluronic acid.

## 1.5. AGE-RELATED CHANGES IN PROTEOGLYCANS/GLYCOSAMINOGLYCANS

The age-related changes in proteoglycans/glycosaminoglycans have been studied primarily in various types of cartilaginous tissue (264-268). The large size and high charge density of proteoglycans in cartilage are largely responsible for the elastic properties of the tissue and decreased elasticity of cartilage with aging is primarily related to changes in proteoglycan content and structure. In general the following changes occur with age:

(i) The proteoglycan content of cartilage decreases, and this is manifested by a decrease in the amount of proteoglycans that can be extracted (264-268), but this is also due to much increased cross-linking of the collagen network.

(ii) The proteoglycan monomers decrease in size (264,268-270). This has been shown to be due to a decrease in the size of the chondroitin sulphate-rich region of the core protein (264,270). Some workers have found a decrease in the average chain length of the chondroitin sulphate chains (266,269) with age; however, others found that the lengths remain constant with age (264).

(iii) There is an increase in the keratan sulphate content in older tissue (265,267,268,270). The keratan sulphate-rich region of the monomer probably becomes larger (264, 267).

(iv) The chondroitin-6-sulphate : chondroitin-4-sulphate ratio generally increases (268,271,272).

(v) There is no change with age in the ability of the monomers to interact with hyaluronic acid to form aggregates (264, 268,269).

(vi) The hyaluronic acid content has been shown to increase with maturation (264,273).

(vii) The protein:glycosaminoglycan ratio of the proteoglycan increases (264,268). The serine and glycine contents of the core protein decrease (268,270) and the arginine

content increases in older tissues (268,270).

(viii) It has been shown in rat costal cartilage that the total sulphotransferase activity decreases and as a result, the sulphation of the chondroitin sulphate chains decreases with an increase in the amount of unsulphated disaccharides being found in the tissue (266).

Most workers favour the synthesis of distinct populations of proteoglycans in different age groups rather than the concept of the smaller proteoglycans in older tissues arising from degradation. This view is supported by the finding of the variation in amino acid composition of core protein with aging.

## 1.6. ARTERIAL PROTEOGLYCANS

The walls of arteries consist of three major layers with an inner lining (Fig. 1.4). This lining is called the endothelium and is made up of a single layer of flattened epithelial cells. It is supported by a basement membrane and connective tissue containing relatively few smooth muscle cells - this constitutes the tunica intima. Below the intima is an intermediate muscle layer, the tunica media, which is comprised of smooth muscle cells embedded in connective tissue. Closer to the media may be a definite external elastic membrane. Adjacent to the media is a thick spongy outer connective tissue layer called the tunica adventitia. The interactions between the different layers and between the cells and their

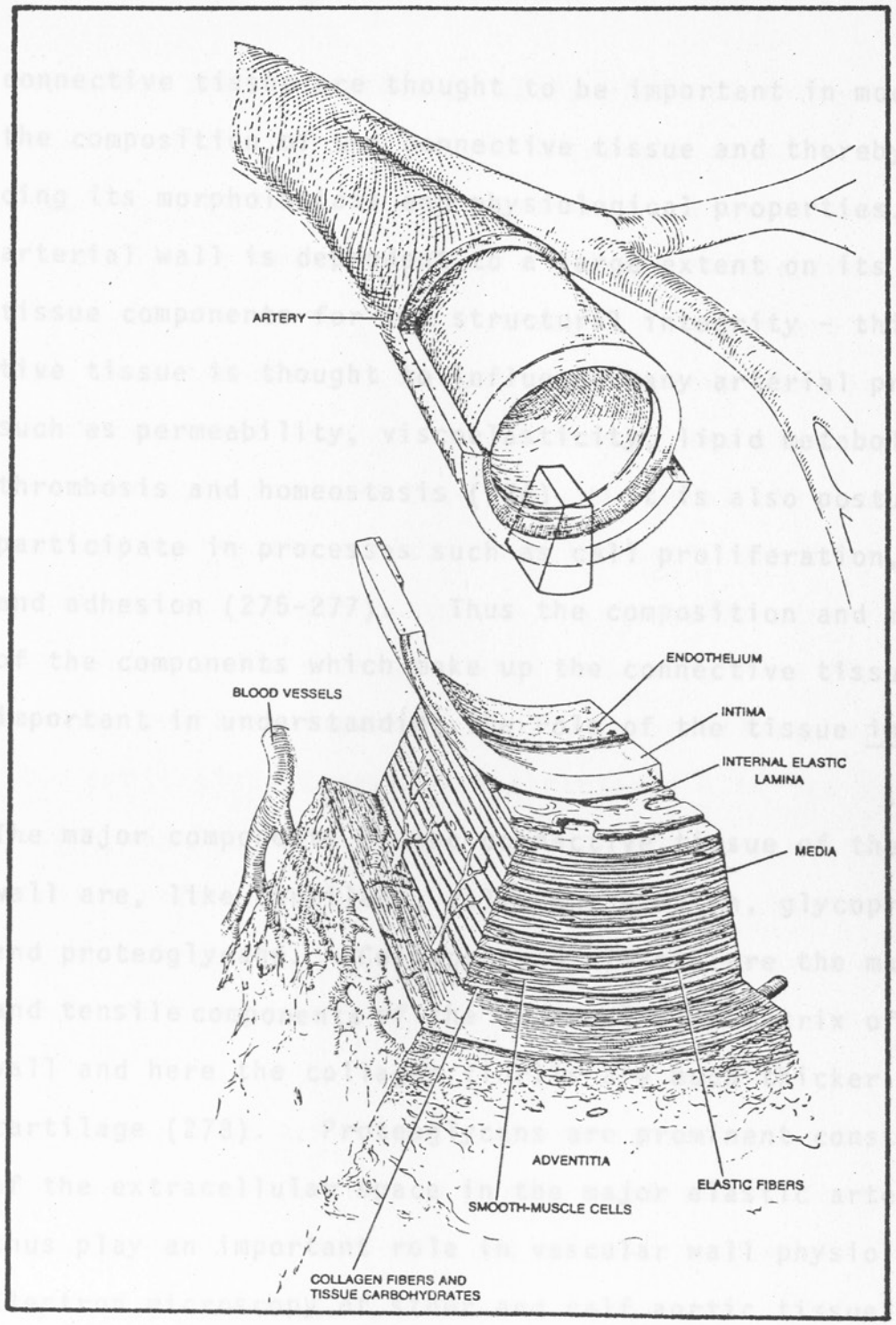



Fig. 1.4. A segment of a major elastic artery of the type of the aorta. (After Benditt, E.P. (1977) Scientific American 236 (2), 74-85).

connective tissue are thought to be important in modulating the composition of the connective tissue and thereby influencing its morphological and physiological properties. The arterial wall is dependent to a large extent on its connective tissue components for its structural integrity - this connective tissue is thought to influence many arterial properties such as permeability, viscoelasticity, lipid metabolism, thrombosis and homeostasis (274). It is also postulated to participate in processes such as cell proliferation, migration and adhesion (275-277). Thus the composition and metabolism of the components which make up the connective tissue are very important in understanding the role of the tissue *in vivo*.

The major components of the connective tissue of the arterial wall are, like cartilage, collagen, elastin, glycoproteins and proteoglycans. Collagen and elastin are the major elastic and tensile components of the extracellular matrix of the aortic wall and here the collagen fibrils are much thicker than in cartilage (278). Proteoglycans are prominent constituents of the extracellular space in the major elastic arteries and thus play an important role in vascular wall physiology. Electron microscopy of steer and calf aortic tissue fixed in the presence of ruthenium red has allowed visualization of proteoglycans within the tissue (279). The proteoglycans are visualized as dense granules, because of the collapse of their carbohydrate side chains during the preparation, and are seen to be closely associated with collagen in older animals and with both collagen and elastin in younger animals. They

are often seen condensed on the surface of the collagen fibrils at the major periodic intervals of 640 Å which suggests that there are ordered interactions between the proteoglycans and the collagen fibrils in the connective tissue of the aorta (280). Recently, Wight and Hascall have used safranin O staining which allows staining without harsh treatment leading to collapse of the side chains, and the proteoglycans have been observed under electron microscopy as having the typical "bottlebrush" structure (281), Proteoglycans in aortic smooth muscle cell cultures have been shown to be entangled with and among fine fibrils, as well as present singularly in the intercellular matrix. Some proteoglycans were also associated with the surface of smooth muscle cells (281). In all electron microscopy studies prior digestion of the tissue with chondroitinase ABC or extraction with 4 M guanidine HCl removed material which stained positively with proteoglycan-specific stains (279,280).

The aorta, like cartilage, undergoes repetitive transient pressure changes and it is likely that the proteoglycans, together with the collagen and elastin components of the connective tissue, buffer these changes and maintain the integrity of the vessel. In addition, the proteoglycans also affect the transport rates of small and large molecules across the arterial wall, they may affect arterial wall hydration and they may be very important in binding macromolecular species such as lipoproteins which have crossed the endothelium from the plasma.

### 1.6.1. Molecular structure of arterial proteoglycans

The polysaccharide moieties of the proteoglycan molecule, the glycosaminoglycans, have been studied extensively in arterial tissue (282-284). However, scant information is available on the native state in which these glycosaminoglycans occur in the arteries i.e. bound to core protein to form proteoglycans. The structural complexity of the vessel wall and the heterogeneity of glycosaminoglycans studied in blood vessels suggest that the composition of the proteoglycans in cardiovascular structures is complex. The proteoglycan content of the arterial wall is low as compared to cartilaginous tissues and they are difficult to extract quantitatively (285,288). In addition, there are strong interactions between some of the proteoglycans and the protein elements of the matrix which makes these more resistant to extraction with buffers of high ionic strength, and solubilization of the fibrous components of the tissue is sometimes required for quantitative extraction (187). Large amounts of the fibrous proteins of the matrix are co-extracted (286) and this makes purification of the proteoglycan an arduous task.

Several different techniques have been described for the isolation of proteoglycans from the arterial wall (287-291). Preparations differing in their chemical composition and in their relative molecular mass from 72000 to $2 \times 10^6$ have been identified (287,288,290). These conflicting results may be explained in part by the variability in proteoglycan size and composition that has been found with differences in species

(292), in age (289,293,294), in the site of origin of the tissue (289,295) and as a consequence of disease (233,282,296). Proteoglycans have been shown to vary from species to species and from age group to age group within a given species. Generally, younger animals have a higher concentration of glycosaminoglycans than older animals (289,293). The distribution of glycosaminoglycans across the arterial wall also varies. There is a radial decrease in the concentration of glycosaminoglycans when assayed at different distances from the endothelium outwards; this is most marked in the case of chondroitin sulphate-dermatan sulphate fractions (289,295). It has also been shown that there are more glycosaminoglycans in the upper than the lower aorta, particularly in younger animals (289). In addition, the diverse isolation methods, a considerable molecular polydispersity, the close association of the proteoglycans with other macromolecular constituents that prevent the dissociation and extraction of proteoglycan complexes account for the variability in proteoglycan structures. Bedevilling all these factors is the ever-present problem of preventing proteolytic degradation of intact proteoglycans by the tissue proteases. It seems hardly surprising that so many and various proteoglycan structures have been described in the literature.

The extraction of proteoglycans from cartilageis based on the ability of a large proportion of the proteoglycan monomers to aggregate specifically with hyaluronic acid in a reversible manner (30). Extraction of the tissue with a solvent of high

ionic strength such as 4 M guanidine HCl results in these aggregates being dissociated, and their components are extracted from the tissue and are subsequently purified by caesium chloride buoyant density centrifugation with resultant high yields (31). Several workers have chosen to adapt these methods to the extraction of proteoglycans from arterial tissue but they pose some problems (290-292). Large amounts of extraneous proteins, largely collagen and actin, are co-extracted with the proteoglycan (286), and in addition substantial amounts of the proteoglycans become irreversibly insoluble during the isolation procedure, due partially to their complexation with these co-extracted proteins (54,289, 298). Thus other techniques such as gel-filtration or ion-exchange chromatography have been introduced in the initial stages to prevent losses during purification (54,288,297,298). Yet other workers have used different techniques to solubilize the proteoglycans from the tissue (53,187,287,299).

The earliest attempt to extract a proteoglycan from an artery was in 1963 when Buddecke et al. described the properties of a proteoglycan extracted from arterial tissue with water (300). The proteoglycan was shown to have a molecular weight of 71000, to contain 16 to 22% protein and to have chondroitin-6-sulphate as the predominant glycosaminoglycan component. It was able to form large molecular weight aggregates in the presence of divalent cations. Kresse et al. reported the characterization of a bovine thoracic aortic proteoglycan isolated by ethanol precipitation of a phosphate buffer-EDTA

extract (287). By light scattering techniques its molecular weight was calculated to be $2 \times 10^6$ and it was shown to contain about eighty polysaccharide chains per core protein, each having a molecular weight of $2 \times 10^4$. The major glycosaminoglycan constituents were chondroitin and dermatan sulphate with the ratio of chondroitin sulphate to dermatan sulphate being 75:25. In addition, the proteoglycan contained chondroitin sulphate-dermatan sulphate hybrid chains with about 50% of the dermatan units arranged in clusters intercalated between glucuronic acid-containing chains. Antonoupolous et al. extracted a proteoglycan fraction from bovine aorta with 4 M guanidine HCl and then chromatographed it on a DEAE cellulose ion-exchange column in the presence of 6 M urea to remove co-extracted proteins from the preparation (288). The resultant proteoglycans were shown to elute on Sepharose CL-2B with a broad peak, indicating molecular heterogeneity. In addition, they had a lower buoyant density compared to cartilage proteoglycans, probably as a result of having a lower glycosaminoglycan to protein ratio. These workers found 4 M guanidine HCl to be a good extractant, releasing about 85% of the tissue uronic acid in 24 to 36 h. Using thoracic aortas of steers and calves, however, Eisenstein et al. found 3 M guanidine HCl extracted 85% of the tissue uronic acid, only after a 7-day incubation and only about 50% was extracted after 2 days (289). The isolated proteoglycan was smaller than cartilage proteoglycan and similar to that described by Kresse and Antonoupolous et al. (287,288). Ehrlich et al. found 3 M magnesium chloride solubilized more proteo-

glycans from bovine aortas than did 4 M guanidine HCl (53% vs 34% after 24 h) (290). The uronic acid-containing material remaining in the tissue after extraction was almost all released after elastase digestion of the residual tissue. Collagenase did not release residual uronic acid in any significant amounts, suggesting that the insoluble proteoglycans were trapped in a network of elastin fibres. Since elastin is lipophilic, a strong ionic medium might cause compaction of the fibres and increase the binding between the proteoglycans and elastin. The proteoglycan extracted with 3 M magnesium chloride was found to consist of 12% protein with chondroitin-6-sulphate making up the bulk of the glycosaminoglycan component (56%), followed by chondroitin-4-sulphate (20%) and then dermatan sulphate (7%). The molecular weight of the proteoglycan was 72000 and it was shown to contain only four glycosaminoglycan chains per core protein, each having a molecular weight of 15000. The size of this proteoglycan monomer is about one-tenth of that of the proteoglycan described by Kresse *et al.* and other workers, and may reflect degradation of the core protein as the extraction was done in the absence of any proteolytic inhibitors. The size of the glycosaminoglycan side chains was similar to that described by some other workers (287).

The hypothesis that the proteoglycans which were resistant to extraction by dissociative solvents were largely linked to elastin fibres was supported by studies by Radhakrishnamurthy *et al.* (187). They digested bovine aortas with elastase or

collagenase. Collagenase solubilized most of the chondroitin sulphates and some dermatan sulphate and hyaluronic acid but no heparan sulphate. Elastase solubilized most of the heparan and about five times more dermatan sulphate than did collagenase. The release of large amounts of proteoglycan from the nonextractable residue by enzymatic digestion indicated a possible general physical entrapment of proteoglycans in the collagen-elastin network. However, it may also imply that cross-linking between proteoglycans and these matrix components plays an important role in the former's solubility and extractability. Based on the specificity of the enzymes involved, it may be postulated that about 50% of chondroitin sulphate and part of the dermatan sulphate are bound to collagen whereas heparan sulphate is bound to elastin in bovine aorta. A sequential extraction method involving initial extraction with 0,15 M sodium chloride followed by digestion with collagenase and elastase solubilized nearly all of the tissue uronic acid (187). The glycosaminoglycans of the proteoglycan solubilized by 0,15 M sodium chloride contained only chondroitin sulphate; those of the proteoglycan solubilized by collagenase contained chondroitin and dermatan sulphate, whereas those of the proteoglycans solubilized by elastase were largely heparan sulphate and some dermatan sulphate.

Recently Oegema et al. have extracted proteoglycans from the inner one-third of bovine thoracic descending aortas, using the methods outlined previously (Section 1.1.2) for the purification of proteoglycans from cartilage i.e. extraction with

4 M guanidinium HCl in the presence of proteolytic inhibitors and caesium chloride buoyant density centrifugation (291). Only 35% of the total tissue uronic acid was extracted with 4 M guanidinium HCl and 60% of this was recovered in the bottom two-fifths (D1) fraction of a dissociative caesium chloride gradient. A chondroitin sulphate-dermatan sulphate proteoglycan and small amounts of a heparan sulphate proteoglycan were identified. The presence of a keratan sulphate-like moiety was suggested by the presence of sialic acid, fucose and mannose. The major chondroitin sulphate-dermatan sulphate proteoglycan was polydisperse and eluted from Sepharose CL-2B with a $K_{av}$ of 0,26 and had a sedimentation equilibrium molecular weight of 1,5 x $10^6$ to 2 x $10^6$ which is similar to that described by Kresse *et al*. (288). It contained approximately 18% protein and glycosaminoglycans with 46% chondroitin-4-sulphate, 47% chondroitin-6-sulphate and 7% dermatan sulphate. The dermatan sulphate content was similar to that found by Ehrlich *et al* (290). The glycosaminoglycan chains had a molecular weight of about 40000 which is longer than those of cartilage proteoglycan, and the average proteoglycan molecule was shown to have nineteen to twenty-five chains per core protein, molecular weight 2 x $10^5$ to 2,4 x $10^5$. The chondroitin sulphate and dermatan sulphate were shown to exist as copolymeric structures. The addition of exogenous hyaluronic acid to the proteoglycan monomer suggested that about 10% of the total was able to form aggregates in a manner analogous to cartilage proteoglycan monomer. This was assayed by quantitating the material eluting in the $V_o$ region of a

Sepharose CL-2B column before and after hyaluronic acid addition to the proteoglycan monomer sample. However, if the initial extract was dialysed against water to associative conditions (where endogenous hyaluronic acid and proteoglycan can aggregate) and then subjected to associative caesium chloride density gradient centrifugation, about 25% of the material appeared as aggregates in the $V_o$ of a Sepharose CL-2B column, which was higher than was detected by the addition of exogenous hyaluronic acid. Thus at least a portion of the aortic proteoglycan monomers has the ability to form aggregates with hyaluronic acid. McMurtrey et al. used similar techniques to those of Oegema and his coworkers to isolate two proteoglycan-hyaluronic acid complexes from bovine aortas (298). Complex I had a higher buoyant density than Complex II. They both had similar amounts of chondroitin-4- and chondroitin-6-sulphate and dermatan sulphate, but differed in their hyaluronic acid content, Complex II having almost twice as much as Complex I. It was suggested that they contained the same proteoglycan subunit with molecular weight of only 80000, but Complex I contained about 7 to 8 proteoglycans per mole of hyaluronic acid which resulted in a total molecular weight of 800000, whereas Complex II had only 1 or 2 proteoglycans per mole of hyaluronic acid resulting in a molecular weight of about 250000.

Using 4 M guanidinium HCl and DEAE-cellulose chromatography in 6 M urea, Kapoor et al. isolated a proteoglycan from the intimal-medial layer of bovine aorta (297). It contained 23% protein and the carbohydrate moiety consisted of some oligosaccharides and glycosaminoglycans which were made up of chondroitin-6-sulphate and dermatan sulphate, which could

be degraded completely by chondroitinase ABC. The stepwise addition of ethanol, in the presence of calcium ions, was used to fractionate the proteoglycan into one separate proteochondroitin-6-sulphate population and two pools containing dermatan sulphate. The chondroitin-6-sulphate fraction was enriched with keratan sulphate-like glycopeptides, the amino acid composition of its core protein was significantly different to that of the two proteodermatan sulphate fractions and its glycosaminoglycan chains were slightly shorter. The dermatan sulphate pools also contained alternating mixed iduronic acid and glucuronic acid-containing units i.e. they existed as copolymers.

Recent characterization of proteoglycans extracted from human aorta with 4 M guanidinium HCl has shown there to be two major sizes (54). Under dissociative conditions the larger population (PGI) eluted near the $V_o$ of a Sepharose CL-4B column ($K_{av}$ 0,06) and was shown to consist predominantly of chondroitin-6-sulphate. The smaller proteoglycan (PGII) eluted at a $K_{av}$ of 0,38 and here dermatan sulphate was shown to be a major glycosaminoglycan component. Small amounts of heparan sulphate were present in both populations and in the area between the two peaks, indicating that it was very heterogeneous. The glycosaminoglycan chains of both proteoglycans were shown to be of similar size. When PGI was chromatographed under associative conditions, about 20% of the uronic acid appeared in the $V_o$ of a Sepharose CL-2B column, indicating its ability to interact with hyaluronic acid to form large

molecular weight aggregates. In contrast, when PG II was chromatographed under associative conditions no uronic acid was detected in the $V_o$ even after the addition of exogenous hyaluronic acid, indicating that this proteoglycan cannot interact with hyaluronic acid to form the typical aggregates. In another study by the same group, the large molecular weight chondroitin-6-sulphate containing PG I of human aorta was shown to have forty-nine glycosaminoglycan chains per core protein, each chain having a molecular weight of 15000. The molecule consisted of 72% glycosaminoglycan and 28% protein (301).

Breton et al. isolated proteoglycans from the intima-media of pig aortas using a sequential associative (0,15 M sodium acetate) and dissociative (2 M calcium chloride) extraction (299). The associative solvent solubilized about 28% of the tissue uronic acid and the dissociative solvent about 57%, and thus resulted in a good total yield of 85%. Furthermore, by the addition of trace amounts of $|^{35}S|$ sulphate labelled proteoglycan during the extraction procedure and by comparison of its profile on Sepharose CL-2B before and after the preparation they were able to show that no proteolysis had occurred during isolation. As the ionic strength increased the relative amount of chondroitin sulphate extracted decreased, and that of hyaluronic acid and heparan sulphate increased. For both extractions the material eluted in two peaks: at the $V_o$ of a Sepharose CL-2B column and at an included position on both Sepharose CL-2B and CL-6B. The material in the $V_o$ was

associated with hyaluronic acid and was only a small proportion of the total extract - about 16%. On chromatography under dissociative conditions the $V_o$ peak became retarded, indicating that it was in a dissociable complex form. The $V_o$ proteoglycans were also characterized by a relatively higher cysteine, arginine, lysine, aspartic acid and tyrosine content than the included proteoglycan. It has been shown that a greater proportion of these amino acids exists for aggregatable proteoglycans from cartilage, reflecting the presence of the hyaluronic acid binding region and link proteins (30,32). It is thus probable that the excluded proteoglycan represented aggregate and the smaller proteoglycan represented monomers that may not be able to interact with hyaluronic acid. Attempts to reaggregate this monomeric proteoglycan with hyaluronic acid were undertaken but the degree of association was very low.

In general, it has been shown that the proteoglycans/glycosaminoglycans synthesized by aortic cells in short-term organ culture or in tissue culture reflect those found in intact tissue (302-305). Schmidt et al. incubated calf arterial tissue freed of adventitia under organ culture conditions for 24 h in the presence of $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine or $|^{3}H|$ mannose (303). Extraction of the tissue with 4 M guanidinium HCl solubilized 84% of the $|^{35}S|$ sulphate and about 82% of the uronic acid with lesser amounts of $|^{3}H|$ glucosamine (67%) and $|^{3}H|$ mannose (35%). Again most chondroitin sulphate and dermatan sulphate were solubilized but some hyaluronic acid and heparan sulphate remained in the tissue. Chromatography

of the extract on Sepharose CL-4B under dissociative conditions gave two major peaks: at $V_o$ (PGA) and an included peak with a $K_{av}$ of 0,33 (PGB). A minor peak containing heparan sulphate was also obtained. Using ion exchange chromatography, hyaluronic acid with a molecular weight of 81000 was shown to be associated with the excluded peak but not with the included peak. The proteoglycan monomers from the two major peaks were different molecular species with differences in protein content (PGA 10,4% vs. PGB 20,3%), molecular weight (PGA 130000 vs. PGB 190000) and glycosaminoglycan content. PGA was almost all chondroitin sulphate, predominantly chondroitin-6-sulphate, with less than 4% dermatan sulphate, whereas PGB was largely dermatan sulphate (46%) which was copolymeric with chondroitin sulphate. Both contained very little heparan sulphate, less than 3%, and some oligosaccharides (about 16%). The number of glycosaminoglycan chains attached to the core protein averaged three to four for both PGA and PGB, with the average molecular weight of the chains being 35000 for PGA and 39000 for PGB. Both PGA and PGB could interact to some degree with exogenous hyaluronic acid to form aggregates. This supports previously mentioned work on proteoglycans isolated from intact tissue, namely the existence of at least two sizes of proteoglycans containing different glycosaminoglycans and differing in their ability to form aggregates with hyaluronic acid (54,299).

Wagner *et al.* characterized $|^{35}S|$ sulphate and $|^{3}H|$ serine labelled proteoglycans synthesized in short-term organ culture by pigeon

aortas and also found two major sizes on Sepharose CL-4B (304). About 25% of the $|^{35}S|$ sulphate labelled proteoglycans eluted at the $V_o$ and the rest with a $K_{av}$ of 0,44. On caesium chloride centrifugation each proteoglycan preparation was shown to be polydisperse and spread throughout the bottom one-third of the gradient. The polydispersity was assumed to reflect a difference in the number of glycosaminoglycan chains attached to the core protein. By examining the ratios of $|^{35}S|$ sulphate and $|^{3}H|$serine specific activities at different times the two proteoglycans were shown to be synthesized at different rates, the smaller one being synthesized faster. This smaller proteoglycan was shown to contain 34% dermatan sulphate and thus was considered to be similar to those proteoglycans described previously (54,299,303), while the larger proteoglycan probably consisted largely of chondroitin-6-sulphate. Thus it seemed possible that dermatan sulphate was synthesized faster than chondroitin sulphate.

Horn *et al.* isolated $|^{35}S|$ labelled proteoglycans from smooth muscle cells of pig thoracic aortic media (305) using the sequential extraction procedure of 0,15 M sodium acetate followed by 2 M calcium chloride described by Breton *et al.* (299). The pattern found for the cell-synthesized glycosaminoglycans was essentially the same as that described for the glycosaminoglycans isolated from the intima-media of pig aorta. The results also suggested the presence of at least two subunit proteoglycan species possibly differing in glycosaminoglycan composition and in their ability to form complexes. About

10% of the proteoglycans from the smooth muscle cells were excluded from a Sepharose CL-2B column under associative conditions compared to 16% from the intima-media tissue. Further interaction of the included proteoglycan with exogenous hyaluronic acid gave very low levels of aggregation - less than 3%.

Wight and Hascall studied proteoglycans synthesized by arterial smooth muscle cells from intimal-medial strips of one to two year old pigtail monkeys (281). After 48 h of labelling with $|^{35}S|$ sulphate, 70 to 80% of macromolecular sulphated molecules were found in the medium. Proteoglycans isolated from the medium and cell layer by 4 M guanidine HCl extraction gave quite similar profiles on Sepharose CL-2B, both containing two major peaks of $|^{35}S|$ sulphate radioactivity at $K_{av}$'s of 0,31 and 0,61 for the medium, and 0,31 and 0,78 for the cell layer proteoglycans, respectively. The smaller proteoglycans ($K_{av}$ 0,61 and 0,78) did not arise from degradation of the larger species since with increased time of labelling the larger peak increased relative to the smaller peak. Caesium chloride density gradient centrifugation showed the proteoglycan molecules from both the media and cell layer to be polydisperse. The larger proteoglycans were predominantly present in the denser fractions but no single population was restricted to any one fraction. On analysing the glycosaminoglycan moieties, after papain digestion or alkaline degradation, the chains were shown to be the same size for proteoglycans from all peaks with a molecular weight of about 40000. Thus

the polydispersity must arise as a result of a different number of chains being attached to the core protein. The large proteoglycan species from both the medium and cell layer were shown to contain 90% chondroitin sulphate and the smaller proteoglycan species were shown to contain 20 to 30% dermatan sulphate, thus agreeing with most previous work on proteoglycans from intact tissue (54,299). When medium and cell layer proteoglycans were extracted under associative conditions only about 5 to 10% of $|^{35}S|$ sulphate radioactivity was excluded from a Sepharose CL-2B column, indicating that the majority of the proteoglycans synthesized and secreted by these cells are not already bound in aggregate structures. This was confirmed by electron microscopy of an associative preparation which showed no evidence of individual monomers aggregating into larger structures. However, if the proteoglycans prepared under dissociative conditions from the medium and cell layer were combined with an excess of carrier prepared associatively, and then returned to associative conditions by dialysis, aggregation was shown to occur with the larger proteoglycan species but not with the smaller populations. Thus it was the large proteoglycan molecule, which contained mostly chondroitin sulphate that could aggregate with hyaluronic acid. Electron microscopy studies also showed that the monomers contained about eight to fourteen glycosaminoglycan side chains per core protein molecule and that it was about one-half to one-third the length of the core protein of cartilage proteoglycan.

Thus several conflicting results on molecular size and structure of aortic proteoglycans have been reported. In general with aortic tissue an average of about half of the total tissue uronic acid is extracted by the various workers (289-291), with a few reports of much higher yields (289,299). The chondroitin sulphate and part of the dermatan sulphate are extracted from the tissues fairly easily but some of the hyaluronic acid and most of the heparan sulphate remain behind in the tissue after extraction with salt solutions, and need to be solubilized by digestion of the fibrous components of the matrix, particularly elastin (187,290). The aortic extracted proteoglycans comprise about 20% protein and contain most types of glycosaminoglycans with condroitin sulphate usually making up the bulk of the carbohydrate moiety. Keratan sulphate has not been conclusively shown to occur in aortic tissues although its presence is suggested by the presence of sialic acid, fucose and mannose, and by digestion with specific endo-β-galactosidase (291,306). The size of the proteoglycan monomer is thought to be slightly smaller than that of cartilage, with the core protein being shorter but the glycosaminoglycan chains being longer than those of the cartilage proteoglycan (281,291,297,303). There is great discrepancy in the reported number of glycosaminoglycan chains attached per protein core with values varying between four and eighty (281,287,290,291,301). In general, at least two different types of proteoglycans appear to exist in the aorta, differing in their glycosaminoglycan composition and in their ability to interact with hyaluronic acid (54,299,303,305).

The larger species usually contains predominantly chondroitin sulphate - largely chondroitin-6-sulphate - and is able to interact with hyaluronic acid, and the smaller proteoglycan contains a large amount of dermatan sulphate with dermatan sulphate and chondroitin sulphate occurring, in part, as copolymers. This species does not seem to be able to form large molecular weight aggregates with hyaluronic acid. In addition, an even smaller proteoglycan species containing predominantly heparan sulphate has been reported by some workers (53,291). Thus it seems as if aortic proteoglycans may contain only one type of glycosaminoglycan chain attached to a core protein, unlike cartilage proteoglycans which have different glycosaminoglycan chains attached to a single core protein in specifically localized areas (15).

As can be seen from the results of several authors, at least some of the proteoglycans from aorta can interact with hyaluronic acid (54,291,298,299). However, the proportion that has been shown to be able to do so is very low compared to the proportion of cartilage proteoglycan monomers that can form aggregates. Cartilage proteoglycan monomer has been shown to have a definite hyaluronic acid-binding region of the core protein which has a specific globular conformation and is rich in certain amino acids (see Section 1.1.2). It has not been established whether such a region exists in aortic proteoglycan monomer. Breton *et al*. showed that the core protein of a proteoglycan from pig aorta, that was able to form aggregates to some degree, was also rich in the amino

acids cysteine, arginine, lysine, aspartic acid and tyrosine (299) which are the amino acids that have been shown to be present in the hyaluronic acid-binding region of the cartilage proteoglycan core protein (30,32). The smaller proteoglycans described by several workers which are unable to form complexes with hyaluronic acid may lack such a binding region. Using immuno-diffusion techniques, Gardell et al. showed that when aortic proteoglycans prepared under dissociative conditions were interacted with antisera prepared against cartilage proteoglycan monomer or against its hyaluronic acid-binding region, a precipitin line was formed, indicating structural similarities between the proteoglycans from aorta and cartilage and thus suggesting that aortic proteoglycan should be capable of forming specific aggregates with hyaluronic acid (307). In cartilage the interaction between proteoglycan monomer and hyaluronic acid is stabilized by the existence of link proteins (43,44). Clear evidence for such species in aortic tissue has not been presented to date. Breton et al. digested their larger and smaller proteoglycan species with chondroitinase ABC and analysed the core proteins on SDS PAGE (299). Both yielded a protein band with a molecular weight of over 300000. In addition, the larger species yielded a few additional bands (about 5% of the total sample protein) which migrated within the gel at the position expected for link protein (308). Gardell et al. also identified link protein from aorta from a low density position after centrifugation of an associative preparation of aortic proteoglycan under dissociative conditions (307). It was identified by cross-reacting with antiserum to

cartilage link protein. The results of Mangkornkanok-Mark et al., also using immunological studies (309), do not entirely support those of Gardell et al. When reacting bovine aorta with antibodies to cartilage proteoglycan monomer and vice versa they found very weak cross-reaction and suggested that possibly the aortic and cartilage core proteins do share some weak antigenic determinants and have some slight similarities, but there are also marked differences between them.

Thus it appears that although some aortic proteoglycans can interact with hyaluronic acid, this interaction and resultant aggregate formation is not as important as in cartilage tissue. In cartilage the aggregation between proteoglycan and hyaluronic acid is thought to be of prime importance in the 'holding' of the proteoglycan within the extracellular matrix. In aorta perhaps the proteoglycan-hyaluronic acid interaction does not have such an important role. There may be other more important ways of trapping the proteoglycans within the matrix such as interaction with the fibrous proteins of the matrix, since such interactions are known to occur (Section 1.4.1.). In addition, several workers have reported the existence of chondroitin sulphate-dermatan sulphate hybrid chains in the aorta (239,287,291). As discussed in Section 1.4.3. these hybrid chains associate with each other and result in the formation of a complex which may have important functions in the aorta. The self-association displayed by copolymeric chondroitin sulphate-dermatan sulphate glycosaminoglycan side chains is particularly sensitive to shear forces (239).

Thus they might physically separate as pressure increases and might endow the matrix with a marked resistance towards compression and deformation.

### 1.6.2. Metabolism of arterial proteoglycans

The aortic proteoglycans are highly heterogeneous and thus the synthesis and turnover of the various different proteoglycan species may be subject to different metabolic controls. The cells in the arterial wall are responsible for the synthesis and secretion of the components of their extracellular connective tissue. Wight and Ross initially studied the synthesis and secretion of proteoglycans by arterial smooth muscle cells in culture (310). Synthesis and secretion was shown to be maximal during the stationary phase of growth. They showed that significant amounts of sulphated glycosaminoglycans were secreted into the culture medium and thus only this compartment was analysed. They found much higher amounts of dermatan sulphate than had been reported previously by other workers (282,284,294). They suggested that this might be a response by the cells to some form of mechanical or chemical stress, or perhaps it might reflect some particular state of development of the cells. Dermatan sulphate has been demonstrated to be endocytosed more rapidly than other glycosaminoglycans (167), and has also been shown to exhibit the most rapid turnover of all the glycosaminoglycans in the rat aorta (311). Thus, even though arterial smooth muscle cells synthesize large quantities of dermatan sulphate, its rapid rate of degradation in non-injured arteries may prevent its accumulation.

Gamse et al. studied the synthesis and secretion of proteoglycans in endothelial cells and in smooth muscle cells derived from the endothelium, intima and media of the same aorta (306). They showed that both the endothelial and smooth muscle cells distribute newly synthesized glycosaminoglycans into the extracellular, pericellular and intracellular compartments but there was a unique pattern of distribution in each compartment for each cell type. They found that endothelial cells synthesized a lower amount of glycosaminoglycan - they only incorporated about one-third of the $|^{35}S|$ sulphate incorporated by smooth muscle cells on the basis of cell number. Both endothelial and smooth muscle cells synthesized chondroitin-4- and chondroitin-6-sulphate, dermatan sulphate, heparan sulphate and small amounts of keratan sulphate, and these were distributed to the various compartments. The distribution of glycosaminoglycans in the endothelial compartments was different to that in the intimal and medial compartments which had a fairly similar glycosaminoglycan distribution. In general, keratan sulphate was not found in the pericellular compartment and heparan sulphate was generally higher in the pericellular and intracellular pools than in the extracellular pool. Dermatan sulphate made up only a small percentage of the pericellular pool but in the cells relatively small amounts of chondroitin-4- and chondroitin-6-sulphate were found. The endothelial cells formed significant quantities of heparan sulphate but considerably less dermatan sulphate, which was predominantly synthesized by the smooth muscle cells. In addition, the heparan sulphate from

endothelial cells differed significantly from that of the smooth muscle cells, since it was larger, had a higher charge density and a greater degree of N-sulphation.

Vijayagopal et al. studied the synthesis and secretion of proteoglycans by bovine aortic tissue in organ culture (312). Proteoglycans were extracted from the tissue by 4 M guanidine HCl followed by sequential collagenase and elastase digestion. The highest content of radioactive sulphated material was noted in the guanidine HCl-extracted proteoglycan, and the least radioactive sulphate was incorporated into the proteoglycan preparation obtained by elastase digestion of the tissue, which may well be due to the presence of large amounts of hyaluronic acid in this fraction. In the culture medium the material with the highest specific radioactivity was heparan sulphate, followed by chondroitin sulphate, then dermatan sulphate. Dermatan sulphate from the preparation obtained by elastase digestion had a lower specific radioactivity than dermatan sulphate obtained from the other extracts, whereas the highest specific radioactivity for heparan sulphate was noted in the elastase-solubilized proteoglycan. The specific radioactivity of the chondroitin sulphate chains in all three preparations from the tissue was considerably lower than those of the other glycosaminoglycans. It was evident from this study that there are differences in the uptake of $|^{35}S|$ sulphate by the different glycosaminoglycan chains of the tissue proteoglycans and in the release of the proteoglycans by the cells into the medium. The very high

specific radioactivity of dermatan sulphate in the tissue suggested that these chains were synthesized relatively faster than the chondroitin sulphate chains. These observations cannot be satisfactorily explained by variations in the rate of sulphation, as sulphation proceeds during or immediately following polymerization (Section 1.2.3.3). Rather there are differences in the rates of synthesis.

The differences in the specific radioactivities in the medium glycosaminoglycans suggested that they were released into the medium at different rates, with heparan sulphate, which was primarily synthesized by the endothelial cells being released faster than dermatan sulphate which was primarily synthesized by the smooth muscle cells (306). The differences in rates of synthesis of different glycosaminoglycans supported earlier work by Kresse and Buddecke, who studied the distribution of $|^{14}C|$ glucose and $|^{35}S|$ sulphate-labelled glycosaminoglycans in calf aortic tissue maintained in organ culture (313). In these studies, following incubation, the aortic tissue was digested with papain and the total glycosaminoglycans were quantified by electrophoresis. The highest rate of synthesis was reported for hyaluronic acid, decreasing for the sulphated glycosaminoglycans in the order dermatan sulphate followed by heparan sulphate followed by chondroitin sulphate. Glycosaminoglycans released into the medium were not studied by these investigators.

The metabolic heterogeneity of proteoglycans from the aortic

wall was also reported by Deudon and Picard (314). They incubated intima-media slices of pig aorta in organ culture and then extracted the tissue with increasing concentrations of guanidine HCl (0,4 M, 4 M then 8 M). They found that the different proteoglycan extracts were very heterogeneous in their rates of precursor uptake. They suggested that the proteoglycans extracted by 8 M guanidine HCl and those remaining in the tissue after extraction (20%) might be tightly bound to cellular structures, whereas those proteoglycans extracted with 0,4 M and 4 M guanidine HCl were associated with the matrix. Pulse-chase experiments by these workers gave some evidence of metabolic relationships between the different proteoglycans and showed that the more insoluble proteoglycans might be precursors of those from the soluble pools, a finding which favours the hypothesis of similar intracellular locations for polymerization and sulphation.

Von Figura *et al*. have shown that metabolic heterogeneities exist in the chondroitin sulphate-dermatan sulphate hybrids found in arterial tissue (315). By stepwise ethanol precipitation they were able to isolate hybrids that are rich in either chondroitin sulphate or dermatan sulphate. After incubating arterial tissue segments with $|^{14}C|$ glucosamine or $|^{14}C|$ glucose they showed that the specific radioactivity of the dermatan-rich hybrids was twice that of the chondroitin-rich hybrids. The specific radioactivity of the disaccharides from dermatan-rich hybrids was also twice that of those from the chondroitin-rich hybrids, but within each fraction the

galactosamine moieties of the chondroitin and dermatan sulphate units and their glucuronic or iduronic acid units exhibited equal specific radioactivity. This supported the theory that epimerization of the carbon-5 in glucuronic acid to form iduronic acid occurs after polymerization to the growing end of the glycosaminoglycan chain. Distinct compartments exist for the formation of hybrid chondroitin sulphate-dermatan sulphate proteoglycans with different proportions of chondroitin sulphate and dermatan sulphate. These findings supported earlier studies by Kresse et al. which provided evidence for the existence of metabolic heterogeneity of chondroitin sulphate and dermatan sulphate units in copolymeric chains from aorta (288). They had shown that the specific radioactivity of the sulphate ester group of dermatan sulphate units was about three times higher than that of the chondroitin sulphate units in a chondroitin sulphate-dermatan sulphate hybrid.

Several workers studying proteoglycans in intact tissue have reported the existence of a large proteoglycan consisting predominantly of chondroitin sulphate and able to interact with hyaluronic acid and of a chondroitin sulphate-dermatan sulphate proteoglycan which is smaller and is unable to form aggregates (54,299). Pulse-chase experiments with [$^{35}$S] sulphate suggested that both forms were primarily produced as monomers and the chondroitin sulphate proteoglycan was subsequently converted to a complex on interaction with hyaluronic acid (303). Wagner et al. suggested that the rates of synthesis

of the two types of proteoglycan were different - that proteoglycan monomer containing dermatan sulphate was synthesized faster than that containing chondroitin sulphate (304).

Gamse et al. showed that radioactive sulphate was incorporated into macromolecular material in the intracellular and pericellular compartments of endothelial and smooth muscle cells in a hyperbolic manner, indicating the achievement of metabolic equilibrium between the entry of labelled macromolecules into the particular pool and their disappearance from it (306). The half-lives of the proteoglycans in the intracellular and pericellular pools were much shorter than those in the extracellular pool which did not reach equilibrium within the 72 h experimental period.

The decay curves of the proteoglycans in the intra- and pericellular pools were complex and indicated the presence of more than one component (306). Endothelial cells exhibited somewhat faster polysaccharide decay than did the smooth muscle cells. Almost 50% of the labelled macromolecules disappeared from the endothelial cells in the first 3 h and there was a component with a slower rate of decay which had a half-life of about 22 h. For smooth muscle cells the half-lives of the components of the intracellular compartment have been calculated to be about 6 h and 72 h. In the pericellular compartment of endothelial cells a minor component exhibited a rapid turnover with a half-life of 6 h and

approximately 60% had a half-life of 14 h, whereas a half-life of 60 h was calculated for the pericellular glycosaminoglycans of the smooth muscle cells. Intracellular proteoglycans disappear by exocytosis or by degradation and pericellular proteoglycans by receptor-mediated endocytosis into the cell or via shedding to the medium. Gel chromatography of intracellular glycosaminoglycans showed them to be smaller than alkali-degraded proteoglycans from the other pools, and in combination with other results obtained the authors concluded that only a small part of intracellular glycosaminoglycans were in the process of biosynthesis and secretion (306). Gel chromatography of labelled material from the extracellular compartment gave rise to the expected elution profiles for proteoglycans. The proteoglycans of the extracellular pool which have been shown to have a glycosaminoglycan composition resembling that of proteoglycans from intact tissue have a much slower rate of decay with a half-life of about 10 days calculated by some workers (315).

Thus, the synthesis, secretion and turnover of aortic proteoglycans are complex processes with the existence of different compartments, each consisting of different pools which exhibit metabolic heterogeneity. It is not yet known whether endothelial cells secrete glycosaminoglycans in vivo and, if so, whether they do so to the lumen or towards the side of the basal membrane, or both. In other words, do the glycosaminoglycans synthesized by the endothelial cells contribute to the whole glycosaminoglycan composition of the endothelial layer and do these cells also influence glycosaminoglycan

secretion by the smooth muscle cells? The proportions of the different glycosaminoglycans secreted by the different cells may reflect the variety of functions for separate layers e.g. the high proportion of heparan sulphate synthesized by endothelial cells may have antithrombin activity whereas the dermatan sulphate, primarily synthesized by the smooth muscle cells, may be important in the organization of collagen in the connective tissue and in the binding of cationic polypeptides and plasma lipoproteins. The synthesis of the different glycosaminoglycans is most likely subjected to different metabolic controls and injury of the artery may disrupt this control, leading to the accumulation of those glycosaminoglycans such as dermatan sulphate which are found associated with atherosclerotic lesions (233). Knowledge of the metabolism of both endothelial and smooth muscle cells under normal and pathological conditions will be very valuable for a better understanding of the pathophysiology of vascular diseases.

# CHAPTER 2

# CHARACTERIZATION OF BOVINE AORTIC MEDIAL PROTEOGLYCANS

## 2.1. INTRODUCTION

The importance of proteoglycans as constituents of the intercellular matrix of the arterial wall has been alluded to already in Chapter 1. Although various glycosaminoglycans, isolated from aorta, have been studied quite extensively in relation to their possible role in the pathology of atherosclerosis, only recently have attempts been made to study the nature of native proteoglycans isolated from arteries. Such studies are important as the functional properties of the proteoglycans are derived from the structure of the molecule as a whole and not only from the component parts.

The studies performed on intact proteoglycans isolated from arteries from different sources have yielded conflicting results. As explained previously, this was partly due to differences that arose with material from different sources. Several workers have documented the differences in glycosaminoglycan composition from aortas of different species and it has also been shown that the glycosaminoglycan composition varies in different regions of the aorta (289,292,293,295). Using cells in culture, Gamse *et al*. demonstrated that the endothelial and smooth muscle cells of bovine aortas have distinct patterns of glycosaminoglycan synthesis (306). Furthermore, glycosaminoglycan patterns change during maturation

and in diseased states (233,282,289,293,294). Changes in the proteoglycan and glycosaminoglycan composition with age have been well studied in cartilage (264-271,273) but not in any great detail for proteoglycans from the aorta.

Here I will describe the procedures used to extract and characterize proteoglycans from bovine aortas of different ages. Methods derived for the preparation of cartilage proteoglycans were essentially employed (30,31), and to avoid variabilities mentioned already the source of aortic material used for each extraction procedure was carefully identified using histological techniques. In order to minimize differences arising as a result of the site of origin of the tissue, only those proteoglycans isolated from the medial layer of the thoracic descending aorta in all age groups have been studied. The medial proteoglycans of foetuses, neonates and adults were characterized in terms of their molecular sizes, the abiility of the monomers to aggregate with hyaluronic acid and the types of glycosaminoglycan chains present.

The data presented in this chapter were also fundamental to the studies described later, where cultured bovine smooth muscle cells from "pure" medial tissue were used to study metabolic aspects of bovine proteoglycans.

## 2.2. METHODS

### 2.2.1. Tissue collection and histology

Bovine thoracic descending aortas from foetuses in the first trimester of foetal life, neonates or adults, were obtained from the local abattoir immediately after slaughter and transported to the laboratory on ice. The intima and adventitia were dissected away and the medial layer was cut into fine pieces. Representative tissue samples were retained for histological identification each time material was extracted. Samples were fixed in 4% phosphate buffered formalin. After dehydration and embedding in wax, sections of each sample were cut with a microtome. These sections were then dewaxed, stained in haematoxylin and eosin and mounted. Tissue samples were also analysed for their content of water, uronic acid and protein, or were subjected to the extraction procedures for proteoglycans as detailed below:-

### 2.2.2. Analysis of water, uronic acid and protein contents

(a) The water contents of weighed tissue samples were determined by freeze drying them repeatedly until the weight of the dry tissue remained constant.

(b) Protein contents were determined in weighed tissue samples following homogenization in water and precipitation with equal volumes of 10% trichloroacetic acid at $4^{o}C$. After centrifugation, the pellets were washed with 96% ethanol and total protein determined using the Biuret method (316).

(c) Tissue uronic acid contents were estimated on samples of

known wet weights suspended by homogenisation in a solution containing 0,1 M sodium acetate and 0,05 M EDTA, pH 7,0. Samples were digested by two sequential additions of papain (1 mg) at 24 h intervals. Digestion was carried out at 65°, and after 48 h, at which stage all tissue was completely digested, two volumes of absolute ethanol containing 0,05 M potassium acetate were added to precipitate glycosaminoglycans. After standing overnight at -15°C, precipitates were collected and taken up in 0,5 M sodium acetate, pH 6,8, and uronic acid concentrations were then determined by the method of Bitter and Muir (317).

2.2.3. Extraction procedure

The extraction procedure was a modification of the procedure previously devised for the extraction of proteoglycans from cartilage (30,31). The tissue was extracted with 4 M guanidine HCl (1 to 5g / 10 ml) in the presence of 0,05 M sodium acetate, 0,1 M aminocaproic acid, 0,005 M benzamidine hydrochloride, 0,01 M EDTA and 0,005 M phenylmethylsulphonylfluoride, all at pH 5,8, for 48 h at 4°C with constant stirring. Extracts were clarified by filtration through glass wool and subsequent centrifugation. At this stage, extracts were divided into two portions: the major part was used for the dissociative preparation of proteoglycan monomers (PGM) and the rest for an associative preparation (PGA). Under dissociative conditions using high molarity salt solutions, aggregate formation between the PGM and hyaluronic acid is inhibited.

2.2.3.1. Dissociative preparation

Solid caesium chloride (0,59 g/g solution) was added to the clarified extract to give an initial density of 1,518. The extract was centrifuged at 40 000 rpm at 10°C for 48 h in a Beckman type 65 rotor, in a Beckman Ultracentrifuge-Model L5-65 or L8-70. Routinely, gradients were separated into two fractions using a Beckman tube cutter, to obtain the top 4/5 and bottom 1/5 of the tube contents. Densities were determined by weighing 200 µl of each fraction in a constriction pipette on a Mettler microbalance. The fractions were dialysed with five changes against 0,5 M potassium chloride (5 ℓ) at 4°C. Dialysis tubing (molecular weight cut-off of 12000) was routinely processed before use by boiling in a solution of 0,005% (w/v) EDTA containing 0,2% (w/v) sodium bicarbonate. The dialysed samples were then freeze-dried and stored at -15°C.

2.2.3.2. Associative preparation

Proteoglycans were prepared under associative conditions by dialysing the initial clarified extract against 10 volumes of water containing protease inhibitors. The dialysed material was freeze-dried and extracted in associative buffer (0,4 M guanidine HCl, 0,05 M sodium acetate, pH 5,8 plus all the proteolytic inhibitors described above). Caesium chloride (0,3 g/g solution) was added to give an initial density of 1,236. The extracts were centrifuged at 40 000 rpm at 10°C for 48 h in a Beckman type 65 rotor, in a Beckman Ultra-

centrifuge-Model L5-65 or L8-70. Gradients were fractionated as described above to yield three samples of equal volume. The samples were dialysed at 5°C against water containing inhibitors. Samples were then freeze-dried and stored at -15°C.

2.2.4. Digestion of PGM

PGM from material of each age was subjected to the following enzymatic digestions and treatments:

(a) Papain digestion: 1,8 mg of PGM dissolved in 0,1 M sodium acetate containing 0,05 M EDTA and 0,005 M cysteine, pH 7,0, were digested with papain at a PGM to enzyme ratio of 40:1. The digestion was carried out at 65°C for 6 hr. After digestion, the samples were boiled to destroy the enzyme and clarified in a Beckman microfuge.

(b) Alkaline borohydride treatment: About 5 mg PGM dissolved in a solution of 1 M sodium borohydride in 0,05 M sodium hydroxide was flushed with nitrogen and incubated at 37°C for 48 h (267,318). Acetic acid (4 to 5 drops/ml) was added dropwise to stop the reaction. If the resultant bubbling was very vigorous, 1 drop of octanol was added to reduce the surface tension. Samples were freeze-dried, desalted on Biogel P-2 columns and freeze-dried again.

(c) Digestion with chondroitinase ABC lyase (EC.4.2.2.5)(39), AC lyase (EC.4.2.2.5) and with keratinase: 3,8 to 4,0 mg PGM, dissolved in 0,1 M Tris acetate, pH 7,3, were digested

with 0,2 units of enzyme at 37°C for 3 h.

(d) Digestion with trypsin and chymotrypsin: After digestion with chondroitinase ABC, samples were sometimes digested with trypsin for 90 min followed by chymotrypsin for a further 90 min. Both digestions were carried out at 37°C, with a PGM:enzyme ratio of 100:1.

(e) Digestion of heparin and heparan sulphate: PGM samples, containing about 500 µg of uronic acid in 200 µl buffer, were treated with 25 µl of glacial acetic acid and 25 µl of 18% sodium nitrite for 90 min at room temperature (320).

(f) Digestion with hyaluronidase (from streptomyces hyaluroliticus, E.C.3.2.1.35): Proteoglycan samples were dissolved in 0,1 M sodium acetate containing 0,65 M sodium chloride pH 6,0, and digested with hyaluronidase (1 mg/mg substrate) for 3 h at 37°C (321).

2.2.5. Interaction with hyaluronic acid

Samples of PGM isolated dissociatively were incubated with hyaluronic acid at a uronic acid:hyaluronic acid ratio of 33:1 by weight, in 600 µl of 0,5 M sodium acetate buffer, pH 7,0, and dialysed for 24 h against the same buffer (291). The source of hyaluronic acid was a commercial preparation which was first chromatographed on Sepharose CL-2B. Material corresponding to a $K_{av}$ of 0,3 or larger was used.

2.2.6. Column chromatography

Columns of Sepharose CL-2B (0,6 x 110 cm or 1,6 x 130 cm),

Sepharose CL-6B (0,8 x 110 cm) or Biogel P-30 (100-200 mesh) (0,8 x 110 cm) were equilibrated with 0,5 M sodium acetate pH 7,0 at room temperature and used to assess the proteoglycan samples prepared as described above. Usually samples (1 to 5 mg) were loaded in about 300 µl equilibration buffer. Column flow rates were approximately 4 ml/h and fractions (0,8 ml) were analysed for hexuronic acid, protein or sialic acid contents by the procedures to be described. The $V_o$ of the columns was determined using aggregated proteoglycan isolated from bovine nasal septum, and the $V_t$ by using radioactive $|^{35}S|$ sulphate. Columns (1,2 x 28 cm) of Biogel P-2 (100-200 mesh) eluted with water were used to desalt samples of proteoglycan after the various treatments.

2.2.7. Ion-exchange chromatography

Columns (1,0 x 6 cm) of DEAE-cellulose were equilibrated with 0,05 M sodium acetate pH 4,0. Samples to be analysed were dissolved in appropriate volumes of the equilibration buffer and applied to the columns in a volume of approximately 1 ml. Columns were eluted initially (10 fractions) with equilibration buffer and subsequently with a linear gradient of lithium chloride (0,2 M to 1,5 M in 0,05 M sodium acetate pH 4,0). The fraction size was approximately 880 µl and the flow rate was about 25 ml/h.

2.2.8. Glycosaminoglycan analysis

The identification and quantitation of the glycosaminoglycan side chains were performed on PGM samples which were papain-

Sepharose CL-6B (0,8 x 110 cm) or Biogel P-30 (100-200 mesh) (0,8 x 110 cm) were equilibrated with 0,5 M sodium acetate pH 7,0 at room temperature and used to assess the proteoglycan samples prepared as described above. Usually samples (1 to 5 mg) were loaded in about 300 µl equilibration buffer. Column flow rates were approximately 4 ml/h and fractions (0,8 ml) were analysed for hexuronic acid, protein or sialic acid contents by the procedures to be described. The $V_o$ of the columns was determined using aggregated proteoglycan isolated from bovine nasal septum, and the $V_t$ by using radioactive $|^{35}S|$ sulphate. Columns (1,2 x 28 cm) of Biogel P-2 (100-200 mesh) eluted with water were used to desalt samples of proteoglycan after the various treatments.

### 2.2.7. Ion-exchange chromatography

Columns (1,0 x 6 cm) of DEAE-cellulose were equilibrated with 0,05 M sodium acetate pH 4,0. Samples to be analysed were dissolved in appropriate volumes of the equilibration buffer and applied to the columns in a volume of approximately 1 ml. Columns were eluted initially (10 fractions) with equilibration buffer and subsequently with a linear gradient of lithium chloride (0,2 M to 1,5 M in 0,05 M sodium acetate pH 4,0). The fraction size was approximately 880 µl and the flow rate was about 25 ml/h.

### 2.2.8. Glycosaminoglycan analysis

The identification and quantitation of the glycosaminoglycan side chains were performed on PGM samples which were papain-

digested and then subjected to the various digestions with chondroitinase ABC, AC, hyaluronidase or nitrous acid as described above. The digests were chromatographed on a Sephadex G-50 (medium) column (1,0 x 120 cm) equilibrated with 0,2 M pyridine acetate, pH 5,0 (297). The flow rate was approximately 8 ml/h and fractions (1 ml) were assayed for hexuronic acid to assess the percentage in the void volume (i.e. undigested material) before and after digestion with the various specific enzymes.

2.2.9. Thin layer chromatography

Thin layer chromatography was used to separate disaccharides formed on digestion of glycosaminoglycan chains. Approximately 50 µg of material was spotted in each case onto cellulose-coated thin layer plates. The plates were desalted by ascending chromatography in 1-butanol:absolute ethanol:water 52:33:15 (v/v/v) at room temperature for 2 h, and then after drying subjected to further ascending chromatography in glacial acetic acid:1-butanol:1 N ammonium hydroxide 6:4:3 (v/v/v), or butyric acid:0,05 M ammonium hydroxide 5:3 (v/v) at room temperature for approximately 4 h (322). The plates were dried and then stained in 0,1 (w/v) toluidine blue in absolute ethanol.

2.2.10. Analytical procedures

Protein was determined by the method of Lowry et al. using bovine serum albumin as standard (323). Hexuronic acid was determined by the automated procedure of Bitter and Muir using D-glucuronolactone as standard (317), and sialic acid was

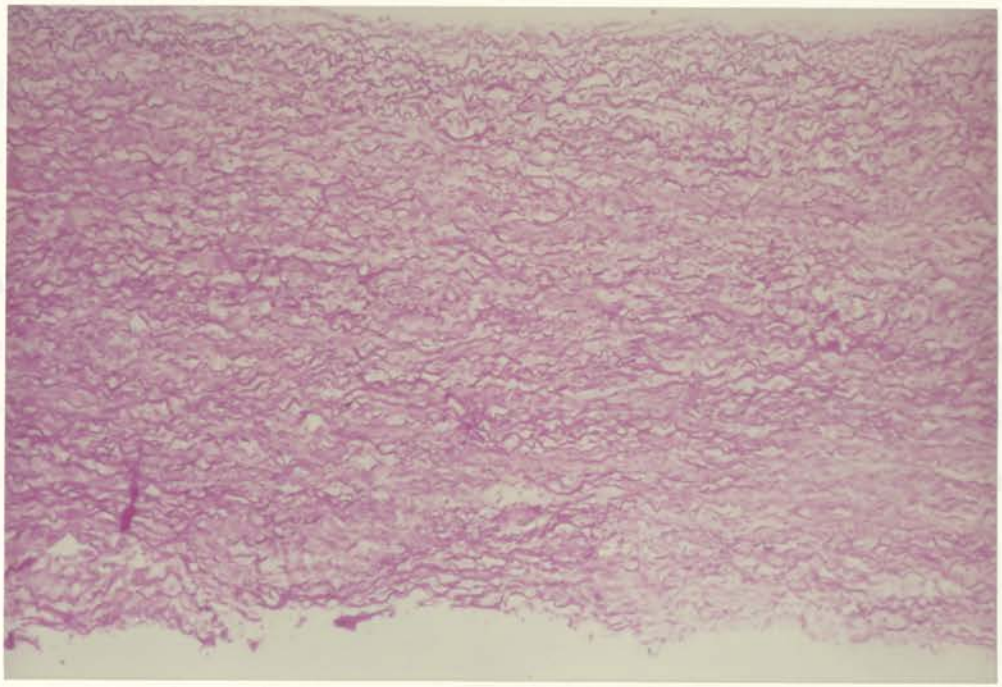
estimated by the periodate-resorcinol procedure using N-acetyl neuraminic acid as standard (324). Sugar analysis for the determination of the glycogen content of proteoglycan preparations was performed on freeze-dried material prepared from bovine medial tissue, obtained from different aged animals, which was subjected to hydrolysis by 2 M trifluoroacetic acid at 100°C for 15 h. The material was prepared from proteoglycans purified by dissociative caesium chloride centrifugation and chromatography on Sepharose CL-2B. The individual peaks from the Sepharose chromatography were treated with papain and resolved into separate charged glycosaminoglycan species by DEAE-cellulose column chromatography (Section 2.2.7). The individual species from the ion-exchange column were freeze-dried, desalted and hydrolysed as described above. After hydrolysis and purification, liberated sugars were converted to alditol acetate derivatives and analysed on a Carlo Erba 420 Gas Liquid Chromatograph. Standard sugars were used to calibrate the column and the glucose content of the unknown samples quantitated using flame detection.

## 2.3. RESULTS

Histological examination of the material used for the isolation of proteoglycans clearly showed it to consist only of medial tissue made up of smooth muscle cells and adherent connective tissue (Plate 2.1).

The water, uronic acid and protein contents of the medial tissues from the different age groups is shown in Table 2.1.

A.



B.

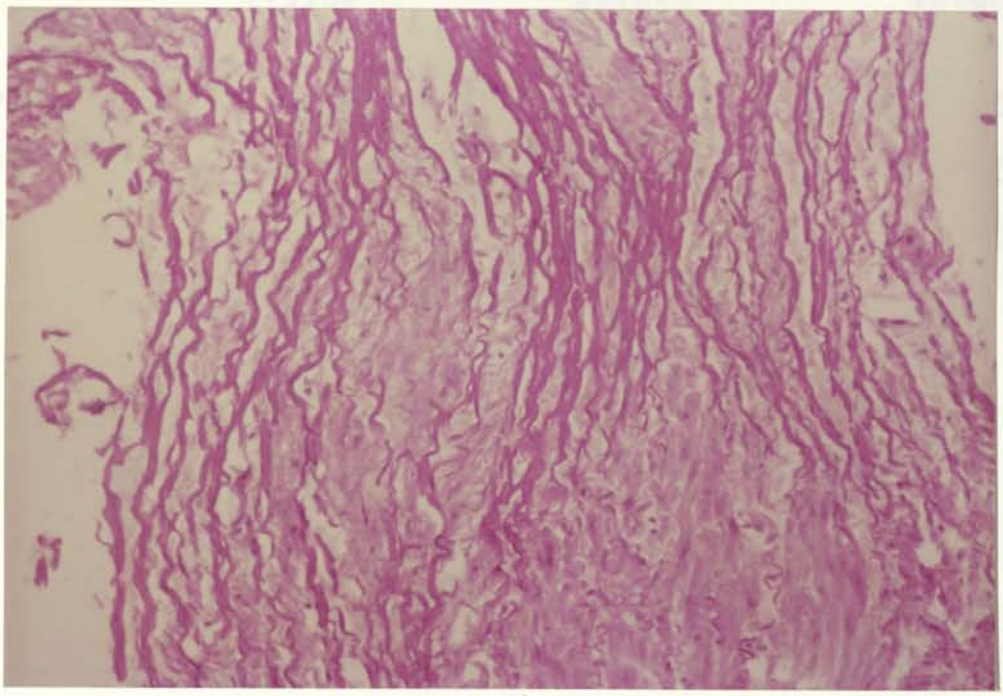


Plate 2.1. Histology of aortic medial tissue.

Samples of adult aortic tissue were fixed in 4% phosphate buffered formalin and stained in haematoxylin and eosin as described in Section 2.2.1.

Magnification A x 35
B x 100

TABLE 2.1

THE WATER, PROTEIN AND URONIC ACID CONTENTS OF AORTIC MEDIAL TISSUE FROM DIFFERENT AGE GROUPS

Samples of bovine aortic media from different aged animals were analysed for their total content of water, protein and uronic acid as described in Section 2.2.2. The data represent the mean ± S.D. for six determinations for one of two similar experiments.

| | Water g/100 g wet tissue | Protein mg/mg dry weight | Uronic Acid µg/mg dry weight |
|---|---|---|---|
| Foetus | 84,36 ± 1,74 | 0,754 ± 0,03 | 27,33 ± 4,56 |
| Neonate | 81,00 ± 1,52 | 0,773 ± 0,06 | 18,70 ± 2,00 |
| Adult | 76,50 ± 1,31 | 0,846 ± 0,17 | 7,36 ± 0,87 |

Both water and uronic acid contents were highest in foetal tissue and decreased for neonatal and adult tissue. Other workers have shown the glycosaminoglycan content to be higher in tissues from younger animals (289,293,294). The relationship between water and uronic acid content is consistent with the concept that proteoglycans play an important physiological role in connective tissue by "trapping" water to maintain fluid homeostasis (55). In the aorta, however, the proteoglycans make up a relatively small proportion of the tissue and may thus play a less significant role in water binding. The protein content of adult tissue was slightly higher than that of foetal or neonatal tissue.

The glycosaminoglycan composition of the intact media of neonate and adult aortas is shown in Table 2.2. Samples of tissue were exhaustively digested with papain to release the glycosaminoglycan chains which were isolated by precipitation with ethanol. The sensitivity of the chains to the different specific glycosaminoglycan-degrading enzymes was then determined. It can be seen that both age groups contained high amounts of hyaluronic acid; at least one-third of the total glycosaminoglycan content of the tissue was hyaluronic acid, with its concentration increasing slightly in older tissue. Chondroitin sulphate was the major sulphated glycosaminoglycan in both ages, accounting for nearly all of the sulphated material in younger tissue. Dermatan sulphate was found in adult tissue but neonatal tissue contained lesser amounts of this glycosaminoglycan. Both ages contained small amounts of

TABLE 2.2

THE GLYCOSAMINOGLYCAN COMPOSITION OF AORTIC MEDIAL TISSUE FROM DIFFERENT AGE GROUPS

Samples of bovine aortic media from adult or neonate animals were digested exhaustively with papain after which the glycosaminoglycans were precipitated by the addition of ethanol as described in Section 2.2.2(c). The sensitivity of the glycosaminoglycans to the different glycosaminoglycan-degrading treatments was determined after digestion and chromatography on Sephadex G-50 as described in Sections 2.2.4 and 2.2.8. The data represent the mean ± S.D. for four determinations for one of two similar experiments.

| Glycosaminoglycan | Glycosaminoglycan Content (% of total) | |
|---|---|---|
| | Adult | Neonate |
| Chondroitin-4- and -6-sulphate | 37,5 ± 4,1 | 54,0 ± 3,7 |
| Dermatan sulphate | 14,2 ± 1,1 | 5,0 ± 0,7 |
| Heparan sulphate/heparin | 8,0 ± 1,2 | 5,3 ± 0,4 |
| Hyaluronic acid | 41,0 ± 2,2 | 35,7 ± 3,2 |

heparan sulphate/heparin.

The extraction of PGM using guanidine HCl has been documented by a number of laboratories (289,293,294,297,298). Some workers have shown that maximum amounts of tissue uronic acid (85% of the total) were extracted from aortas only when 3,0 M guanidine HCl was used for prolonged periods (7 days) (289). I found that shorter extraction periods of 48 h yielded at least 50% of the total uronic acid from the tissue and minimized artifactual degradation of the material. Increasing time of extraction to 4 days did not significantly increase the amount of uronic acid extracted from adult tissue; about 40% was not extractable even after 9 days. Our 48 h extraction procedure using 4,0 M guanidine HCl containing proteolytic inhibitors, resulted typically in yields of 3,5 μg uronic acid/mg dry weight (± 50%) for adult tissue and 19 μg uronic acid/mg dry weight (± 70%) for foetal tissue. Prior digestion of tissue with bacterial collagenase (EC.3.4.24.3) did not increase the yield of uronic acid obtained by subsequent extraction with 4,0 M guanidine HCl but pretreatment with pancreatic elastase (EC.3.4.21.36) did result in slightly higher yields - about 8% more tissue uronic acid was extracted. Radhakrishnamurthy *et al*. showed that elastase digestion of aortic tissue residue after extraction with 0,15 M sodium chloride yielded most of the heparan sulphate, some dermatan sulphate and hyaluronic acid (187). The tissue I used contained little heparan sulphate (Table 2.2), and low amounts of dermatan sulphate, but large amounts of hyaluronic acid, thus

the increased yield of uronate with elastase was probably due to an increase in released hyaluronic acid. Other authors have included detergent treatment prior to guanidine HCl extraction to obtain better yields (107) but in our hands no improvement in extraction of material was obtained in the presence of 1% (w/v) zwittergent 3-12.

The uronic acid and protein profiles for the dissociative and associative extraction procedures obtained following caesium chloride density gradient centrifugation are shown in Figures 2.1 and 2.2. The recoveries of uronic acid from the gradients were routinely about 90% of the material loaded. With adult material approximately 50% of the uronic acid was found in the bottom 1/5 of the gradient under dissociative conditions (Fig. 2.1a), whereas some 75% of the uronic acid was found in this area of the gradient in the case of neonatal or foetal material (Fig. 2.1b). The majority of protein is found in the top of the gradient with less than 5% in the bottom 1/5. Thus, using this procedure, the separation of PGM from the co-extracted protein was successfully carried out. In the case of the adult, the material appeared to be more heterogeneous as a considerable amount of uronic acid was dispersed throughout the rest of the gradient (Fig. 2.1a). This heterogeneity may arise as a result of a decreased glycosaminoglycan:protein ratio which would result in a decreased buoyant density for the proteoglycan monomers. Analysis by Sepharose CL-2B chromatography of the various gradient fractions obtained from adult tissue showed that the bottom 1/5 of the caesium

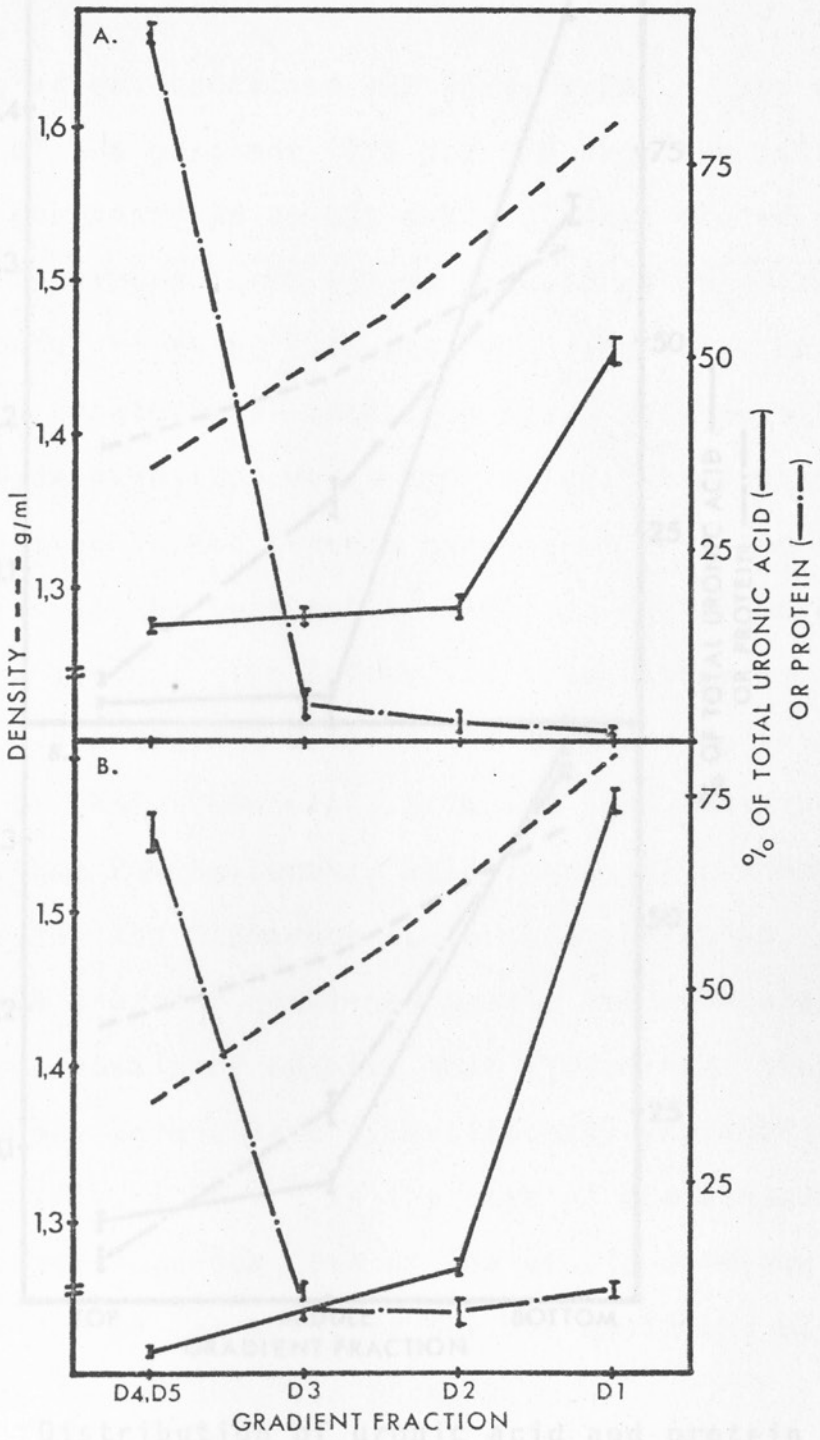



Fig. 2.1. Distribution of uronic acid and protein following caesium chloride density gradient centrifugation of extracted material from aortic media under dissociative conditions.

Gradients were fractionated from the top (D4,D5) to the bottom (D1) of centrifuge tubes, using a Beckman tube slicer (see Methods) after centrifugation under conditions described in Section 2.2.3.1. Aliquots of each fraction were taken to determine the protein and uronic acid concentration in each sample. The profiles represent analysis of adult material (A) and foetal or neonatal material (B) and the data represent the mean ± S.D. for triplicate determinations for one of several similar experiments.

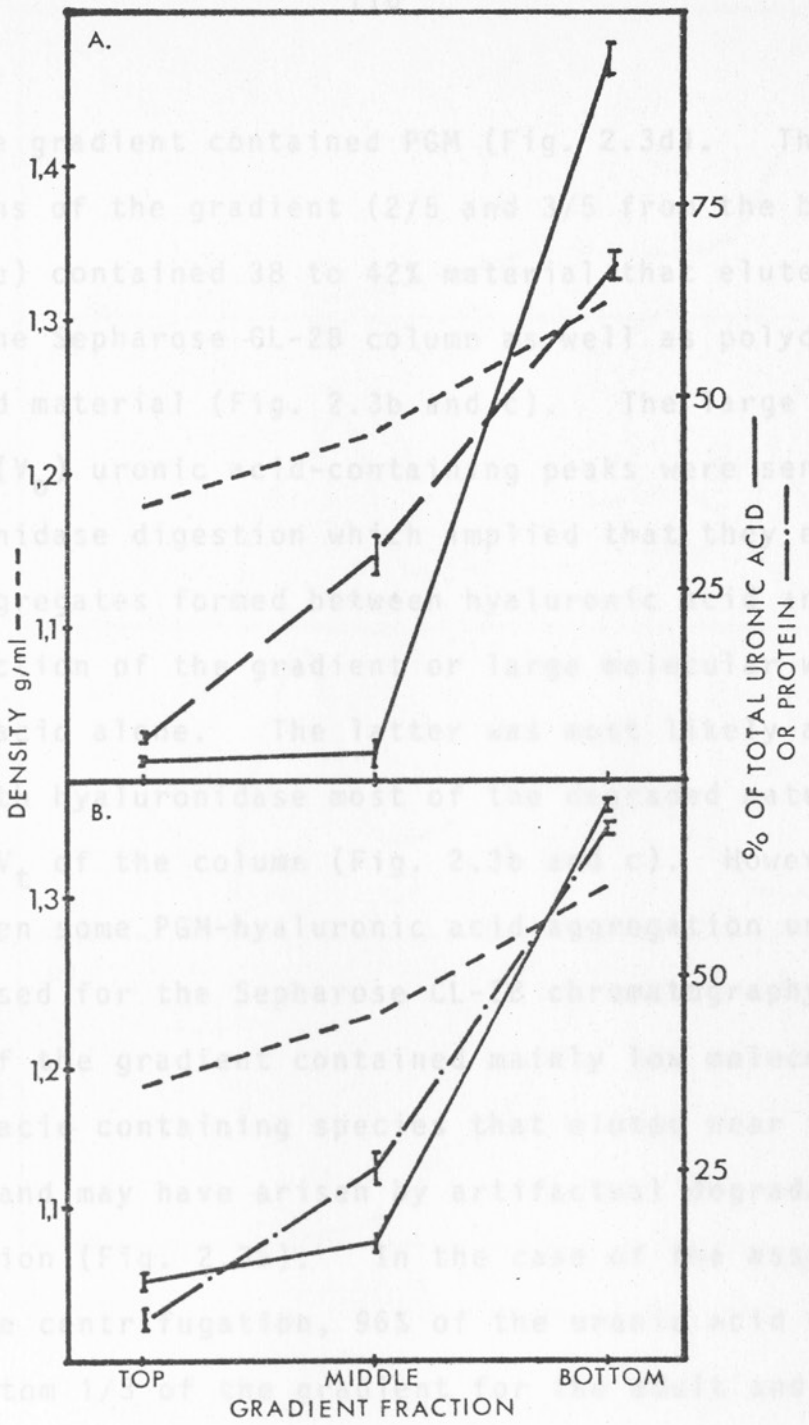



Fig. 2.2. Distribution of uronic acid and protein following caesium chloride density gradient centrifugation of extracted material from aortic media under associative conditions.

Fractions from the top, middle and bottom one-third of the tubes after associative caesium chloride density gradient centrifugation of material extracted from adult (A) and foetal or neonatal (B) aortic medial tissue were analysed for their uronic acid and protein contents as described in Section 2.2.3.2. The data represent the mean + S.D. for triplicate determinations for one of several similar experiments.

chloride gradient contained PGM (Fig. 2.3d). The middle fractions of the gradient (2/5 and 3/5 from the bottom of the tube) contained 38 to 42% material that eluted at the $V_o$ of the Sepharose CL-2B column as well as polydisperse-included material (Fig. 2.3b and c). The large molecular weight ($V_o$) uronic acid-containing peaks were sensitive to hyaluronidase digestion which implied that they either represent aggregates formed between hyaluronic acid and the PGM in that section of the gradient or large molecular weight hyaluronic acid alone. The latter was most likely as after digestion with hyaluronidase most of the degraded material was eluted at the $V_t$ of the column (Fig. 2.3b and c). However, there may have been some PGM-hyaluronic acid aggregation under the conditions used for the Sepharose CL-2B chromatography. The top fractions of the gradient contained mainly low molecular weight uronic acid containing species that eluted near the $V_t$ of the column and may have arisen by artifactual degradation during extraction (Fig. 2.3a). In the case of the associative caesium chloride centrifugation, 96% of the uronic acid was found in the bottom 1/3 of the gradient for the adult and 80% for the neonate and foetal material (Fig. 2.2).

When a comparison of the uronic acid profiles, obtained after Sepharose CL-2B chromatography of the bottom 1/5 fraction of the dissociative caesium chloride gradients of material from different ages, was made, there was evidence for two sizes of PGM in the younger tissues (Fig. 2.4). All ages showed a peak of material with a $K_{av}$ of 0,56, whereas neonatal and foetal tissue had a second larger species of PGM with a $K_{av}$ of

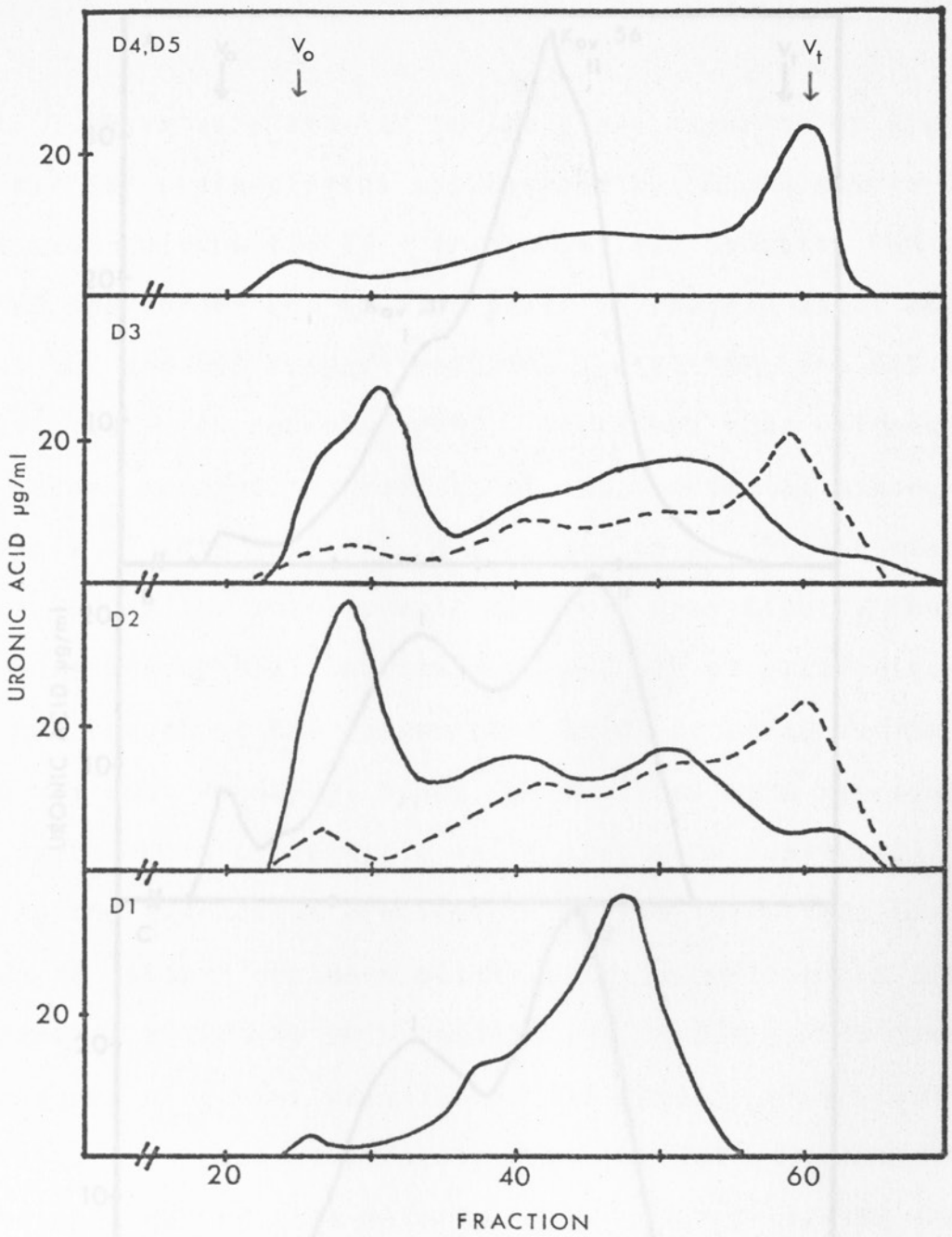



Fig. 2.3. Sepharose CL-2B chromatography of dissociative caesium chloride density gradient fractions containing uronic acid.

Proteoglycans were extracted dissociatively from adult aortic medial tissue and subjected to dissociative caesium chloride density gradient centrifugation as described in Section 2.2.3.1. After centrifugation, gradients were fractionated as described in Fig. 2.1 and each fraction was dialysed and lyophilised. Samples were dissolved in 0.5 M sodium acetate buffer pH 6.8 and chromatographed on a Sepharose CL-2B column as described in Section 2.2.6 either before (—) or after (--) digestion with hyaluronidase. Column fractions were assayed for their uronic acid content as described in Section 2.2.10.

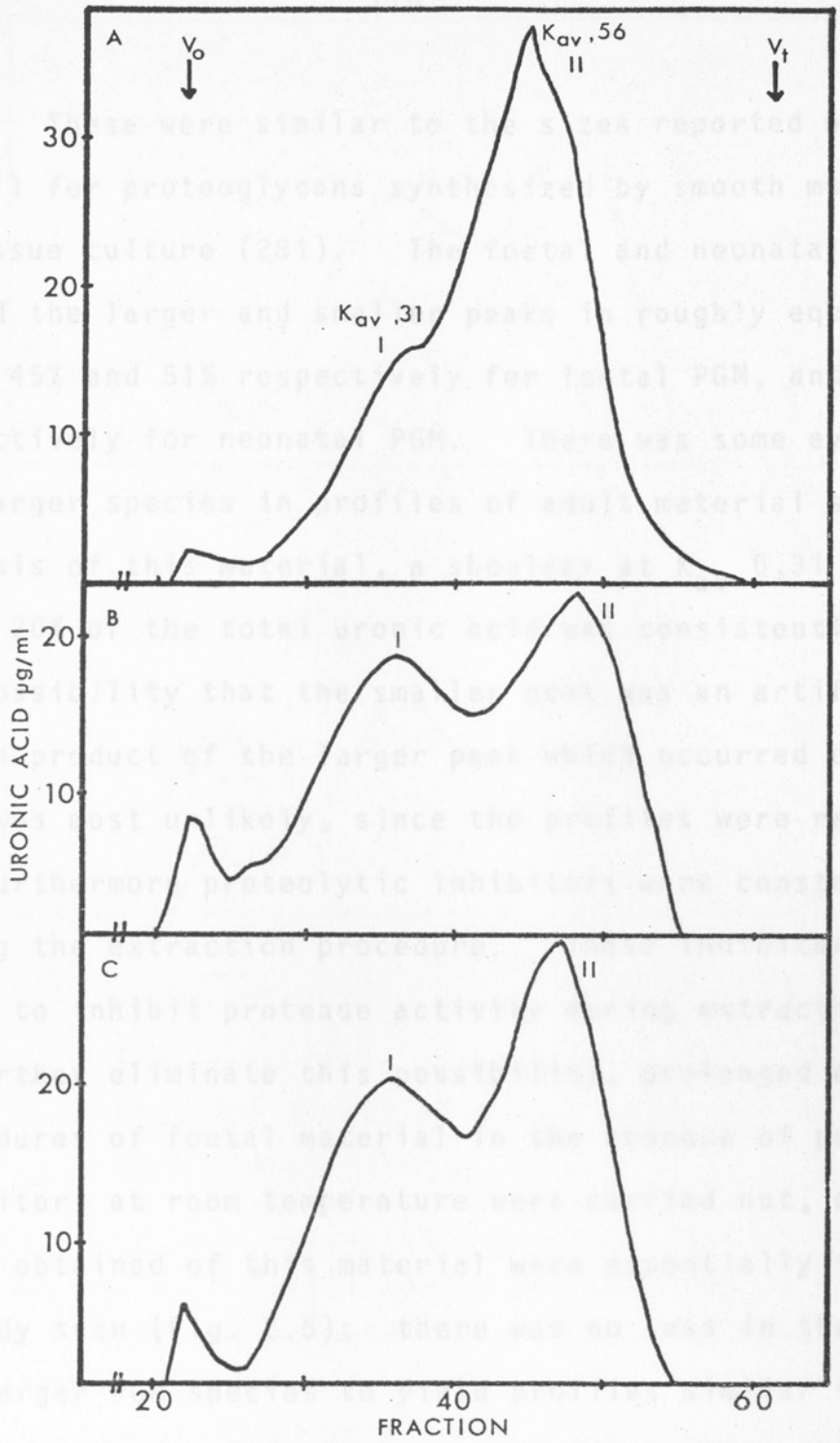



Fig. 2.4. Sepharose CL-2B chromatography of bottom (D1) fractions after dissociative caesium chloride density gradient centrifugation of material extracted from media of different aged animals.

Proteoglycans were extracted dissociatively from the media of adult (A), neonatal (B) or foetal (C) aortas and subjected to dissociative caesium chloride density gradient centrifugation as described in Section 2.2.3.1. Aliquots of the bottom (D1) fraction obtained after centrifugation, dialysis, freeze-drying and redissolution in acetate buffer (see under Fig. 2.3) were chromatographed on a Sepharose CL-2B column as described in Section 2.2.6 and column fractions were assayed for their uronic acid content.

0,31. These were similar to the sizes reported by Wight and Hascall for proteoglycans synthesized by smooth muscle cells in tissue culture (281). The foetal and neonatal PGM contained the larger and smaller peaks in roughly equal amounts: about 45% and 51% respectively for foetal PGM, and 44% and 49% respectively for neonatal PGM. There was some evidence for the larger species in profiles of adult material since, on analysis of this material, a shoulder at $K_{av}$ 0,31 containing about 20% of the total uronic acid was consistently found. The possibility that the smaller peak was an artifactual degradation product of the larger peak which occurred during isolation was most unlikely, since the profiles were reproducible, and furthermore proteolytic inhibitors were constantly present during the extraction procedure. These inhibitors have been shown to inhibit protease activity during extraction (325). To further eliminate this possibility, prolonged extraction procedures of foetal material in the absence of proteolytic inhibitors at room temperature were carried out, and the profiles obtained of this material were essentially the same as already seen (Fig. 2.5); there was no loss in the amount of the larger PGM species to yield profiles similar to the adult species. Even after 72 h at room temperature the two peaks were still present in roughly equal amounts (39% and 41%) and about 16% of the total material appeared in the $V_t$ of a Sepharose CL-2B column (Fig. 2.5b); thus both peaks had degraded to a similar extent; the amount of material in the smaller peak did not increase relative to that in the larger peak, even after prolonged periods at room temperature.

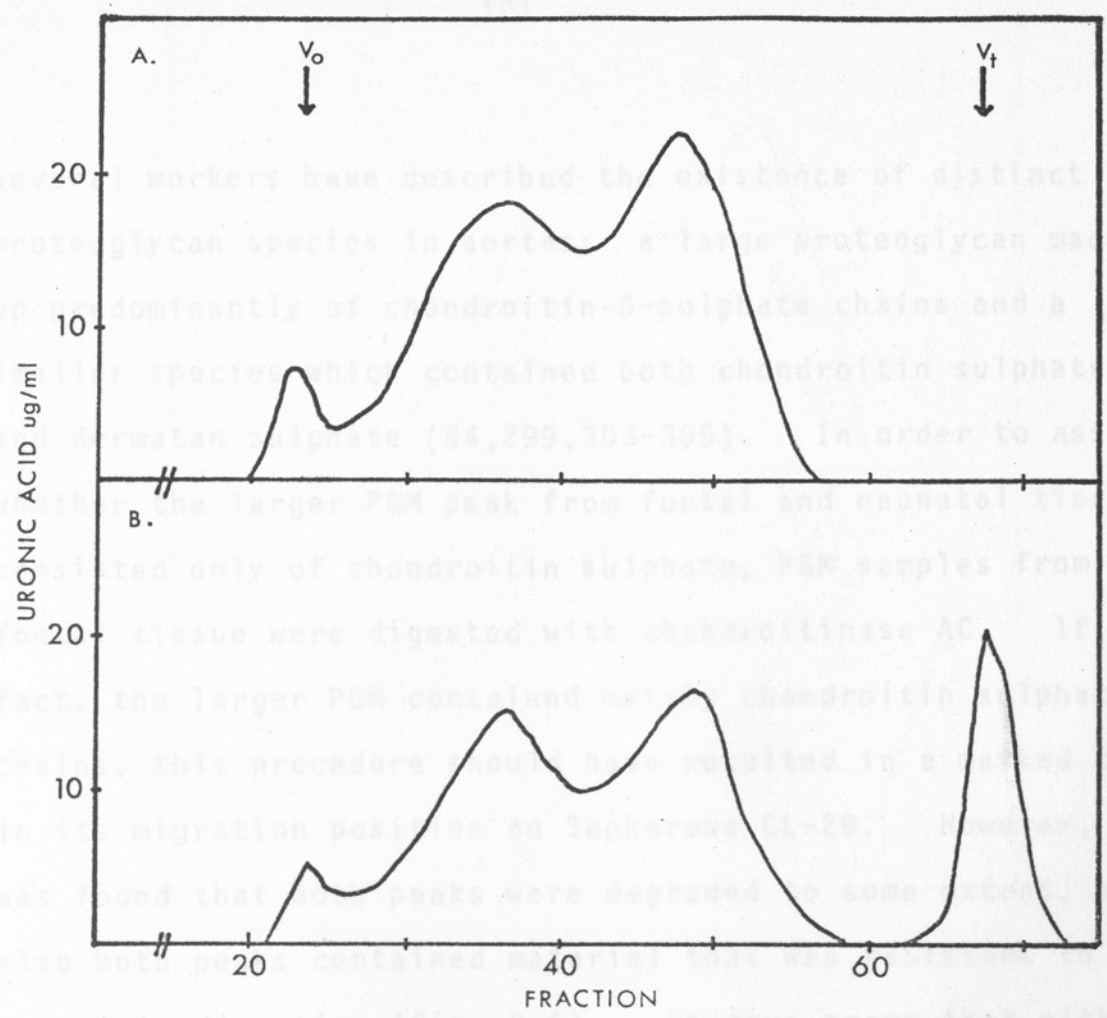



Fig. 2.5. Sepharose CL-2B chromatography of proteoglycans from foetal medial tissue extracted at 4°C or at room temperature.

Proteoglycans were extracted under dissociative conditions from the media of foetal aortas as described in Section 2.2.3.1. Aliquots of extracted material were then allowed to stand at room temperature for a further 3 days. The two extracts (4° and room temperature) were then subjected to dissociative gradient centrifugation as described in Section 2.2.3.1; the bottom (D1) fractions were collected, and after processing as described under Fig. 2.3, were chromatographed on a Sepharose CL-2B column as described in Section 2.2.6. Column fractions were assayed for their uronic acid content and the profiles represent(A) proteoglycans extracted at 4°C for 48 h and (B) proteoglycans extracted at room temperature for 3 days.

Several workers have described the existence of distinct proteoglycan species in aortas; a large proteoglycan made up predominantly of chondroitin-6-sulphate chains and a smaller species which contained both chondroitin sulphate and dermatan sulphate (54,299,303-305). In order to assess whether the larger PGM peak from foetal and neonatal tissue consisted only of chondroitin sulphate, PGM samples from foetal tissue were digested with chondroitinase AC. If, in fact, the larger PGM contained mainly chondroitin sulphate chains, this procedure should have resulted in a marked change in its migration position on Sepharose CL-2B. However, it was found that both peaks were degraded to some extent, and also both peaks contained material that was resistant to the enzymatic digestion (Fig. 2.6). It thus seems that either the larger PGM species does not contain only chondroitin sulphate side chains, or that there are more than one species of PGM present in that peak having similar molecular weights.

Both foetal and neonatal preparations contained small peaks of uronic acid containing material (5 to 7%) that were excluded from Sepharose CL-2B under dissociative conditions (Fig. 2.4b and c). This may be due to aggregate formation with hyaluronic acid since some hyaluronidase-sensitive material was found in the bottom 1/5 of the dissociative gradient (see Table 2.3). However, it is much more likely to be due to interactions between PGM (self-association) or between PGM and those proteins which were found in the bottom of the gradient. This belief was reinforced by the fact that

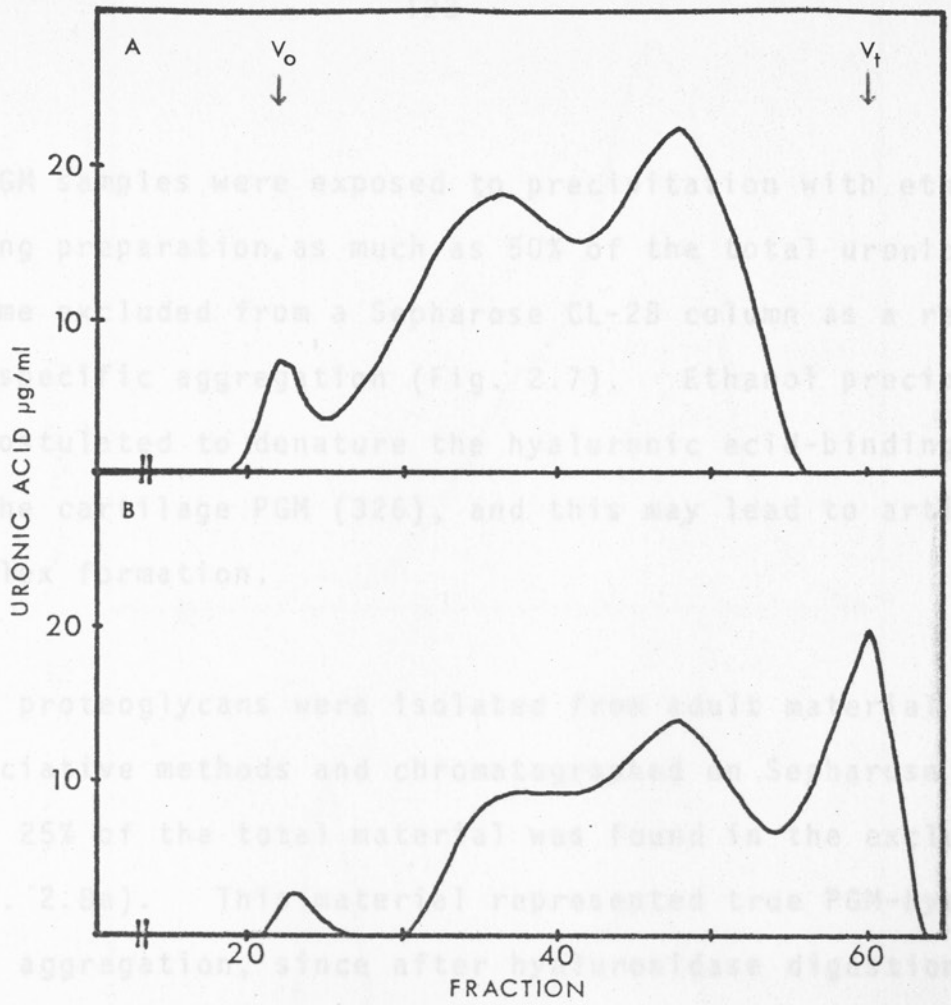



Fig. 2.6. Sepharose CL-2B chromatography of proteoglycans from foetal medial tissue before and after digestion with chondroitinase AC.

Proteoglycans were extracted under dissociative conditions from the media of foetal aortas and subjected to dissociative caesium chloride density gradient centrifugation as described in Section 2.2.3.1. The bottom (D1) fractions were collected and after processing as described under Fig. 2.3 were chromatographed on a Sepharose CL-2B column as described in Section 2.2.6. Column fractions were assayed for their uronic acid content before and after treatment with chondroitinase AC, and profiles represent samples before treatment (A) and after enzymatic digestion (B).

if PGM samples were exposed to precipitation with ethanol during preparation, as much as 50% of the total uronic acid became excluded from a Sepharose CL-2B column as a result of non-specific aggregation (Fig. 2.7). Ethanol precipitation is postulated to denature the hyaluronic acid-binding region of the cartilage PGM (326), and this may lead to artificial complex formation.

When proteoglycans were isolated from adult material using associative methods and chromatographed on Sepharose CL-2B, some 25% of the total material was found in the excluded peak (Fig. 2.8a). This material represented true PGM-hyaluronic acid aggregation, since after hyaluronidase digestion it disappeared to give an increased included peak. Neither foetal nor neonatal-derived material showed any hyaluronidase sensitive excluded peaks when isolated under associative conditions, and the profiles obtained on Sepharose CL-2B were similar to those seen in Fig. 2.4 b and c. Dialysis of the initial dissociative extract to associative conditions resulted in the formation of precipitates of PGM and coextracted proteins, and thus a large amount of uronic acid-containing material was lost due to the formation of these insoluble complexes. As a consequence high yields of PGA could not be obtained. Some workers have shown that dialysis must not be allowed to proceed beyond a concentration of 0,4 M guanidine HCl (307). However, even with limited dialysis, large amounts of material were lost during the associative preparations as described above.

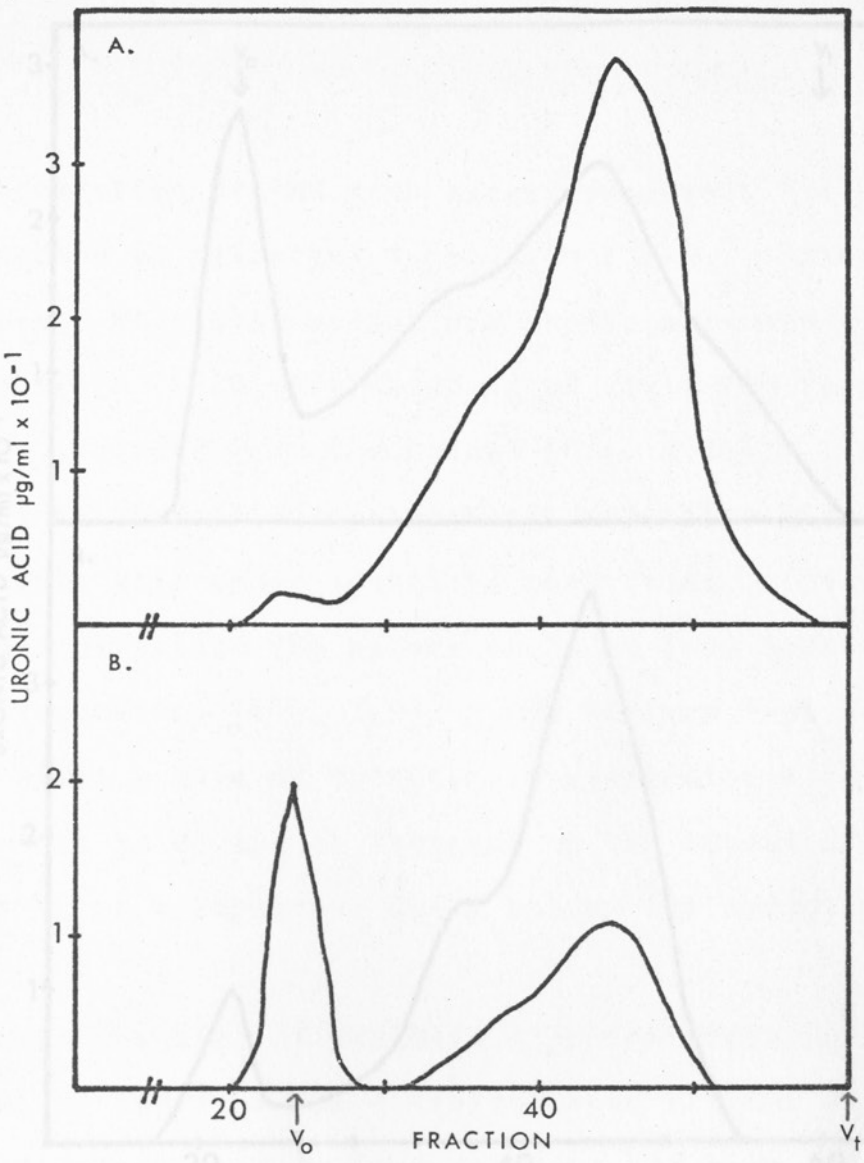



Fig. 2.7. The effect of an ethanol precipitation step during isolation of proteoglycans on the Sepharose CL-2B profile of such material.

Proteoglycans were extracted under dissociative conditions from adult medial tissue and subjected to dissociative caesium chloride density gradient centrifugation as described in Section 2.2.3.1, either before (A) or after (B) precipitation with two volumes of cold absolute ethanol. The bottom (D1) fractions were collected and chromatographed on a Sepharose CL-2B column as described under Fig. 2.3 and in Section 2.2.6. Column fractions were analysed for their uronic acid content.

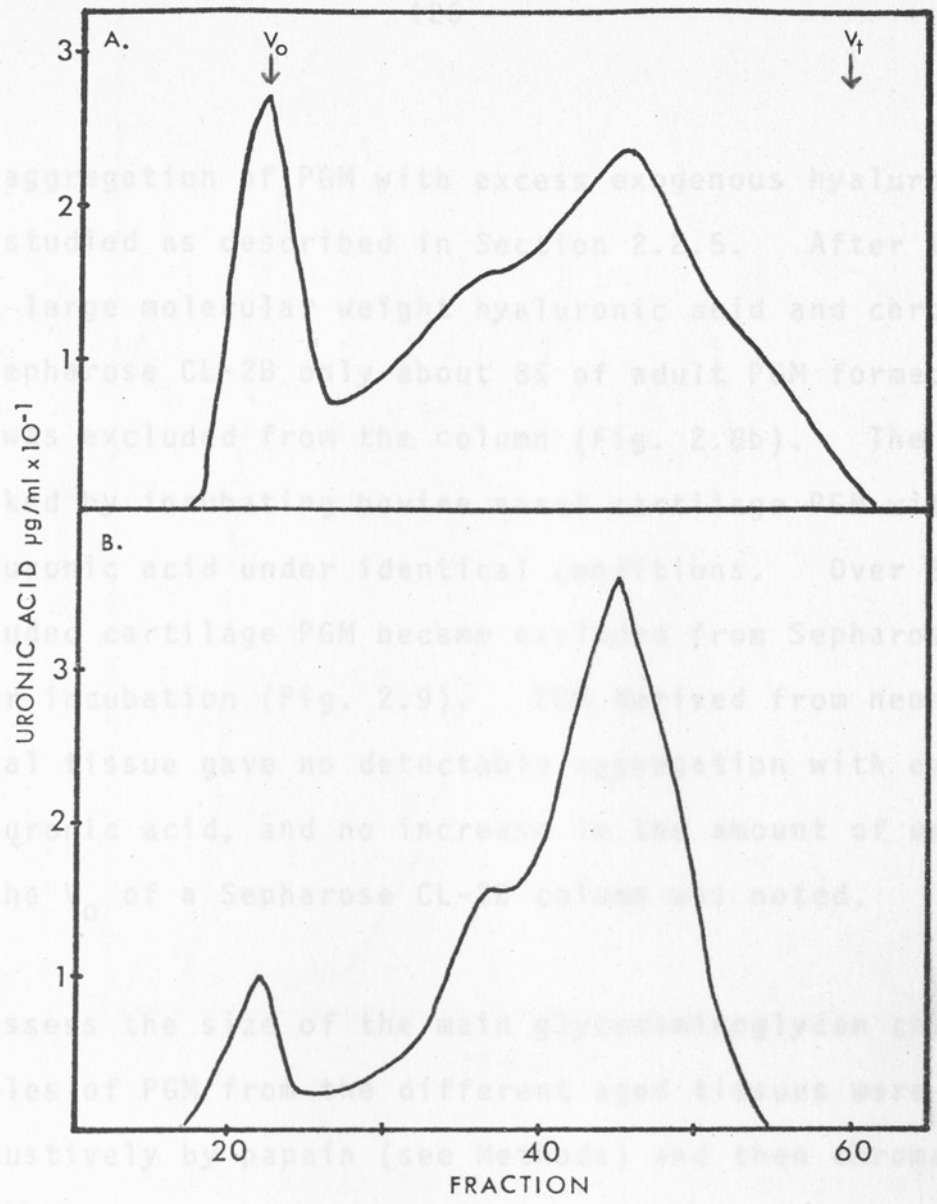



Fig. 2.8. Sepharose CL-2B chromatography of proteoglycans from adult medial tissue after incubation in the presence of endogenous or exogenous hyaluronic acid.

A. Proteoglycans were isolated from adult medial tissue and subjected to associative caesium chloride density gradient centrifugation as described in Section 2.2.3.2. A sample of the bottom one-third fraction after centrifugation was chromatographed on Sepharose CL-2B as described in Section 2.2.6. Column fractions were assayed for their uronic acid content.

B. Proteoglycans were isolated dissociatively from adult medial tissue as described in Section 2.2.3.1. A sample of the bottom (D1) fraction after dissociative density gradient centrifugation was mixed with an excess of exogenous large molecular weight hyaluronic acid as described in Section 2.2.5 and chromatographed on Sepharose CL-2B as described in Section 2.2.6. Column fractions were assayed for their uronic acid content.

The aggregation of PGM with excess exogenous hyaluronic acid was studied as described in Section 2.2.5. After incubation with large molecular weight hyaluronic acid and chromatography on Sepharose CL-2B only about 8% of adult PGM formed aggregates and was excluded from the column (Fig. 2.8b). The system was checked by incubating bovine nasal cartilage PGM with the same hyaluronic acid under identical conditions. Over 85% of the included cartilage PGM became excluded from Sepharose CL-2B after incubation (Fig. 2.9). PGM derived from neonatal and foetal tissue gave no detectable aggregation with exogenous hyaluronic acid, and no increase in the amount of uronic acid in the $V_o$ of a Sepharose CL-2B column was noted.

To assess the size of the main glycosaminoglycan chains, samples of PGM from the different aged tissues were digested exhaustively by papain (see Methods) and then chromatographed on a Sepharose CL-6B column (Fig. 2.10). The average molecular weight of the released glycosaminoglycans was estimated by comparing the elution position ($K_{av}$) of the sample fraction to that of known chondroitin sulphate standards as described by Wasteson (327). From the uronic acid profiles obtained, it appeared that there was a decrease in the bulk glycosaminoglycan chain size with age, with the $K_{av}$ on Sepharose CL-6B being 0,27 for chains from foetal and neonatal PGM and 0,34 for chains from adult PGM. The $K_{av}$s suggest molecular weights of greater than 50000 for foetal and neonatal glycosaminoglycans, and about 42000 for those from adult PGM. These sizes are at least double the sizes found for glycos-

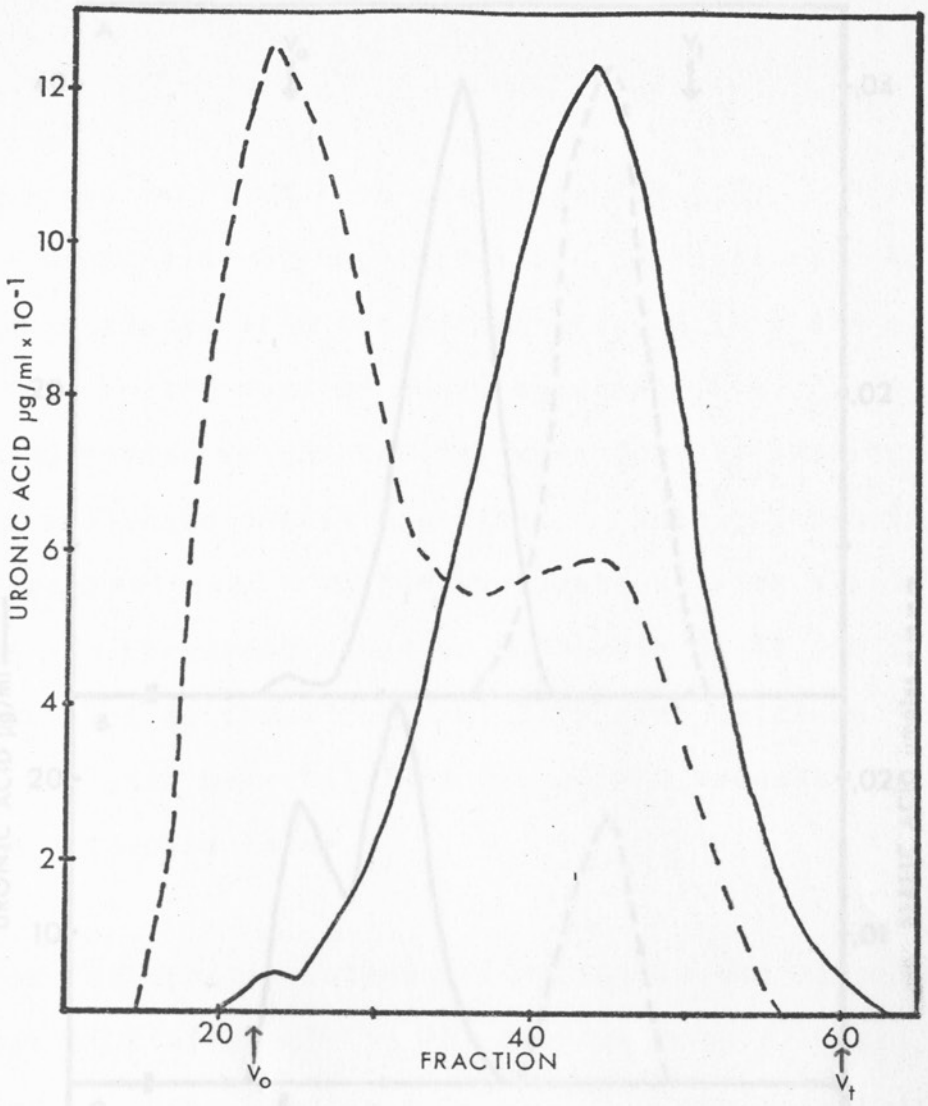



Fig. 2.9. Sepharose CL-2B chromatography of proteoglycans from bovine nasal cartilage incubated in the presence and absence of exogenous hyaluronic acid.

Proteoglycans were extracted under dissociative conditions from bovine nasal cartilage and subjected to dissociative caesium chloride density gradient centrifugation as described in Section 2.2.3.1. A sample of the bottom (D1) fraction was processed (see under Fig. 2.3) and chromatographed on Sepharose CL-2B as described in Section 2.2.6, either in the absence (—) or presence (--) of an excess of exogenous large molecular weight hyaluronic acid (Section 2.2.5). Column fractions were assayed for their uronic acid content.

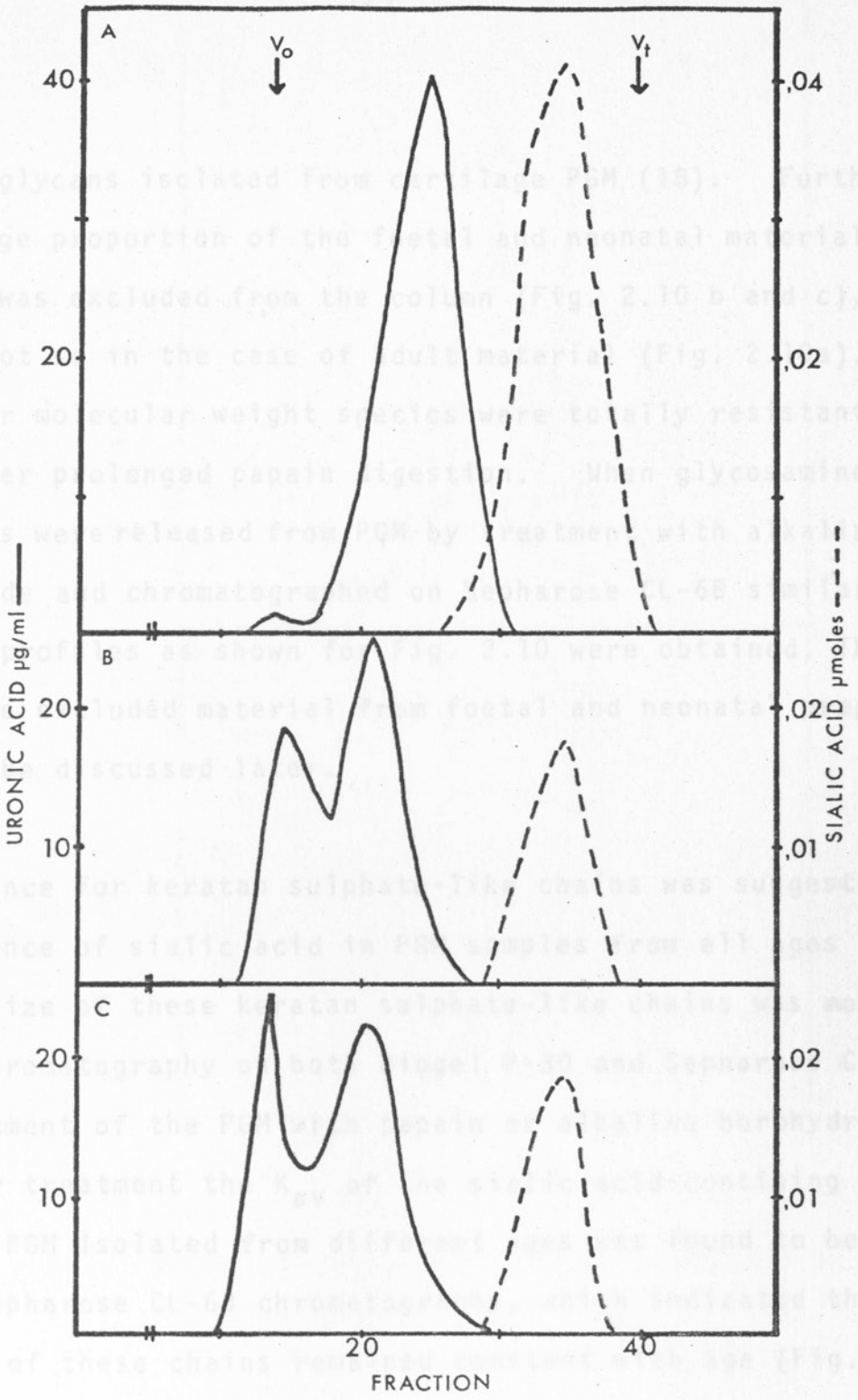



Fig. 2.10. Sepharose CL-6B chromatography of free glycosaminoglycan chains obtained from proteoglycans isolated from aortic medial tissue of different aged animals.

Proteoglycans were isolated under dissociative conditions from the aortic media of adult (A), neonatal (B) or foetal (C) animals. Free glycosaminoglycan chains were obtained either by papain digestion or treatment with alkaline borohydride as described in Section 2.2.4 and chromatographed on Sepharose CL-6B as described in Section 2.2.6. Column fractions were assayed for their uronic or sialic acid content.

aminoglycans isolated from cartilage PGM (18). Furthermore, a large proportion of the foetal and neonatal material (nearly 30%) was excluded from the column (Fig. 2.10 b and c), which was not so in the case of adult material (Fig. 2.10a). These larger molecular weight species were totally resistant to further prolonged papain digestion. When glycosaminoglycan chains were released from PGM by treatment with alkaline borohydride and chromatographed on Sepharose CL-6B similar uronic acid profiles as shown for Fig. 2.10 were obtained. The nature of the excluded material from foetal and neonatal preparations will be discussed later.

Evidence for keratan sulphate-like chains was suggested by the presence of sialic acid in PGM samples from all ages (Fig. 2.10). The size of these keratan sulphate-like chains was monitored by chromatography on both Biogel P-30 and Sepharose CL-6B after treatment of the PGM with papain or alkaline borohydride. After treatment the $K_{av}$ of the sialic acid-contining moiety from PGM isolated from different ages was found to be 0,82 by Sepharose CL-6B chromatography, which indicated that the size of these chains remained constant with age (Fig. 2.10). Chromatography on Biogel P-30 yielded the profiles shown in Fig. 2.11, namely two sialic acid-containing peaks identified for all ages with a small proportion of the chains having a $K_{av}$ of 0,49 and the rest a $K_{av}$ of 0,67. These results suggested the existence of keratan sulphate-like oligosaccharides on PGM from bovine aortas. The amount of material in the smaller peak relative to that in the larger species was

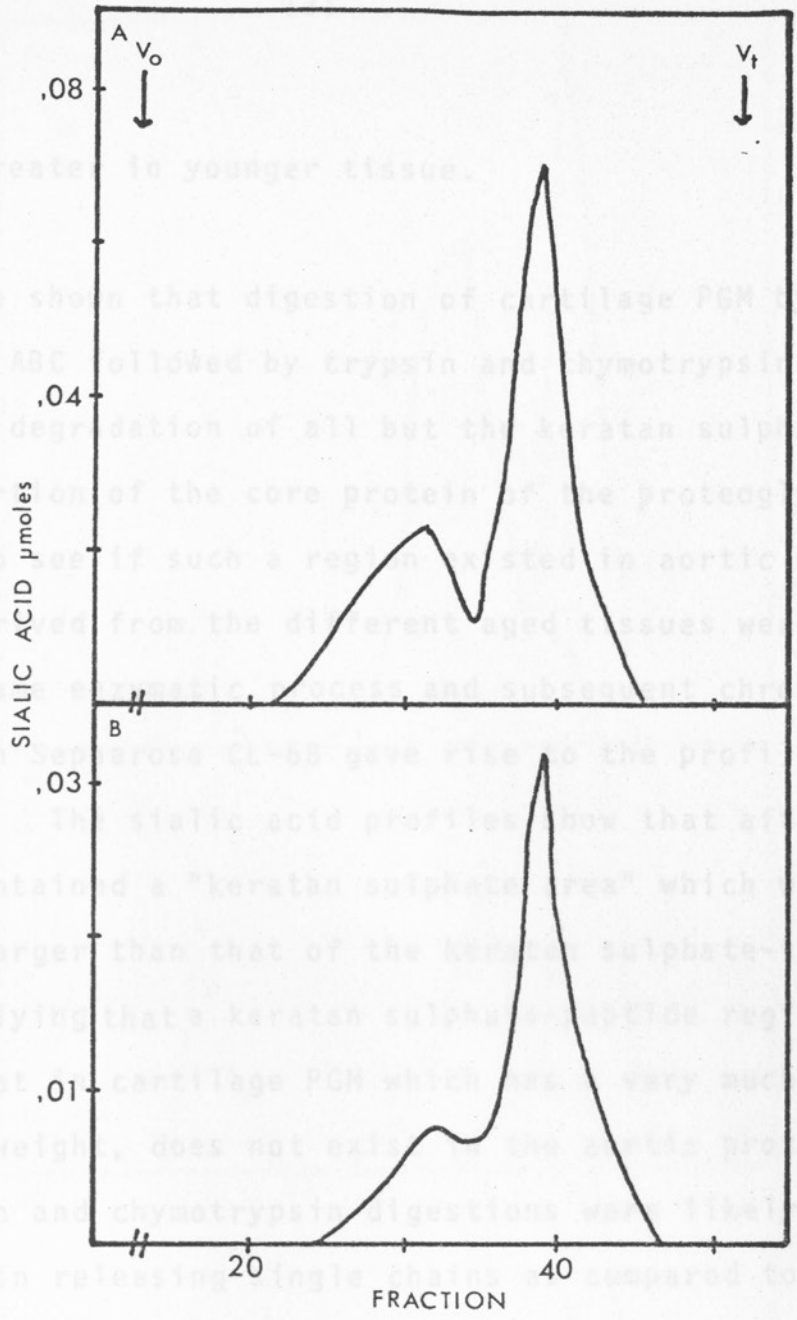



Fig. 2.11. Biogel P-30 chromatography of sialic acid-containing material obtained from proteoglycans isolated from aortic medial tissue of different aged animals.

Proteoglycans were isolated under dissociative conditions from the aortic media of adult (A) and neonatal or foetal (B) animals and treated with alkaline borohydride as described in Section 2.2.4. Samples were chromatographed on a Biogel P-30 column as described in Section 2.2.6. Column fractions were assayed for their sialic acid content.

slightly greater in younger tissue.

It has been shown that digestion of cartilage PGM by chondroitinase ABC followed by trypsin and chymotrypsin digestion results in degradation of all but the keratan sulphate-rich peptide portion of the core protein of the proteoglycan (15). In order to see if such a region existed in aortic PGM, samples derived from the different aged tissues were treated with the same enzymatic process and subsequent chromatographic analysis on Sepharose CL-6B gave rise to the profiles shown in Fig. 2.12. The sialic acid profiles show that after digestion, the PGM contained a "keratan sulphate area" which was only slightly larger than that of the keratan sulphate-type chains alone, implying that a keratan sulphate-peptide region, comparable to that in cartilage PGM which has a very much larger molecular weight, does not exist in the aortic proteoglycans. The trypsin and chymotrypsin digestions were likely to be less effective in releasing single chains as compared to papain digestion or alkaline borohydride treatments. This might explain the slightly larger size of the product on Sepharose CL-6B. It was possible that the peak material represented two chains joined by a peptide. However, it was clear that a keratan sulphate-peptide region containing a large number of keratan sulphate chains joined together by a peptide, such as found in cartilage, does not occur in aortic PGM.

Again, both neonatal and foetal-derived PGM contained a large amount of uronic acid-containing material that was insusceptible to chondroitinase ABC digestion and eluted at the $V_o$ of

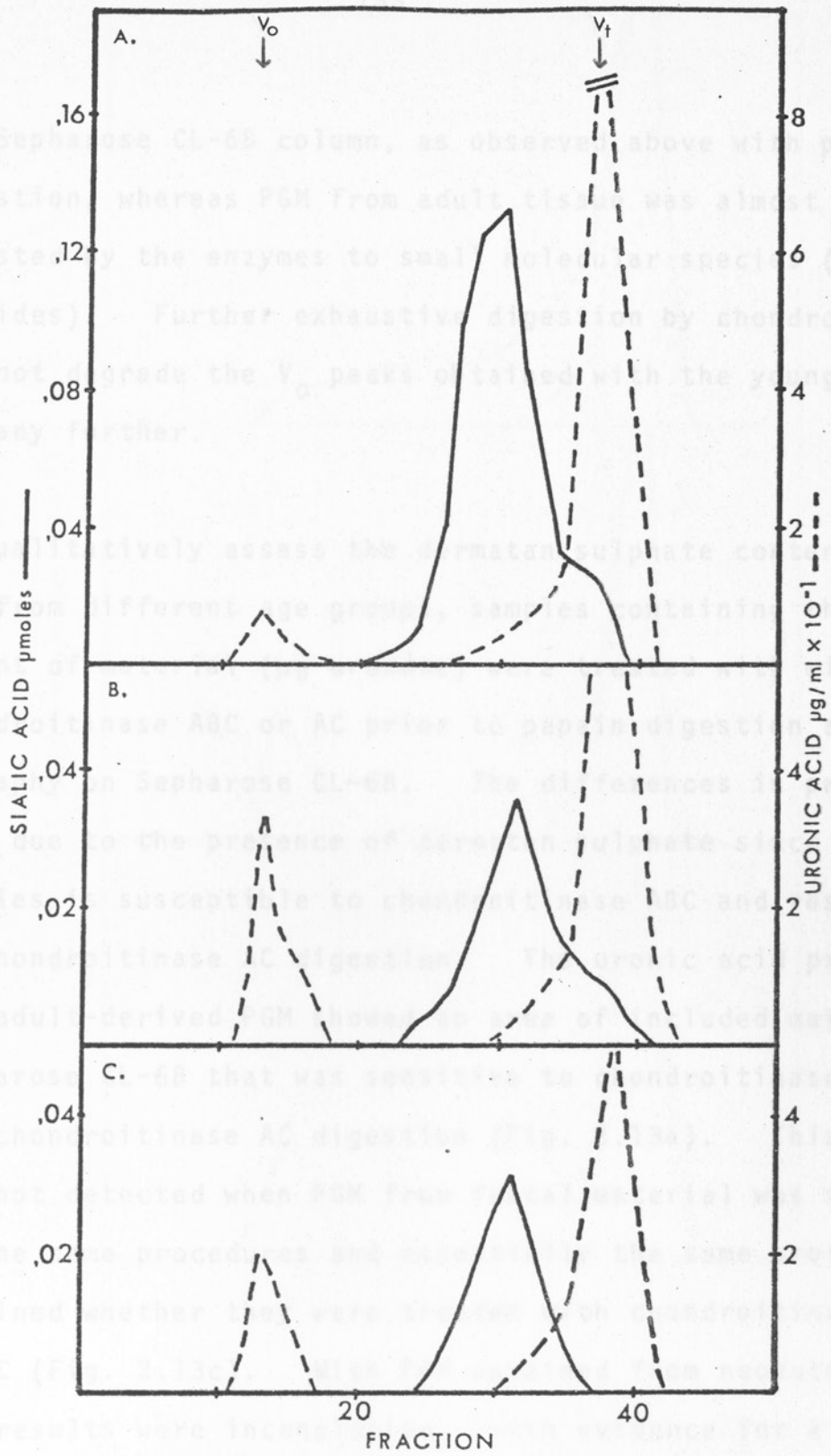



Fig. 2.12. Sepharose CL-6B chromatography of proteoglycans from aortic medial tissue of different aged animals after sequential enzyme treatment.

Proteoglycans were isolated under dissociative conditions from the aortic media of adult (A), neonate (B) and foetal (C) animals as described in Section 2.2.3.1. Samples of D1 fractions processed as described under Fig. 2.3 were digested with chondroitinase ABC followed by trypsin and chymotrypsin, and chromatographed on Sepharose CL-6B as previously described. Column fractions were assayed for their uronic and sialic acid content.

the Sepharose CL-6B column, as observed above with papain digestion, whereas PGM from adult tissue was almost totally digested by the enzymes to small molecular species (disaccharides). Further exhaustive digestion by chondroitinase did not degrade the $V_o$ peaks obtained with the younger tissue PGM any further.

To qualitatively assess the dermatan sulphate content of the PGM from different age groups, samples containing the same amount of material (μg uronate) were treated with either chondroitinase ABC or AC prior to papain digestion and chromatography on Sepharose CL-6B. The differences in profiles were due to the presence of dermatan sulphate since this species is susceptible to chondroitinase ABC and resistant to chondroitinase AC digestion. The uronic acid profiles for adult-derived PGM showed an area of included material on Sepharose CL-6B that was sensitive to chondroitinase ABC but not chondroitinase AC digestion (Fig. 2.13a). This, however, was not detected when PGM from foetal material was subjected to the same procedures and essentially the same profiles were obtained whether they were treated with chondroitinase ABC or AC (Fig. 2.13c). With PGM obtained from neonatal tissue the results were inconclusive, with evidence for a small amount of chondroitinase AC-resistant material (Fig. 2.13b). The data indicated that only adult PGM contained any appreciable amount of dermatan sulphate, and the absence of dermatan sulphate in foetal PGM was confirmed when the analysis of the different glycosaminoglycan species was performed on PGM from the different aged material (see Table 2.3). Furthermore,

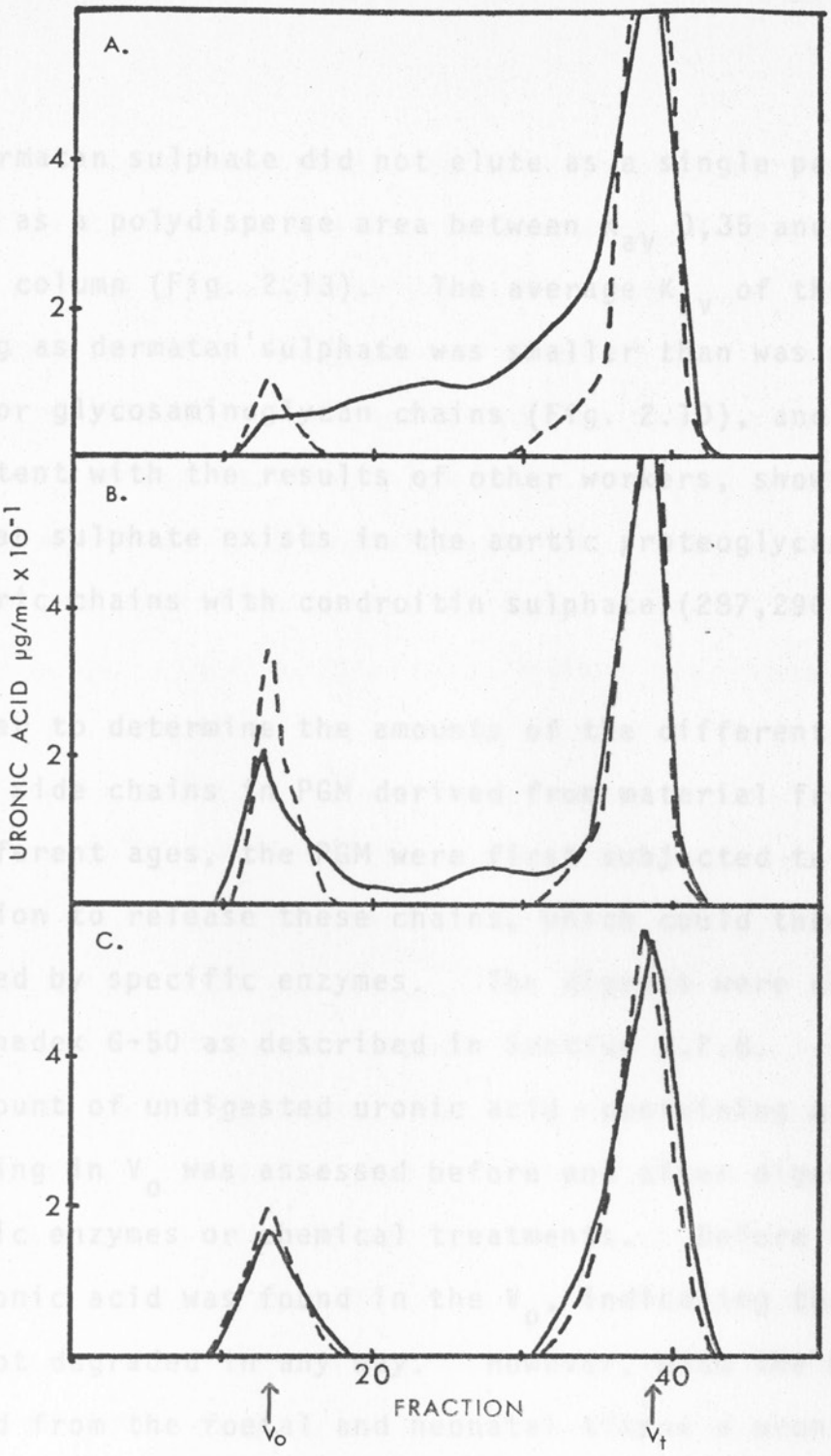



Fig. 2.13. Sepharose CL-6B chromatography of proteoglycans from aortic medial tissue of different aged animals after sequential digestion by chondroitinase ABC and papain or chondroitinase AC and papain.

Proteoglycans were isolated under dissociative conditions from the aortic media of adult (A), neonate (B) and foetal (C) animals as described in Section 2.2.3.1. The bottom (D1) fractions were collected after dissociative density gradient centrifugation and processed as described under Fig. 2.3. Samples from these fractions were digested with either chondroitinase ABC followed by papain (---) or with chondroitinase AC followed by papain (——) and then chromatographed on Sepharose CL-6B as described in Section 2.2.6. Column fractions were assayed for their uronic acid content.

the dermatan sulphate did not elute as a single peak but rather as a polydisperse area between $K_{av}$ 0,35 and the $V_t$ of the column (Fig. 2.13). The average $K_{av}$ of the material eluting as dermatan sulphate was smaller than was normally seen for glycosaminoglycan chains (Fig. 2.10), and thus was consistent with the results of other workers, showing that dermatan sulphate exists in the aortic proteoglycan as co-polymeric chains with condroitin sulphate (287,290).

In order to determine the amounts of the different glycosaminoglycan side chains in PGM derived from material from animals of different ages, the PGM were first subjected to papain digestion to release these chains, which could then be further digested by specific enzymes. The digests were chromatographed on Sephadex G-50 as described in Section 2.2.8. In each case the amount of undigested uronic acid - containing material remaining in $V_o$ was assessed before and after digestion with specific enzymes or chemical treatments. Before any digestions all uronic acid was found in the $V_o$, indicating that the chains were not degraded in any way. However, with the material derived from the foetal and neonatal tissue a uronic acid-containing species which resisted digestion by all of the specific enzymes was routinely noted. This was consistent with our previous observations that some of the foetal and neonatal PGM contained material resistant to digestion with papain (Fig. 2.10) and chondroitinase ABC (Fig. 2.12). In order to characterize this material, the following tests were carried out. Firstly, exhaustive digestion with hyaluronidase using

times and enzyme concentrations that totally destroyed 100 µg commercial hyaluronic acid. Furthermore, from quantitation of the various glycosaminoglycans in the bottom 1/5 fraction of the caesium chloride gradient (Table 2.3), it was already known that, although some hyaluronic acid was present, it could not account for the large proportion of uronic acid that was excluded from Sepharose CL-6B. The second approach involved the digestion of the excluded material with amylase (EC.3.2.1.1), since neutral sugars have been shown to give positive results with the carbazole reaction used for quantitating uronic acids. It was found after brief digestion (10 min at 37°C) of PGM preparations with amylase that indeed some of the uronic acid positive material appeared in the $V_t$ of the Sephadex G-50 column. This indicated that PGM preparations, in spite of the rigorous isolation procedure, were contaminated with glycogen. It was not, however, feasible to include as routine an amylase digestion step in the isolation procedure, since all the amylase preparations invariably contained some proteases which resulted in degradation of our PGM preparation. Precipitation of the initial 4 M guanidine HCl extract with cetylpyridinium chloride has been shown by some workers to rid the preparation of some of its contaminants, including glycogen (291), but in our hands such precipitation made little difference to the amount of glycogen present in the final preparation. Therefore, in order to quantitate the amount of different glycosaminoglycan types in PGM samples, the amount of contaminating glycogen was first determined, and then the values corrected accordingly. The

glycogen contaminant of the adult PGM preparation was only a small proportion (less than 8%), but was higher in neonatal and foetal PGM preparations. It was also important to show which of the peaks found on chromatography of the PGM samples on Sepharose CL-2B (Fig. 2.4) were contaminated with glycogen. This analysis was carried out as follows: large amounts of the two peaks (Fig. 2.4) were prepared, using a preparative Sepharose CL-2B column (1,6 x 130 cm). Material from the different peaks was pooled separately, dialysed and freeze-dried. They were then digested with papain and then subjected to ion-exchange chromatography on DEAE-cellulose (Section 2.2.7). The uncharged glycogen was not bound by the cellulose, whereas the highly anionic glycosaminoglycan chains bound and eluted only at high salt concentrations. The profiles of the individual peaks from neonatal and foetal PGM after chromatography on DEAE-cellulose are shown in Fig. 2.14. Both peak I ($K_{av}$ 0,31) and peak II ($K_{av}$ 0,56) from Sepharose CL-2B contained a portion of material not bound to the column as well as material that eluted later. The majority of the bound material eluted at a position corresponding to that where standard chondroitin sulphate elutes; this agreed with results obtained on the quantitation of glycosaminoglycans by susceptibility to different enzymes, which showed that the majority of glycosaminoglycans were chondroitin and dermatan sulphate (Table 2.3). Small peaks eluted at a position corresponding to that of standard hyaluronic acid, indicating that the dissociative preparation still contains hyaluronic acid associated with it. The proportion of unbound material

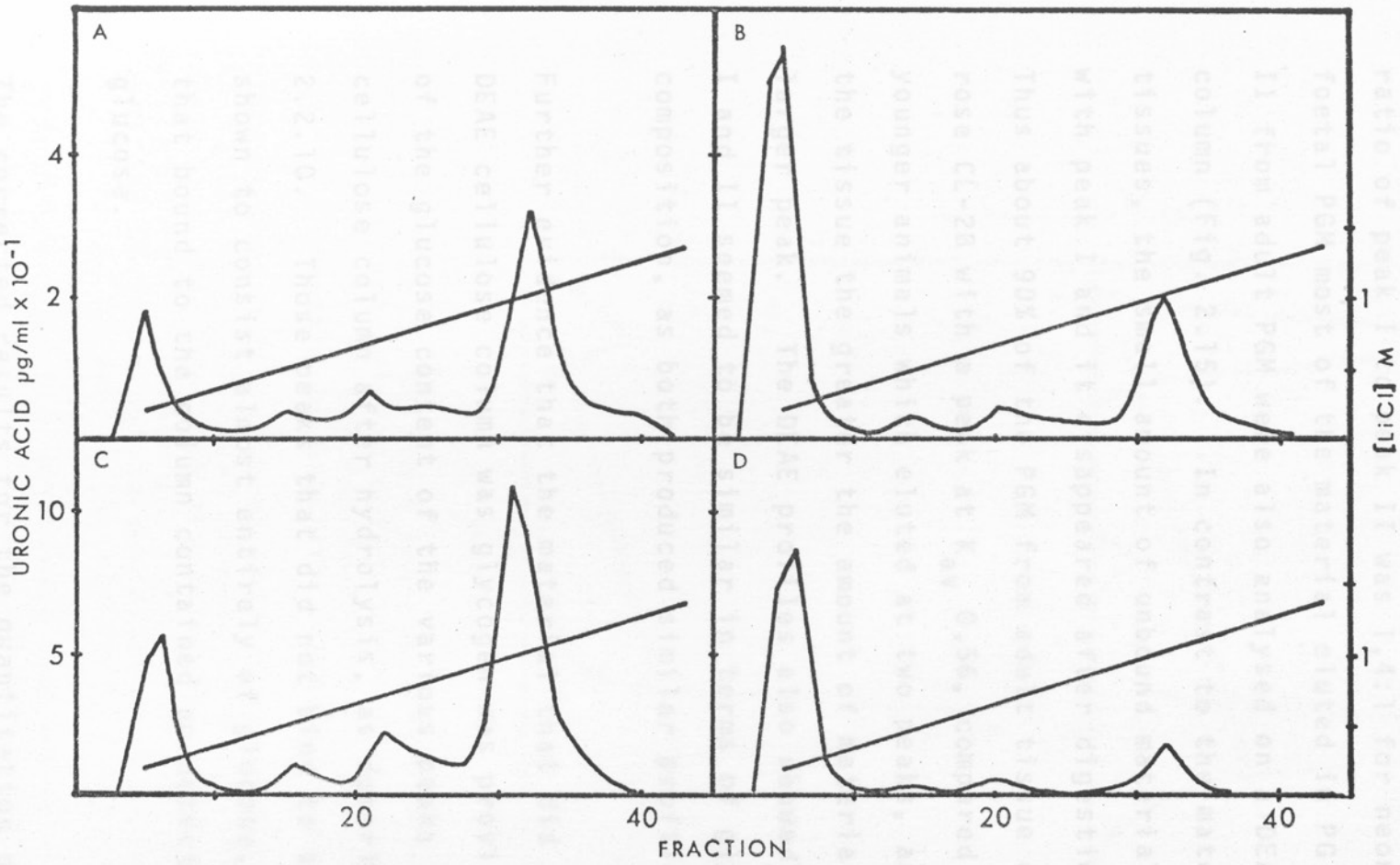



Fig. 2.14. DEAE-cellulose ion-exchange chromatography of glycosaminoglycan chains associated with individual proteoglycan species separated by Sepharose CL-2B chromatography of material extracted from neonatal or foetal medial tissue.

Proteoglycans were isolated under dissociative conditions from the aortic media of neonate (A,B) and foetal (C,D) animals as described in Section 2.2.3.1. The bottom (D1) fractions were collected after dissociative density gradient centrifugation and chromatographed on a preparative Sepharose CL-2B column as described in Section 2.2.6. Material corresponding to peak I (A,C) and peak II (B,D) (see Fig. 2.4) was collected, dialysed, digested with papain and then chromatographed on DEAE-cellulose columns as described in Section 2.2.7. Column fractions were assayed for their uronic acid content.

was greater in peak II for young material (Fig. 2.14) and, after correction for the amount of glycogen in each peak, the ratio of peak I to peak II was 1,4:1 for neonatal PGM and for foetal PGM most of the material eluted in PG 1. Peaks I and II from adult PGM were also analysed on a DEAE-cellulose column (Fig. 2.15). In contrast to the material from younger tissues, the small amount of unbound material was associated with peak I and it disappeared after digestion with amylase. Thus about 90% of the PGM from adult tissue eluted on Sepharose CL-2B with a peak at $K_{av}$ 0,56, compared to material from younger animals which eluted at two peaks, and the younger the tissue the greater the amount of material eluting in the larger peak. The DEAE profiles also showed that the peaks I and II seemed to be similar in terms of glycosaminoglycan composition, as both produced similar profiles on the columns.

Further evidence that the material that did not bind to the DEAE cellulose column was glycogen was provided by analysis of the glucose content of the various peaks off the DEAE cellulose column after hydrolysis, as described in Section 2.2.10. Those peaks that did not bind to the column were shown to consist almost entirely of glucose, whereas those that bound to the column contained no detectable amounts of glucose.

The corrected results for the quantitation of glycosaminoglycans from PGM from different ages are shown in Table 2.3. The results parallel those from the intact media (Table 2.2) in that the majority of the sulphated glycosaminoglycan

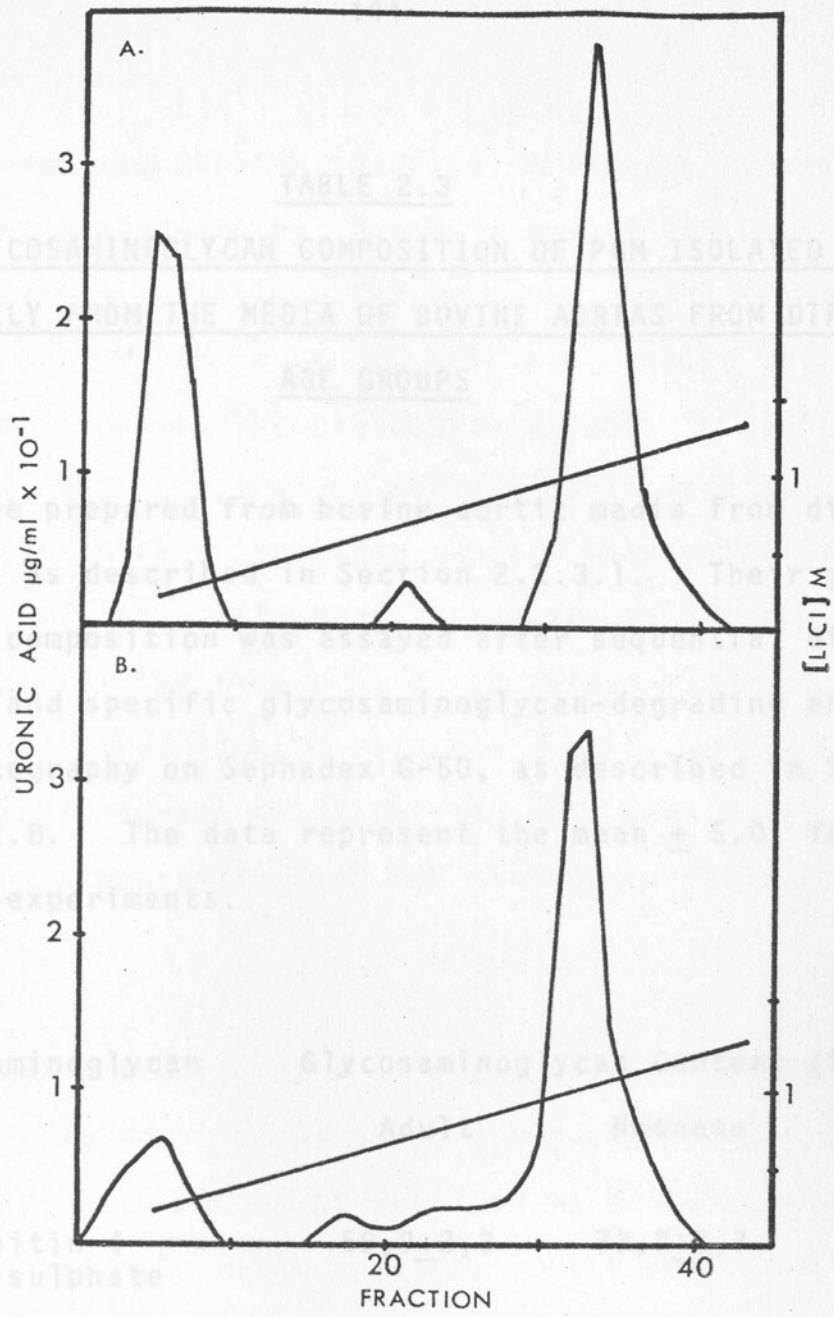



Fig. 2.15. DEAE-cellulose ion-exchange chromatography of glycosaminoglycan chains associated with individual proteoglycan species separated by Sepharose CL-2B chromatography of material extracted from adult medial tissue.

Proteoglycans were isolated under dissociative conditions from the aortic media of adult animals as described in Section 2.2.3.1. The bottom (D1) fraction was collected after dissociative density gradient centrifugation and chromatographed on a preparative Sepharose CL-2B column as described in Section 2.2.6. Material corresponding to peak I (A) and peak II (B) (see Fig. 2.4a) was pooled and processed as described under Fig. 2.14, prior to chromatography on DEAE-cellulose columns as described in Section 2.2.7. Column fractions were assayed for their uronic acid content.

TABLE 2.3

THE GLYCOSAMINOGLYCAN COMPOSITION OF PGM ISOLATED DISSOCIATIVELY FROM THE MEDIA OF BOVINE AORTAS FROM DIFFERENT AGE GROUPS

PGM were prepared from bovine aortic media from different aged animals as described in Section 2.2.3.1. Their glycosaminoglycan composition was assayed after sequential digestion with papain and specific glycosaminoglycan-degrading enzymes and chromatography on Sephadex G-50, as described in Sections 2.2.4 and 2.2.8. The data represent the mean ± S.D. for three different experiments.

| Glycosaminoglycan | Glycosaminoglycan Content (% of total) | | |
|---|---|---|---|
| | Adult | Neonate | Foetus |
| Chondroitin-4- and -6-sulphate | 59,9±3,3 | 73,2±2,7 | 88,6±4,5 |
| Dermatan sulphate | 17,6±1,1 | 12,0±1,1 | 0 |
| Heparan sulphate/ heparin | 10,3±2,0 | 6,4±0,6 | 4,8±0,4 |
| Hyaluronic Acid | 12,2±2,8 | 8,4±1,8 | 6,6±0,6 |

chains were chondroitin sulphate in all age groups. Only chondroitin sulphate was found in foetal PGM with no significant dermatan sulphate being detected. The dermatan sulphate content of the PGM increased with age, making up 12% of the uronic acid-containing glycosaminoglycans in neonate and 18% in adult PGM. Heparan sulphate/heparin made up 10% or less of the PGM in each age group and its concentration seemed to increase slightly with age. In spite of the PGM having been isolated from the tissue by dissociative extraction techniques and dissociative caesium chloride density gradient centrifugation, small amounts of hyaluronic acid were still found in the final PGM preparation from the most dense fraction of the gradient.

Thin layer chromatography of disaccharides released from the glycosaminoglycans of each age group after digestion with chondroitinases ABC or AC resulted in the disaccharides being fractionated into three species: those sulphated at the 4-position, those sulphated at the 6-position and unsulphated disaccharides. Quantitation of these species proved difficult as the spots were not sufficiently defined to yield reproducible results. However, it was consistently noted that in the disaccharides derived from adult tissue the spot corresponding to chondroitin-6-sulphate took up more stain than did that corresponding to chondroitin-4-sulphate, whereas in the disaccharides from the younger tissue they stained with about the same intensity.

## 2.4. DISCUSSION

In this study proteoglycans have been isolated from the medial layer of bovine aortas from different age groups. Histology showed that the tissues used for extraction were pure media and thus the proteoglycans isolated were only those synthesized and secreted by the smooth muscle cells of the media. The total glycosaminoglycan content of the media was shown to decrease with age, a change that has been noted by other workers (289,293). In addition, the water content of the aorta paralleled its glycosaminoglycan content, supporting the theory that proteoglycans are important for trapping water in tissues (55).

When the different glycosaminoglycans of the media were quantified it was noted that hyaluronic acid made up a large proportion of the glycosaminoglycans in tissues from all age groups and its amount increased slightly with age (Table 2.2). This finding agrees with those of other workers who noted that hyaluronic acid made up almost one-third of the glycosaminoglycans in the aorta (284). Chondroitin sulphate made up the bulk of the sulphated glycosaminoglycans in all age groups studied, with the amount of chondroitin-6-sulphate increasing with age. Studies of changes with maturation have also shown an increase in chondroitin-6-sulphate relative to chondroitin-4-sulphate for both cartilage and aortas (268.271,272,292). Toledo and Mourão have shown that chondroitin-6-sulphate is more abundant in adult human aortas, whereas in young aortas chondroitin-4- and -6-sulphate are present in approximately

equal amounts (292). Small amounts of heparan sulphate/ heparin were found in tissues from all ages and the amount increased with age, a change that has been noted by some workers (284). Dermatan sulphate was hardly detected in the younger tissue but its concentration increased with age, a finding also reported earlier (284).

The extraction techniques devised for the isolation of proteoglycans from cartilage seemed to be effective in the isolation of proteoglycans from the aorta. With foetal and neonatal tissue high yields of uronic acid (70% or higher) were obtained after extraction for 48 h with 4 M guanidine HCl containing proteolytic inhibitors. With the thick adult tissue, the yields were not so good (about 50%) but still compared favourably to yields obtained by other workers using several different techniques (289-291,298). The dissociative caesium chloride density gradient centrifugation was effective in separating the PGM from the majority of coextracted proteins, since most of the latter (90%) were found in the top of the gradient after centrifugation, whereas less than 15% of the uronic acid was found in this fraction. Precipitation with ethanol should be avoided during isolation since it resulted in the formation of artifactual complexes which were excluded from Sepharose CL-2B (Fig. 2.7), and thus can give false representation of the amount of material able to form aggregates with HA.

Analysis of extracted PGM, following caesium chloride buoyant density centrifugation, on Sepharose CL-2B showed them to be

heterogeneous in size. They were smaller and exhibited lower buoyant densities than cartilage proteoglycans and there was a broader distribution throughout the bottom half of a caesium chloride gradient as compared to cartilage PGM (30,31). On Sepharose CL-2B chromatography, two sizes of PGM were found; the larger had a $K_{av}$ of 0,31 and was thus similar in size to that described by Oegema et al. (291) and Wight and Hascall (281). The amounts of each species varied within the different ages, the larger species predominated in younger tissue and the smaller species ($K_{av}$ 0,56) became predominant in older tissue. In agreement with some other workers (281,291,303), we have found the glycosaminoglycan chains to be at least twice as long as those from cartilage PGM (18). Analysis of these chains on Sepharose CL-6B showed them to be longer in PGM from younger tissue and this may, in part, account for the differences noted in the sizes of PGM. There may be the same number of chains attached to a comparable core protein in both young and older animals, but the decreased chain length in older animals would lead to a decrease in PGM size. However, it was possible that the number of chains also varied, perhaps as a consequence of an increased core protein length making available in increased number of glycosaminoglycan initiation sites. This would lead to changes in monomer size, particularly in the younger animals where two monomer sizes were noted.

The presence of keratan sulphate chains has not been demonstrated conclusively, but has been suggested by some authors

(291,306). The presence of keratan sulphate-like glycosaminoglycans/oligosaccharides was suggested by the presence of sialic acid in PGM preparations from all ages. These chains were shown to be much smaller than the uronic acid-containing glycosaminoglycan chains and did not seem to occur as a specific keratan sulphate-rich peptide cluster region on core protein as is the case in cartilage (15). They may represent potential oligosaccharide-keratan sulphate initiation sites.

Comparison on Sepharose CL-6B of PGM digested with either chondroitinase ABC or AC followed by papain demonstrated that adult and probably neonatal material, but not foetal material, contained dermatan sulphate that was resistant to digestion with chondroitinase AC. In addition, the dermatan sulphate was very heterogeneous in size and generally was much smaller than the size determined for intact glycosaminoglycan chains. These observations support the findings of several laboratories that dermatan exists in aortic PGM as copolymers, with lengths of iduronic acid-containing dermatan sulphate units interspersed among glucuronic acid-containing chondroitin sulphate units to form chondroitin sulphate-dermatan sulphate hybrids (287,290). The absence of dermatan sulphate in foetal PGM was confirmed after quantitation of the glycosaminoglycans found in PGM preparations from the different ages. We have used susceptibility to specific glycosaminoglycan degrading enzymes to quantitate these chains in our proteoglycan samples. We consider this to be a better method for analysis than electrophoretic separation, as many factors can alter the electro-

phoretic mobility of the glycosaminoglycans (e.g. degree of sulphation) and thus electrophoretic mobility may not be definite proof of chemical identity. The foetal PGM contained only chondroitin sulphate with less than 1% dermatan sulphate, but the amount of dermatan sulphate increased with age with a concomitant decrease in the amount of chondroitin sulphate. The PGM derived from adult tissue contained 18% dermatan sulphate. This level was similar to that found for proteoglycans from bovine aortas as described by Kresse et al. (287) but is higher than the amount found by Oegema et al. (291), and Ehrlich et al. (290). The pattern of glycosaminoglycans found in the PGM samples paralleled that found in the tissue and it seems that 4 M guanidine HCl extracted a representative proportion of the total glycosaminoglycans and not only one type. Heparan sulphate has been shown to most resist extraction by dissociative solvents, but the aortic medial tissue was shown to contain only small amounts of this glycosaminoglycan (Table 2.2) so this did not pose a serious problem for extraction.

In spite of having undergone a dissociative extraction and caesium chloride gradient centrifugation, our final PGM preparations contained significant amounts of hyaluronic acid. The hyaluronic acid content of the media was found to be very high and, although it was broadly dispersed throughout the middle fractions of caesium chloride gradients during purification. It was not therefore surprising that some was found associated with material at the bottom of the tube. The high content of

hyaluronic acid in the media of all ages suggests that the PGM should be able to interact with it to form large molecular weight aggregates as has been found in the case of cartilage PGM. However, this does not appear to occur with aortic material since little aggregation occurred with adult PGM and no evidence for aggregate formation with material from foetal and neonatal aortas was obtained. In spite of the large concentration of hyaluronic acid in the young tissues, when proteoglycans were extracted dissociatively and then dialysed to associative conditions, no evidence of aggregate formation with endogenous hyaluronic acid was observed. Addition of an excess of exogenous large molecular weight hyaluronic acid also did not result in the formation of aggregates. Thus it seems as if the PGM from young aortic medial tissue are not able to aggregate with hyaluronic acid. Dialysis of a dissociative extraction of proteoglycans from adult medial tissue to associative conditions, however, resulted in about 25% of the material being excluded from a Sepharose CL-2B column, indicating that at least a portion of the adult PGM was able to form large molecular weight aggregates with endogenous hyaluronic acid. Addition of exogenous hyaluronic acid, however, resulted in lower amounts of aggregate formation (less than 10%). This difference between the amount excluded from the column in associative preparations and after addition of exogenous hyaluronic acid to a dissociative preparation was also observed by Oegema et al. (291). Perhaps some additional factors are needed which are separated from the PGM during the dissociative caesium chloride spin and in their absence the PGM cannot aggregate with exogenous hyaluronic acid to any great extent. Such factors may also be lacking in young

tissue, accounting for the inability of the PGM from these tissues to aggregate. The potential to form aggregates may be present but may not be expressed until later, when all the necessary components are present. Our findings support those of several other authors who have all reported some degree of aggregate formation between aortic proteoglycans and hyaluronic acid (291,299,305). The results also support the view that such aggregation may not be as physiologically important as it is in cartilage tissue. Other interactions such as those between PGM and matrix proteins or with other PGM or between hybrid chondroitin sulphate-dermatan sulphate chains, as discussed in Section 1.4, may be just as important or even more so in organizing the proteoglycans within the extracellular matrix of the aorta. On dialysis of the initial dissociative extract to associative conditions, large amounts of proteoglycan precipitated with coextracted proteins which caused significant losses of material. This has not been observed during the extraction of proteoglycans from cartilage and may reflect a tendency of the aortic proteoglycans to interact with these proteins at low ionic strength.

One problem we have not been able to solve is the contamination of our PGM preparations with glycogen, which gives a positive reaction with the carbazole method used for detecting uronic acids. This contamination was negligible in PGM material prepared from adult tissue but is significant in PGM isolated from young tissues. Neither digestion with amylase nor precipitation with cetylpyrididinium chloride proved satisfactory as additional routine preparative procedures. However, the quantitation of the glycogen content of our preparations and the

subsequent correction of data by the amount determined has enabled accurate analysis to be performed. Glycogen has been shown to be mostly associated with the species eluting later on a Sepharose CL-2B column.

The proteoglycans isolated from aortas are similar to cartilage proteoglycans in that they contain chondroitin sulphate as the major sulphated glycosaminoglycan. However, they are smaller than cartilage proteoglycans, have longer glycosaminoglycan side chains and are unable to interact with hyaluronic acid to the same extent. These findings agree with those of several workers regarding the polydispersity, the glycosaminoglycan chain length and the ability to interact with hyaluronic acid (281,288,291). However, the existence of different species of PGM, each containing side chains with a specific glycosaminoglycan composition and differing in their ability to interact with hyaluronic acid as described by other workers (54, 299.303), was not found. In our system, material from adult tissues eluted predominantly as one peak on Sepharose CL-2B and contained chondroitin sulphate, dermatan sulphate, heparan sulphate/heparin and sialic acid-containing oligosaccharide/keratan sulphate chains. The smaller species was presumed to be the one that interacted preferentially with hyaluronic acid since it was found that only PGM from adult tissue could form aggregates to any degree. PGM from foetal tissue which contained the large proteoglycan species predominantly, could not form aggregates with hyaluronic acid. These findings were in direct contradiction to findings of others

(54,303-305) who describe their large proteoglycan species as being the one able to aggregate with hyaluronic acid.

The changes observed with age, such as a decreased glycosaminoglycan content in older tissue and possibly increased chondroitin-6-sulphate content, were similar to those observed with cartilage (265-271,273). The proteoglycans from aortas of different ages were fairly similar in glycosaminoglycan composition but differed strikingly in terms of their ability to interact with hyaluronic acid. The appearance of dermatan sulphate in older tissues may be physiologically important, as it is this species that has the greatest ability to interact with macromolecules around it. It is considered to play important roles in processes such as atherosclerosis and anticoagulation, and it has also been shown to bind to several proteins in human tissue. It may be important in the response of the arterial wall to certain stimuli, and may help to organize collagen and proteoglycans within the matrix.

# CHAPTER 3

# CHARACTERIZATION OF CULTURED BOVINE AORTIC SMOOTH MUSCLE CELLS

## 3.1. INTRODUCTION

The characterization of the proteoglycans isolated from bovine aortic media of different age groups enabled me to approach the task of culturing aortic smooth muscle cells and the study of their proteoglycan synthetic capacity with a base-line of knowledge regarding the types of proteoglycans I should expect to find. The use of cultured cells for the study of metabolic pathways such as biosynthesis, secretion and turnover has contributed greatly to our general biochemical knowledge; however, it is necessary to clearly establish whether the cells in culture are representative of the situation *in vivo*. The characterization, biosynthesis and secretion of proteoglycans have been studied using cultured chondrocytes and fibroblasts from various sources, but information concerning the production of proteoglycans by cultured smooth muscle cells is as yet limited. In this study two lines of foetal bovine aortic medial smooth muscle cells have been used to characterize the proteoglycans synthesized and secreted by these cells. Cells were isolated from medial tissue of thoracic aortas from foetuses having crown-to-tail lengths of 45 to 50 cm. This was the same size of foetus used for the preparation of foetal PGM as described in Chapter 2. Thus the direct comparison of proteoglycans produced by cultured cells and extracted from medial tissue could be undertaken. Before the characterization of the

proteoglycans synthesized by the cultured cells could be undertaken, it was important to thoroughly quantitate various parameters of the culture system such as growth rate, matrix production and proteoglycan synthesis and secretion in the absence or presence of added ascorbic acid.

## 3.2. METHODS

### 3.2.1. Preparation of cell cultures

$AS_1Cl_6$ was a clonal cell line derived from explants obtained from the media of the thoracic aorta of a bovine embryo and prepared according to the method of Ross (328). $A_3$ was an uncloned mass culture also obtained from foetal bovine aortic medial explants. The embryos used were in the first trimester of foetal life (crown-to-tail lengths 45 to 50 cm). Hearts and thoracic aortas were excised from foetuses using sterile techniques. A portion of the aorta was cleaned of adherent fat, rinsed and cut into smaller circular segments in 100 mm Petri dishes containing small amounts of medium: Eagle's minimum essential medium, buffered with Earle's salts and containing 10% tryptose phosphate broth (Difco), 10% heat-inactivated foetal calf serum (inactivated at 56°C for 30 min), 60 µg/ml penicillin G and 100 µg/ml streptomycin sulphate. The portions of aorta were cut open to expose the lumen. The intimal surface was scraped with a blade and medial shreds of less than one-third of the aortic wall thickness were stripped from the surface with fine-tipped forceps. These shreds were cut into approximately 1 mm squares for explants. The explants

were transferred into 100 mm Petri dishes and covered with a small amount of medium. The medium was used sparingly in order to promote adherence to the plastic surface. Explants were maintained at 37°C in a humidified atmosphere of 95% air, 5% carbon dioxide. After a few days, additional medium was added and thereafter medium was changed carefully every 3 days. After about 7 to 10 days, outgrowths of cells from explants could be clearly seen using a Nikon inverted microscope. The lumps of explanted medial tissue were washed free from the outgrowing cells and removed from culture dishes at this stage. When outgrowths appeared confluent they were trypsinized (0,05% (w/v) Difco trypsin 1:250 containing 0,002% (w/v) EDTA), and either grown to large numbers (uncloned mass culture-$A_3$) or plated into 60 mm Petri dishes at a clonal density of between 100 and 400 cells/4 ml medium. These were left for one to two weeks to allow colony formation. Dishes were marked to identify single cells that gave rise to viable colonies. During this time care was taken not to disturb the dishes in order to prevent secondary seeding. These colonies were later harvested using trypsin and the clonal ring technique; harvested cells were grown to large numbers. Subsequent divisions were performed when growth reached confluency. Cells were passaged by trypsination and stored in liquid nitrogen in 1 ml aliquots containing $10^6$ cells in medium containing 10% (v/v) dimethylsulphoxide (DMSO). For initiation of new cultures, frozen cells were thawed in a waterbath at 37°C and transferred to 75 $cm^3$ tissue culture flasks containing 14 ml of fresh medium. The medium was

changed the following day to ensure the removal of the DMSO. Cells were used between passages 6 and 13 in culture and were cultured in plastic tissue culture flasks at a seeding density of $10^6$ cells/75 $cm^3$ flask or in 35 mm plastic Petri dishes at a seeding density of $10^5$ cells/35 mm dish.

### 3.2.2. Determination of cell number

For the determination of cell numbers, medium was removed from the cultures and the cell layers were washed with phosphate-buffered saline (PBS). A mixture of 0,25% (w/v) Viokase and 0,05% (w/v) crude collagenase was added to the cell layers, and they were incubated at 37°C for 30 min. Cell numbers were determined in a Coulter Counter - Model Zf (Coulter Electronics Inc., Hialeah, Florida, USA) after dispersion of the cells. Trypsin was inadequate for cell number determinations late in culture, especially in those cultures grown in the presence of ascorbic acid, since the cells produced an extensive cross-linked trypsin-insensitive matrix which enmeshed cells and resulted in erroneous cell numbers.

### 3.2.3. Determination of cellular protein

To determine the amount of cellular protein, the medium was removed from the cultures and the cell layers washed with PBS. Cells were lysed by the addition of 1% (w/v) sodium dodecyl sulphate (SDS) for 30 min at room temperature. The lysates were collected and a sample analysed for the protein content by the method of Lowry *et al.* (323) using bovine serum albumin

in 1% SDS as standard. The matrix proteins were not solubilized by SDS.

3.2.4. Determination of matrix collagen production

Cells were cultured in 35 mm dishes as described in Section 3.2.1. for the requisite number of days in the presence or absence of ascorbic acid (50 µg/ml culture medium) which was added daily. During the final 24 hours of culture, medium contained, in addition to ascorbic acid (see above), 10 µCi L-|5$^{3}$H| Proline. At the end of incubation, culture medium was removed, cell layers washed 3 times with PBS and cells were solubilized by the addition of 1 ml of 0,5 M ammonium hydroxide for 20 min. Culture dishes with their adherent matrix proteins, which were not solubilized by the above procedure (329), were washed extensively with distilled deionized water, followed by 70% (v/v) ethanol prior to air-drying. Dishes containing dry radioactively labelled matrix material were digested with 1 ml of solution which contained 20 µg pure collagenase, 20 mM Tris/HCl pH 7,4, 2,5 mM N-ethylmaleimide and 10 mM calcium chloride. After digestion for 12 h at 37$^{o}$C, 500 µl aliquots were removed and assayed for released radioactivity. Samples were counted using scintillation mixutre 299 (see Appendix) in a Beckman Liquid Scintillation Counter Model LS 9000.

3.2.5. Determination of matrix protein composition

Radioactively-labelled matrix components were quantified according to the procedure of Jones et al. (330). Cell cultures

were grown for 7 days in the presence or absence of ascorbic acid. On the seventh day 10 μCi of |$^{3}$H| Proline was added with fresh medium and the cells incubated for a further 24 h. Alternatively, |$^{3}$H| Proline was present throughout the culture period. Following incubation, the medium was removed and the radiolabelled matrices prepared as described in Section 3.2.4. The dried matrices were subjected to sequential proteolytic digestion, first with trypsin (EC.3.4.21.4), then elastase and finally collagenase, all at 37°C for 3 h. The enzymes were all used at concentrations of 20 μg/ml in 0,1 M Tris/HCl pH 7,4, containing 10 mM calcium chloride. After each 3 h digestion period aliquots were removed for counting and the dishes were washed 3 times with distilled deionized water before the next enzyme was added. Samples were counted in scintillation mixture as described in Section 3.2.4.

### 3.2.6. Assay of macromolecular |$^{35}$S| sulphate incorporation into the medium and cell-matrix layer

Cells were cultured as described in Section 3.2.1 for the requisite number of days in either the presence or absence of ascorbic acid, prior to the addition of 10 μCi of |$^{35}$S| sulphate/ml culture medium for 24 h. At the end of the labelling period the culture medium was collected and its volume measured. Cell layers were washed 3 times with PBS and then extracted with 4 M guanidine HCl containing 0,05 M sodium acetate, 0,1 M 6-aminocaproic acid, 0,01 M EDTA, 5,0 mM benzamidine HCl and 250 mM phenylmethylsulphonylfluoride, all at pH 5,8 for 2 h.

Medium samples were made 4 M with respect to guanidine HCl by addition of solid material and a mixture of proteolytic inhibitors was added from stock solutions. The separation of macromolecular sulphated molecules from unincorporated radioactive sulphate was carried out by chromatography of samples of medium and cell layer extracts on Pharmacia PD-10 columns equilibrated and eluted with 4 M guanidine HCl, 0,05 M sodium acetate pH 5,8. The columns were calibrated with respect to the $V_o$ and $V_t$ using dextran blue and $|^{35}S|$ sulphate, respectively. Fractions were counted for radioactivity using "Instagel" (see Appendix).

### 3.2.7. Analysis of macromolecular $|^{35}S|$ sulphate incorporation into the extracellular, pericellular-matrix and intracellular culture compartments

Cells were cultured in 35 mm dishes as described in Section 3.2.1 for the requisite number of days, in the presence or absence of ascorbic acid, prior to the addition of $|^{35}S|$ sulphate as described above. At the end of the labelling period culture medium was collected, its volume measured and it was retained as the extracellular compartment for later analysis. The cell layers were washed 3 times with PBS and then incubated for 30 min at 37°C with 1 ml of a mixture of 0,25% (w/v) Viokase and 0,05% (w/v) crude collagenase containing 30 mM sodium fluoride and potassium cyanide to prevent further secretion of macromolecules from the intracellular compartment (331). The cell suspensions obtained after Viokase/collagenase treatments were transferred to centrifuge tubes

after gentle aspiration with a Pasteur pipette. Samples were centrifuged for 2 min in a bench centrifuge at 2000 rpm in order to pellet cells, and supernatants were retained as the pericellular-matrix compartment. Cell pellets were lysed in 500 µl PBS by sonication for 30 sec with a Branson Sonifier, microtip 50 W (Branson Sonic Company, USA). This fraction was designated as the intracellular compartment. The separation of macromolecular sulphated molecules from unincorporated precursor isotope was routinely carried out as follows: Aliquots (100 µl) of samples from all compartments were subjected to descending paper chromatography on Whatman 3 MM chromatography paper (25 x 45 cm). Chromatograms were eluted overnight using 1-butanol:glacial acetic acid:1 N ammonium hydroxide 2:3:1,5 (v/v/v) as solvent (332). After elution, chromatograms were dried and the macromolecular material that remained at the origin was cut out and counted in scintillation mixture as described in Section 3.2.4. Under these chromatographic conditions, radioactive precursors ($|^{3}H|$ glucosamine or $|^{35}S|$ sulphate) were eluted from the origin but macromolecular labelled material remained. Corrections for background counts due to non-specific binding of the labelled precursor to macromolecules at the origin were routinely carried out on each chromatogram by counting 100 µl aliquots of fresh medium containing the requisite amount of radioactive isotope as used in any experiment. The values obtained for radioactivity remaining at the origin under these conditions were subtracted from those found for test samples. They were routinely less than 5% of the value for macromolecular labelled material.

## 3.3. RESULTS AND DISCUSSION

Bovine aortic smooth muscle cells showed an initial fast rate of proliferation during the first few days of culture when plated at $10^5$ cells per 35 mm Petri dish (Fig. 3.1). Once cells reached confluency, about 6 to 8 days after plating, their rate of growth slowed down; however, they still continued to divide, and formed thick multilayers of characteristic "hills and valleys" (328,333) when viewed under light microscopy (Plate 3.1). In addition, they displayed the typical morphology of modulated smooth muscle cells under electron microscopy (334), namely an abundance of thin filament bundles containing many dense bodies and also a large number of plasmalemmal vesicles (Plate 3.2). Ascorbic acid-supplementation of the cultures had no gross effects on their morphology, which is contrary to the results of Schwartz *et al*. (335), but resulted in the cells showing a faster rate of proliferation. Cultures supplemented with ascorbic acid exhibited higher cell numbers than the corresponding non-supplemented cultures.

The amount of matrix collagen produced per day for cultures grown either in the presence or absence of ascorbic acid was determined as described in Section 3.2.4. Production of collagen in the matrix started early in culture with vitamin-supplemented cells and increased steadily until day 10, when it peaked and thereafter declined slowly (Fig. 3.2). This is at variance with the findings of Schwartz *et al*. (335), who showed that the amount of collagen in the extracellular

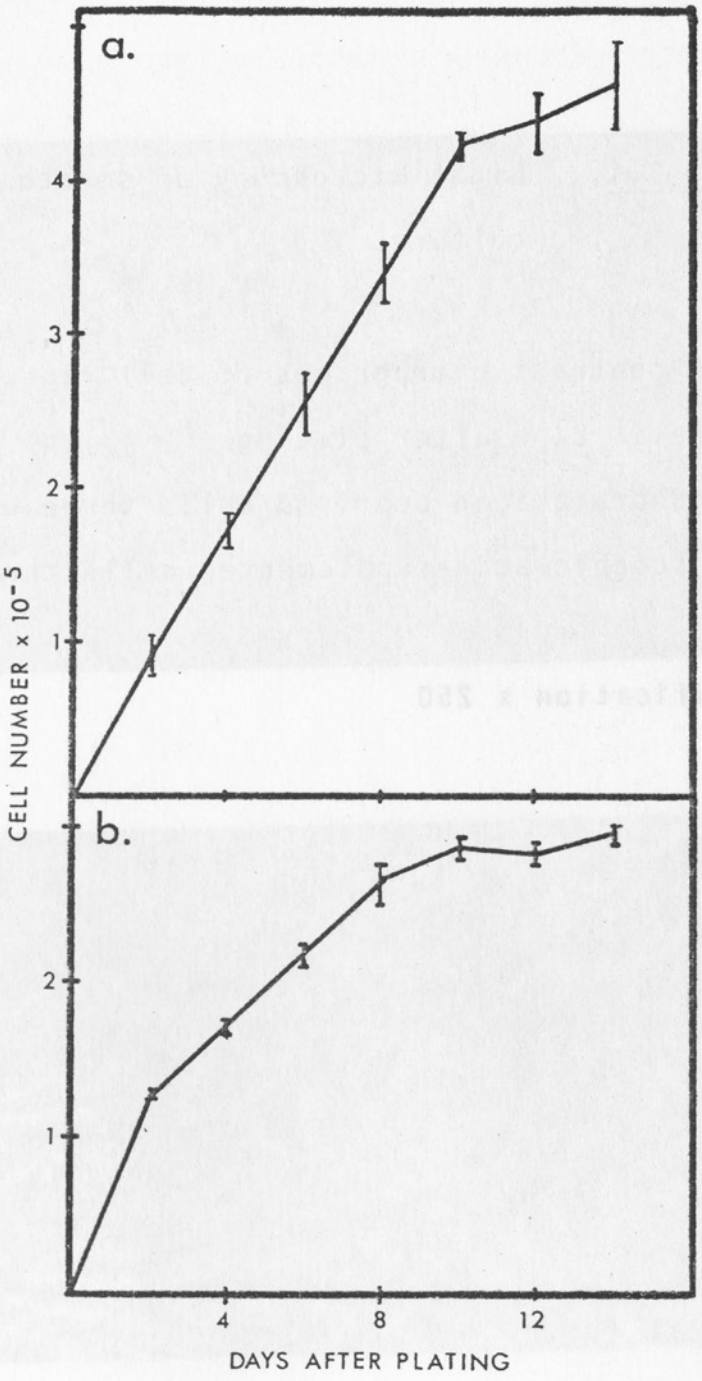



Fig. 3.1. Growth curve of $AS_1Cl_6$ in culture.

$AS_1Cl_6$ smooth muscle cells, plated at $10^5$ cells/35 mm dish, were grown in the presence (a) or absence (b) of ascorbic acid, and harvested every second day for determination of cell number as described in Section 3.2.2. Each time point represents the mean ± S.D. for triplicate dishes. These results represent one of several experiments.

Plate 3.1. Light microscopy of smooth muscle cells in culture.

Phase contrast micrographs of cell line $A_3$ in culture:

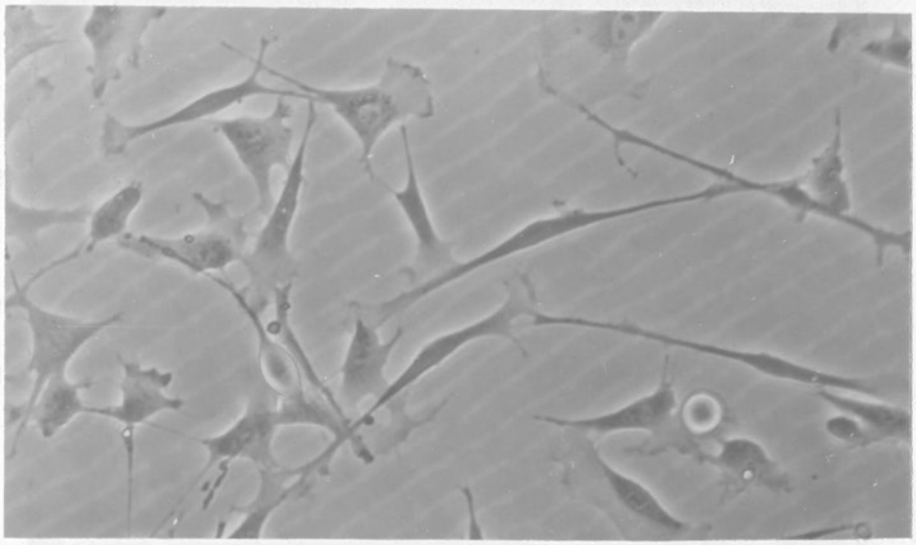
A. Cells 24 h after plating

B. Ascorbic acid-deprived cells three days after plating.

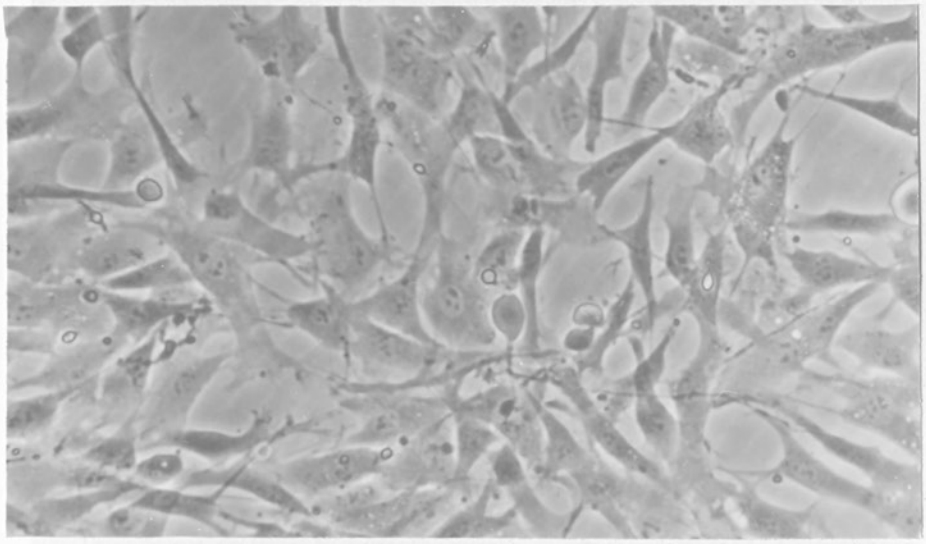
C. Ascorbic acid-supplemented cells three days after plating.

Magnification x 250

A.



B.



C.

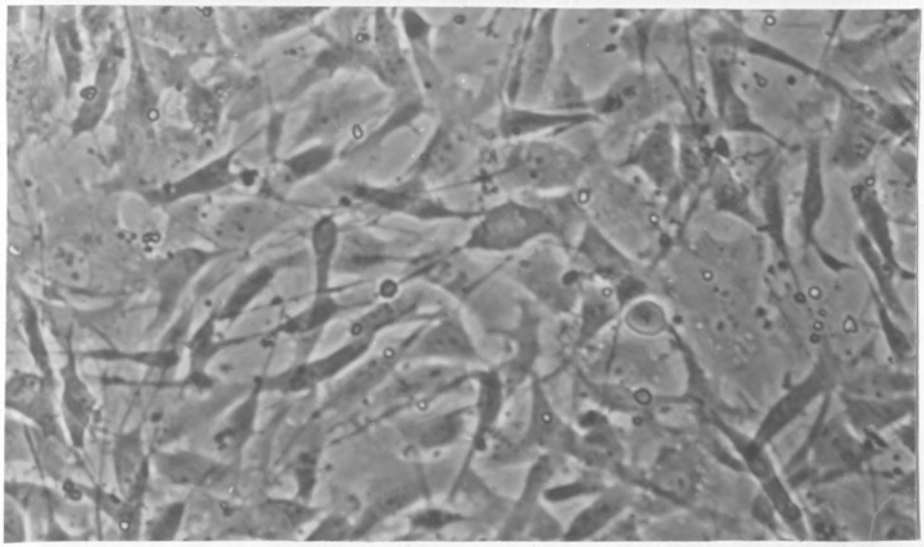


Plate 3.1. continued

D. Ascorbic acid-deprived cells five days after plating.

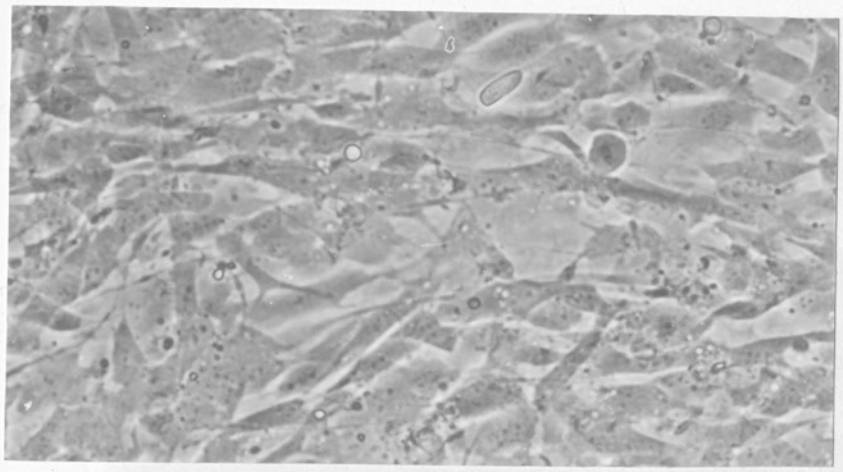
E. Ascorbic acid-supplemented cells five days after plating.

F. Ascorbic acid-deprived cells eight days after plating.

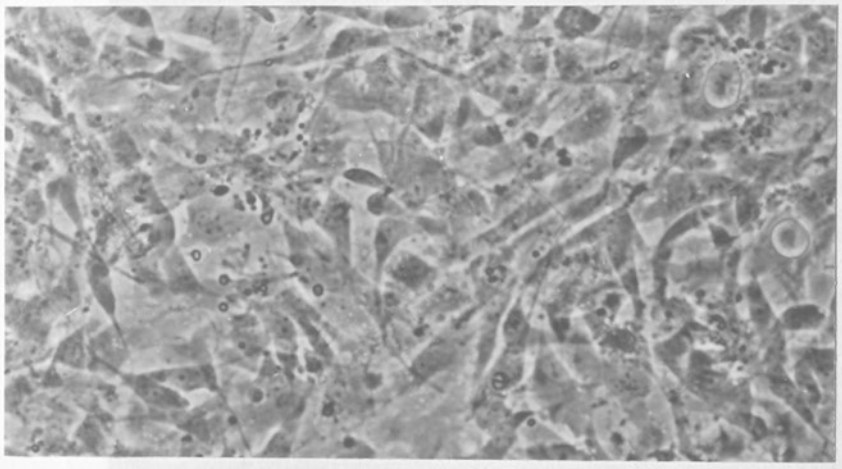
G. Ascorbic acid-supplemented cells eight days after plating.

Magnification x 250

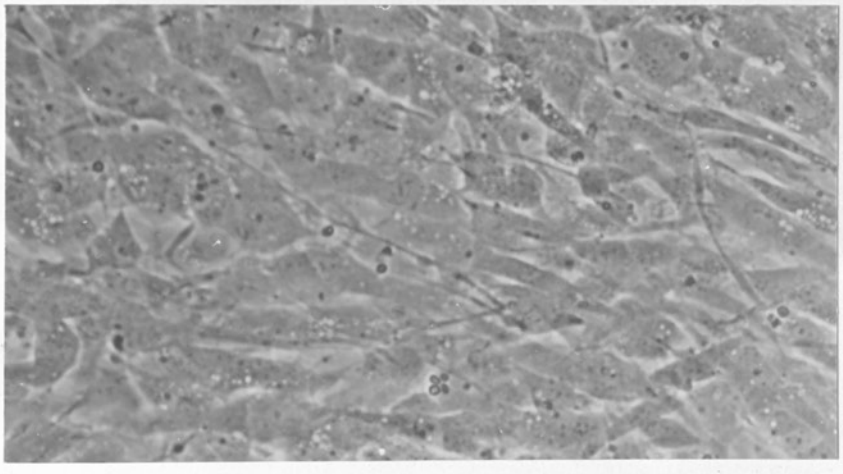
D.



E.



F.



G.

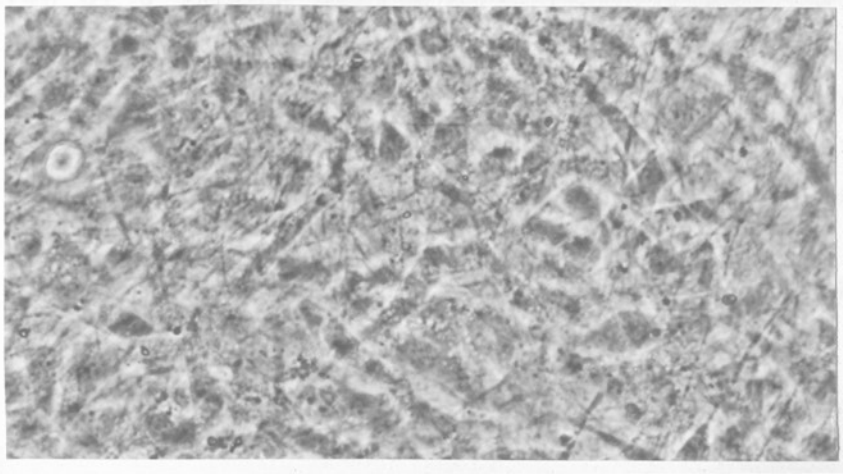



Plate 3.2. Transmission electron micrograph of cell line $AS_1Cl_6$ in culture (transverse section):

A portion of a smooth muscle cell in culture. The cell can be seen to be rich in thin filament bundles (f) and dense bodies (d), together with numerous other organelles, such as rough-surfaced endoplasmic reticulum (rer) and lysosomes (L). Note the presence of plasmalemmal vesicles (pv) and extracellular collagen (c).
This is a negative print, final magnification x 80000.

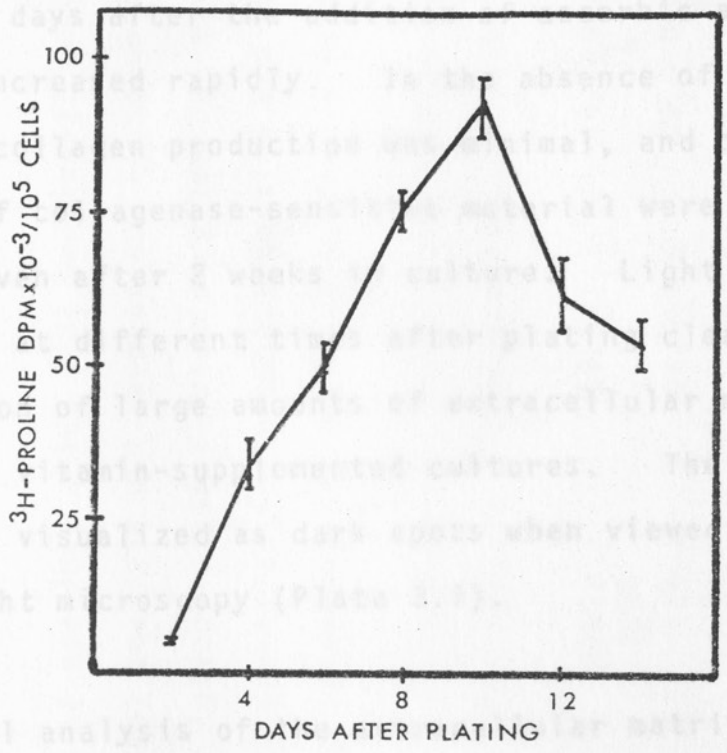



Fig. 3.2. Matrix collagen production by $AS_1Cl_6$ cultures grown in the presence of ascorbic acid.

$AS_1Cl_6$ smooth muscle cells (plated at $10^5$ cells/35 mm dish) were grown in the presence of ascorbic acid and harvested every second day for the quantitation of matrix collagen as described in Section 3.2.4. Each time point represents the mean $\pm$ S.D. for triplicate dishes. The results represent one of three similar experiments.

matrix of cultured calf aortic smooth muscle cells changed little for 6 days after the addition of ascorbic acid and thereafter increased rapidly. In the absence of ascorbic acid matrix collagen production was minimal, and only very low levels of collagenase-sensitive material were found in the matrix even after 2 weeks in culture. Light microscopy of the cells at different times after plating clearly showed the deposition of large amounts of extracellular matrix, particularly in vitamin-supplemented cultures. The matrix material was visualized as dark spots when viewed by phase contrast light microscopy (Plate 3.1).

Compositional analysis of the extracellular matrix proteins secreted by vitamin supplemented or deprived smooth muscle cells at confluency yielded the data shown in Table 3.1. It is clear that in both the $A_3$ and $AS_1Cl_6$ cell lines there was a marked increase in total matrix protein laid down in the presence of ascorbic acid. For $A_3$ and $AS_1Cl_6$ cell lines the ascorbic acid-supplemented cultures produced 8 times and 14 times more matrix proteins, respectively, than did the scorbutic cells. In addition, there was a marked difference in the types of matrix proteins synthesized by the cells with and without ascorbic acid. In the absence of the vitamin most of the $|^3H|$ proline in the matrix was sensitive to trypsin digestion (86 to 88%), which indicated the presence of non-cross linked glycoproteins and proteoglycans, whereas elastin (elastase-sensitive material) made up about 10% and collagen only 2 to 4% of the matrix proteins. In supplemented

TABLE 3.1

PROTEIN COMPOSITION OF EXTRACELLULAR MATRIX MATERIAL LAID DOWN BY SMOOTH MUSCLE CELLS IN THE PRESENCE AND ABSENCE OF ASCORBIC ACID

Cells were grown for 8 days in the presence or absence of ascorbic acid; $|^3H|$ proline (5 μCi/ml) was present during the final 24 h of culture. Compositional analysis was carried out by sequential enzymatic digestion as described in Section 3.2.5. The "glycoproteins" fraction given under the "percentage composition" reading refers to the material sensitive to trypsin digestion and is so described after the data of Jones et al. (330). The data represent the mean ± S.D. from 8 determinations.

| Smooth Muscle Cell line | Ascorbate | Total Radioactivity (dpm) | Radioactivity (dpm) released by | | | Composition (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | | Trypsin | Elastase | Collagenase | Glycoproteins | Elastin | Collagen |
| $AS_1Cl_6$ | + | 122007 ± 8530 | 33391 ± 1793 | 5698 ± 312 | 82918 ± 3986 | 27,3±1,1 | 4,7±0,2 | 68,0±1,0 |
| | - | 8487 ± 537 | 7470 ± 527 | 853 ± 144 | 164 ± 31 | 88,0±1,6 | 10,0±0,4 | 1,9±0,1 |
| $A_3$ | + | 526472 ± 42435 | 144380 ± 11186 | 31537 ± 2837 | 350555 ± 33491 | 27,5±1,2 | 6,0±0,4 | 66,5±1,5 |
| | - | 65589 ± 3580 | 56248 ± 3279 | 6510 ± 357 | 2831 ± 169 | 85,8±0,7 | 9,9±0,4 | 4,4±0,3 |

cultures, however, only about 27% of the |$^{3}$H| proline-labelled proteins were trypsin-sensitive, and 4 to 6% elastase-sensitive, but there was a large increase in the amount of material released by collagenase (66 to 68% of the total) (Table 3.1). This was a net increase of about 20-fold, and was consistent with the accepted role of ascorbic acid in collagen biosynthesis; namely, its role as a cofactor in the posttranslational conversion of proline and lysine residues in the newly formed collagen chain to hydroxyproline or hydroxylysine. These reactions are catalysed by the enzymes prolyl hydroxylase (EC,1.14.11.2) and lysyl hydroxylase (EC.1.14.11.4), respectively, and are necessary for correct structural organization of the procollagen molecule, leading to its incorporation into cross-linked collagen fibrils. A relative decrease in the elastin component of the extracellular matrix in the presence of ascorbic acid has been reported previously for rat heart smooth muscle cells (336).

The synthesis and secretion of proteoglycans by the cells was assessed over a period of 14 days in culture. Cultures grown in the presence of ascorbic acid cannot be maintained for much longer than 14 days, as the matrix becomes very thick and cell-matrix layers start to peel off dishes, and eventually round up and detach. This phenomenon has also been reported by Schwartz *et al.* (335). On alternate days until day 13, cultures were labelled with |$^{35}$S| sulphate for 24 h. At the end of the 24 h labelling period, the medium and guanidine HCl extracts of the cell layer were prepared as described in Section 3.2.6 and analysed for |$^{35}$S| sulphate incorporated into macro-

molecular material. Both $A_3$ and $AS_1Cl_6$ cell lines produced similar patterns of synthesis and secretion. The results obtained with $AS_1Cl_6$ cells are shown in Fig. 3.3. As may be seen from the figure, proteoglycan synthesis and secretion followed a similar pattern in both ascorbic acid-supplemented and non-supplemented cultures, with the amount of material being synthesized and secreted increasing until a peak was reached at 6 to 8 days after plating, and thereafter the rate of synthesis and secretion declined. The $|^{35}S|$ sulphate radioactivity in macromolecular material in the medium accounted for the majority of the total $|^{35}S|$ sulphate incorporated into macromolecular material. In ascorbic acid-supplemented cultures the amount of radioactivity in macromolecular material in the medium after 6 to 8 days was approximately 1,5 times higher than the amount of $|^{35}S|$ sulphate associated with macromolecular material in medium of cells grown without ascorbic acid for the same period. Likewise, the amount of radioactive macromolecular material in the extracts of cell layers from cells grown in the presence of ascorbic acid was about 1,5 to 1,8 times that found in extracts of cell layers from cells grown without ascorbic acid. Wight and Ross have reported that the rate of incorporation of labelled precursors was maximal during the stationary phase of growth and less during logarithmic growth periods (310). My results showed that incorporation rates were maximal just after the cells had reached confluency but thereafter they were not maintained at this high level and incorporation of $|^{35}S|$ sulphate into macromolecular material declined late in culture.

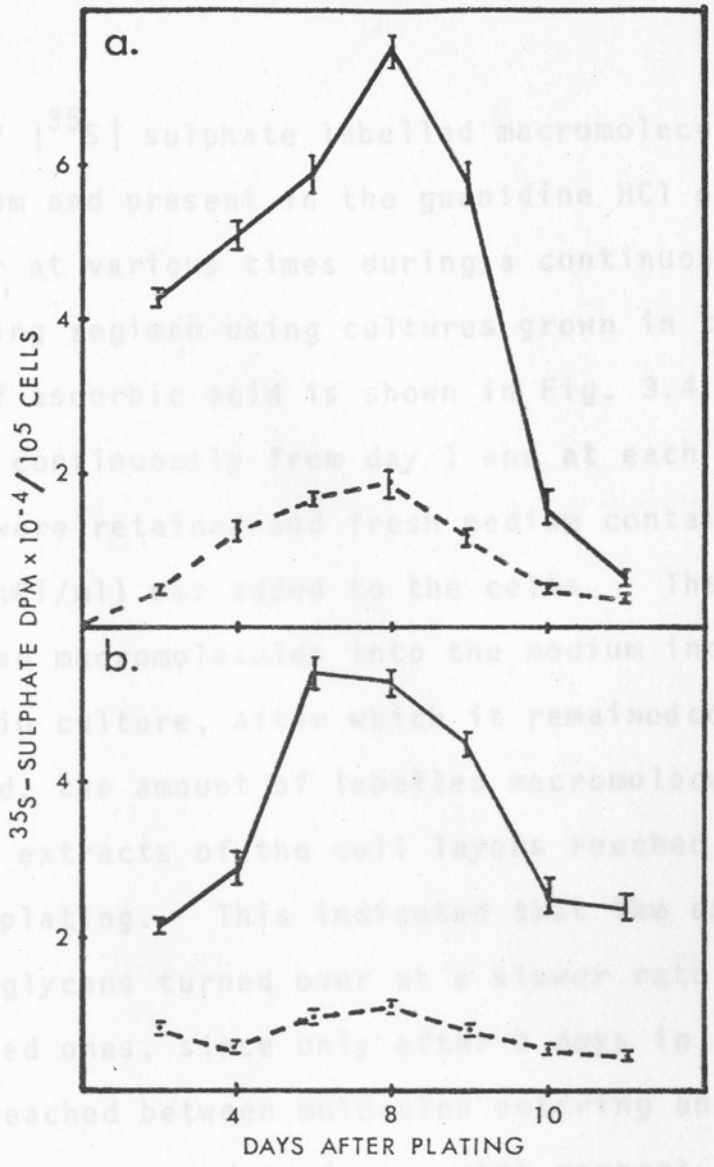



Fig. 3.3. Incorporation of $|^{35}S|$ sulphate into proteoglycans.

$AS_1Cl_6$ smooth muscle cells were plated at $10^5$ cells/35 mm dish and grown in the presence (a) or absence (b) of ascorbic acid. Cultures were labelled for 24 h with 10 µCi/ml of $|^{35}S|$ sulphate. At each time point the medium (-) and guanidine HCl extract of the cell layer (---) were collected and assayed for their total incorporated radioactive sulphate as described in Section 3.2.6. Each point represents the mean ± S.D. for triplicate dishes.

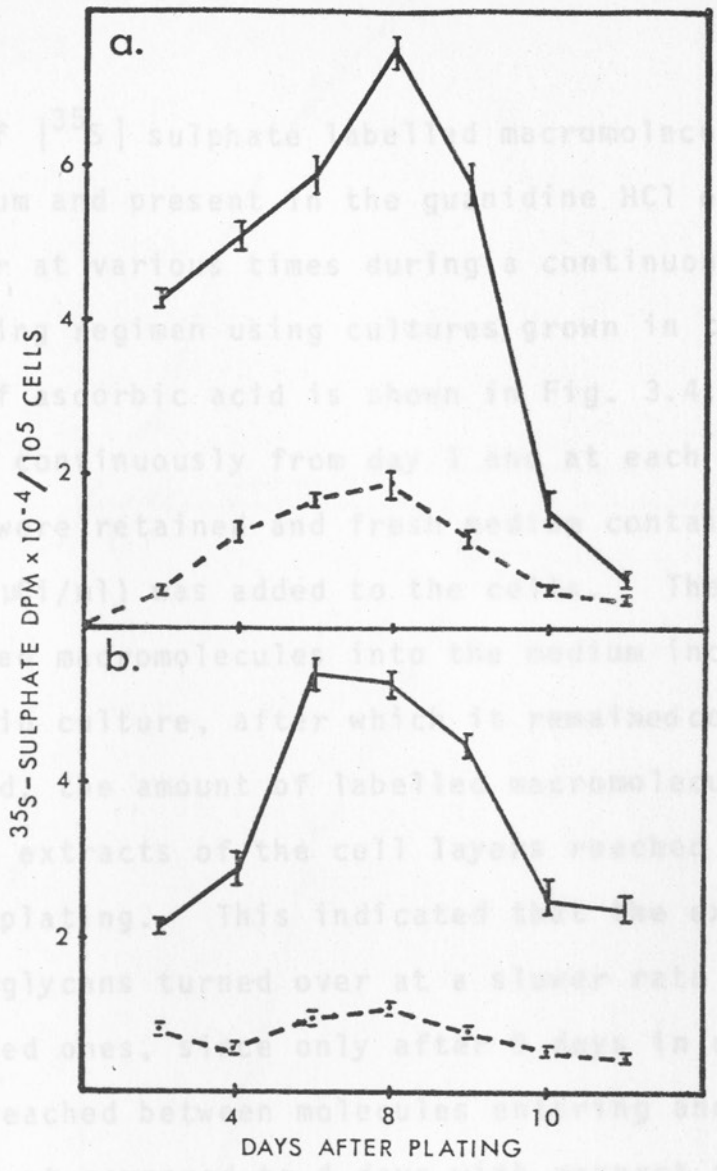



Fig. 3.3. Incorporation of $|^{35}S|$ sulphate into proteoglycans.

$AS_1Cl_6$ smooth muscle cells were plated at $10^5$ cells/35 mm dish and grown in the presence (a) or absence (b) of ascorbic acid. Cultures were labelled for 24 h with 10 µCi/ml of $|^{35}S|$ sulphate. At each time point the medium (-) and guanidine HCl extract of the cell layer (---) were collected and assayed for their total incorporated radioactive sulphate as described in Section 3.2.6. Each point represents the mean ± S.D. for triplicate dishes.

The amount of $|^{35}S|$ sulphate labelled macromolecules released into the medium and present in the guanidine HCl extract of the cell layer at various times during a continuous radioactive labelling regimen using cultures grown in the presence and absence of ascorbic acid is shown in Fig. 3.4. Cultures were labelled continuously from day 1 and at each medium change media were retained and fresh medium containing $|^{35}S|$ sulphate (10 µCi/ml) was added to the cells. The secretion of the labelled macromolecules into the medium increased up until 9 days in culture, after which it remained constant. On the other hand, the amount of labelled macromolecules in the guanidine HCl extracts of the cell layers reached equilibrium 4 days after plating. This indicated that the extracellular medium proteoglycans turned over at a slower rate than the cell-associated ones, since only after 9 days in culture was equilibrium reached between molecules entering and leaving this compartment compared to 4 days with respect to the proteoglycans found in the matrix/cell layer. It was routinely noted that the presence of ascorbic acid resulted in a greater incorporation of $|^{35}S|$ sulphate into macromolecular material in the medium and extracts of the cell layer of supplemented smooth muscle cell cultures as compared to scorbutic cultures. However, since ascorbic acid also resulted in increased cell numbers, it was of interest to see if there was a net increase in total $|^{35}S|$ sulphate incorporation in the presence of ascorbic acid, or whether the increased $|^{35}S|$ sulphate incorporation merely occurred as a result of a general increase in metabolic activity in the presence of the vitamin. As already mentioned, there were marked differences in the composition

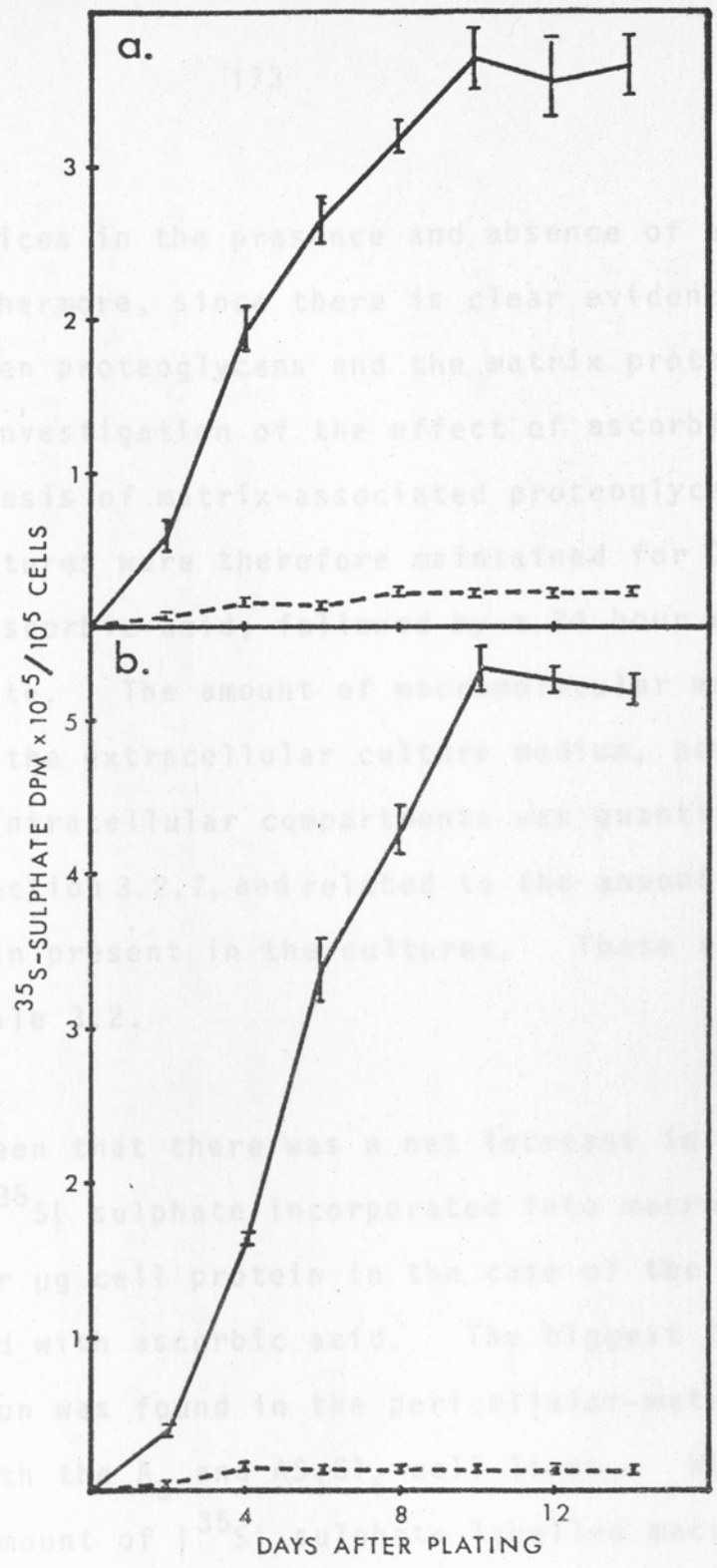



Fig. 3.4. Accumulation of $|^{35}S|$ sulphate labelled macromolecules in $AS_1Cl_6$ cultures.

$AS_1Cl_6$ smooth muscle cells were plated at $10^5$ cells/35 mm dish and grown in the presence (a) or absence (b) of ascorbic acid. Cultures were labelled continuously with $|^{35}S|$sulphate. At each time point the medium (-) and guanidine HCl extracts of the cell layer (---) were collected and assayed for their total incorporated radioactive sulphate as described in Section 3.2.6. Each point represents the mean ± S.D. for triplicate dishes.

of the matrices in the presence and absence of ascorbic acid. Furthermore, since there is clear evidence for interaction between proteoglycans and the matrix proteins (Section 1.4.1), an investigation of the effect of ascorbic acid on the biosynthesis of matrix-associated proteoglycans was undertaken. Cultures were therefore maintained for 7 days with or without ascorbic acid, followed by a 24 hour exposure to $|^{35}S|$ sulphate. The amount of macromolecular material associated with the extracellular culture medium, pericellular-matrix and intracellular compartments was quantitated as described in Section 3.2.7, and related to the amount of total cellular protein present in the cultures. These results are shown in Table 3.2.

It can be seen that there was a net increase in the total amount of $|^{35}S|$ sulphate incorporated into macromolecular material per µg cell protein in the case of the cultures supplemented with ascorbic acid. The biggest increase in incorporation was found in the pericellular-matrix compartment for both the $A_3$ and $AS_1Cl_6$ cell lines. With the $A_3$ cells the amount of $|^{35}S|$ sulphate labelled macromolecular material found in the extracellular and intracellular compartments did not change significantly in the presence of ascorbic acid, but for the $AS_1Cl_6$ cells an increased amount of incorporation of $|^{35}S|$ sulphate into macromolecular material was found in these compartments as well, although the increase was not as dramatic as that found with the pericellular-matrix compartment (Table 3.2). Thus, addition of

TABLE 3.2

THE EFFECTS OF ASCORBIC ACID ON THE INCORPORATION OF $|^{35}S|$ SULPHATE INTO THE DIFFERENT CULTURE COMPARTMENTS

Cells were grown for 8 days in the presence or absence of ascorbic acid; $|^{35}S|$ sulphate (10 μCi/ml) was present during the final 24 h of culture. The incorporation of $|^{35}S|$ sulphate into the medium (extracellular), pericellular-matrix and intracellular compartments of the culture system was carried out as described in Section 3.2.7. The data represent the mean ± S.D. of triplicate determinations in one of several similar experiments.

| Smooth Muscle Cell Line | Ascorbate | Incorporation of $\|^{35}S\|$ sulphate (dpm/μg cell protein) | | | |
|---|---|---|---|---|---|
| | | Total | Extracellular | Pericellular-Matrix | Intracellular |
| $AS_1Cl_6$ | + | 515,8±19,8 | 352,6±21,2 | 115,7±5,4 | 47,5±1,4 |
| | - | 300±12,4 | 238,7±9,3 | 33,6±2,3 | 27,7±1,1 |
| $A_3$ | + | 465,3±21,2 | 289,4±15,4 | 154,9±6,5 | 21,0±2,8 |
| | - | 368,6±17,5 | 311,5±15,7 | 37,6±2,6 | 19,4±1,6 |

ascorbic acid resulted in a net increase in the amount of $|^{35}S|$ sulphate incorporated into macromolecular material with the biggest effect being in the pericellular-matrix compartment. The nature of this effect will be discussed in detail in Chapter 5.

Therefore, both the $A_3$ and $AS_1Cl_6$ cell lines isolated from bovine foetal aortic media were shown to have the growth patterns and morphology characteristic of smooth muscle cells in culture. They were shown to produce an extracellular matrix, the amount and composition of which was greatly affected by the addition of ascorbic acid to the cultures. In addition, they were also shown to incorporate $|^{35}S|$ sulphate into macromolecular material, and the synthesis and secretion of such material reached a maximum just after the cells attained confluency. The amount of $|^{35}S|$ sulphate incorporated by the cultures was increased in the presence of ascorbic acid, with the biggest effect being in the pericellular-matrix compartment.

# CHAPTER 4

# ANALYSIS OF PROTEOGLYCANS SYNTHESIZED AND SECRETED BY AORTIC MEDIAL SMOOTH MUSCLE CELLS IN CULTURE

## 4.1. INTRODUCTION

Although a number of studies on the biosynthesis and secretion of proteoglycans by cultured cells has been reported (13,23,306, 310,337), few of these are concerned specifically with cultured smooth muscle cells. Wight and Ross were among the first workers to study glycosaminoglycan secretion by primate arterial smooth muscle cells in culture (310). They noted that the incorporation of labelled precursors into glycosaminoglycans was maximal during the stationary phase of growth and was much reduced during the logarithmic growth phase. The majority of labelled material was found associated with culture medium and thus in their early work they only analysed this material. Smooth muscle cell cultures were shown to synthesize dermatan sulphate principally, and some chondroitin sulphate, but little or no hyaluronic acid. Gamse *et al*. showed that arterial smooth muscle cells synthesized chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate, heparan sulphate and small amounts of material identified as keratan sulphate (306). The different glycosaminoglycan species were also shown to be distributed between the extracellular, pericellular and intracellular compartments in a distinct manner.

Information on the size of the intact proteoglycan monomer synthesized by smooth muscle cells in culture is scarce.

Recently, Wight and Hascall studied cultured arterial smooth muscle cells obtained from intimal-medial strips of pigtail monkey aorta, with respect to proteoglycan monomer size, the size of the glycosaminoglycan side chains and the ability of the proteoglycan to interact with hyaluronic acid (281). They found two sizes of proteoglycan monomer separated on Sepharose CL-2B which they could isolate from both the culture medium and the cell layer. The two species had the same size glycosaminoglycan side chains but differed in their glycosaminoglycan composition and in their ability to aggregate with hyaluronic acid.

The smooth muscle cell lines $AS_1Cl_6$ and $A_3$ were shown to behave in tissue culture as typical smooth muscle cells, exhibiting the morphology and growth patterns characteristic of such cells (Chapter 3). By 7 to 8 days after plating, the cells had reached confluency and their rate of division slowed down. At this stage their rates of synthesis and secretion of proteoglycans was shown to have reached a maximum (Fig. 3.3).

The addition of ascorbic acid to these cells resulted in their producing much larger amounts of extracellular matrix, with collagen making up the greatest proportion of this matrix (about 70%), whereas the matrix of cells that had not been supplemented with ascorbic acid contained less than 5% collagen (Table 3.1). Proteoglycans have been shown to interact with the structural protein elements of the matrix

and such interactions are thought to be important in the maintenance of the integrity of the matrix (see Section 1.4.1). The addition of ascorbic acid to smooth muscle cells was also shown to result in an increased incorporation of $|^{35}S|$ sulphate into macromolecular material in these cultures with the most marked effect being in the pericellular-matrix compartment (Table 3.2). Thus it was of prime importance to ascertain whether the presence of ascorbic acid and hence the presence of a more copious collagenous extracellular matrix had an effect on the type or size of proteoglycans synthesized by these cultured cells.

In this study proteoglycans synthesized and secreted by medial smooth muscle cells during their maximum synthetic phase, i.e. at days 6 to 8 after plating, were studied. Cells were cultured both in the presence and in the absence of ascorbic acid, and proteoglycans produced under these conditions characterized with respect to their monomer size, the size of their glycosaminoglycan side chains, their ability to interact with hyaluronic acid and their glycosaminoglycan composition. The data so obtained would enable the full evaluation of the tissue culture model for the study of proteoglycan synthesis to be made. A full comparison between the proteoglycans synthesized by cultured cells and those extracted from medial tissue could be made since cells were obtained from the same tissue as used for PGM extraction and characterization (Chapter 2).

## 4.2. METHODS

### 4.2.1. Preparation of proteoglycan monomer from smooth muscle cell cultures

Proteoglycan monomers were prepared from cultures that had been seeded into 75 $cm^3$ tissue culture flasks at a density of 1 x $10^6$ cells per flask, and grown either in the absence or presence of daily additions of ascorbic acid (50 µg/ml) for 8 days. Medium was changed on days 3 and 6 after plating, and |$^{35}$S| sulphate (10 µCi/ml) was added, together with fresh medium on day 6. After the 48 h labelling period, the culture medium was removed and retained, cell layers were washed 4 times with PBS and the washes that did not contain significant amounts of macromolecular labelled material were discarded. Washed cell layers were then extracted at -5°C with 6 mls of 4 M guanidine HCl containing 0,05 M sodium acetate, 0,1 M 6-aminocaproic acid, 0,01 M EDTA, 5 mM benzamidine HCl and 250 mM phenylmethylsulphonylfluoride, all at pH 5,8, for 24 h. Medium samples were made 4 M with respect to guanidine HCl by addition of the solid compound, a mixture of proteolytic inhibitors was added from concentrated stock solutions and the pH was adjusted to 5,8 by addition of glacial acetic acid. After extraction, the cell layers were centrifuged at 10000 rpm for 5 min in a Beckman J-21C centrifuge (JA-21 rotor) to remove any insoluble residue.

In a parallel experiment the amount of |$^{35}$S| sulphate-labelled macromolecules released into the medium by the cultured cells

and that present in the 4 M guanidine HCl extracts of the cell layers at various times after the beginning of continuous labelling were determined, using cells plated in 35 mm Petri dishes. At various times after the initiation of the radioactive labelling procedure the culture media and guanidine HCl extracts of cell layers from triplicate cultures were analysed with respect to the amount of $|^{35}S|$ sulphate in macromolecular material, using chromatography on PD-10 columns as described in Section 3.2.6.

Solid caesium chloride (0,59 g/g solution) was added to treated culture medium and guanidine HCl extracts of the cell layer to bring them to an initial density of 1,51 g/ml. The cell layer extracts were clarified by centrifugation at 8000 rpm for 10 min in a Beckman J-21C centrifuge, and then both extracts (medium and cell layer) were centrifuged at 40000 rpm for 48 h at 10°C in a Beckman type 65 rotor using a Beckman model L5-65 or L8-70 ultracentrifuge. After centrifugation tubes were fractionated using a Beckman tube cutter, and the collected fractions dialysed against distilled-deionized water, containing the proteolytic inhibitors mentioned above, at 4°C for 48 h. The protein content of each fraction together with its mean density were determined as described in Section 2.2.3.1. The amount of macromolecular $|^{35}S|$ sulphate radioactivity was determined after descending paper chromatography of samples. This procedure removed any remaining unincorporated isotope (see Section 3.2.7). Dialysed fractions were then freeze dried and stored at -15°C for further analyses.

4.2.2. Treatments of the PGM

The PGM isolated from the culture medium and cell layers of smooth muscle cells grown both in the presence and absence of ascorbic acid were subjected to the enzymatic degradative treatments described in Section 2.2.4, i.e. digestion with papain, chondroitinases ABC or AC, keratanase and hyaluronidase. Furthermore, samples were also subjected to treatment with alkaline borohydride or nitrous acid.

4.2.3. Interaction with hyaluronic acid

Samples of PGM isolated using dissociative techniques from the culture medium and cell layers of smooth muscle cells grown either in the presence or absence of ascorbic acid were dissolved in 0,5 M sodium acetate pH 6,8, mixed with high molecular weight hyaluronic acid and dialysed for 24 h against the same buffer (Section 2.2.5). Mixtures were applied to a Sepharose CL-2B column and eluted with 0,5 M sodium acetate pH 6,8. The percentage of radioactivity eluting in the $V_o$ was used to provide an estimate of the amount of aggregate formation.

4.2.4. Column chromatography

Columns of Sepharose CL-2B and CL-6B prepared as described in Section 2.2.6 were used to assess the sizes of the isolated proteoglycan samples. Biogel P-2 columns (100-200 mesh) were also used for the removal of salts from proteoglycan samples. Quantitation of the glycosaminoglycan side chains

was performed on PGM samples which were papain-digested and then digested with specific enzymes and chromatographed on a Sephadex G-50 (medium) column equilibrated with 0,2 M pyridine acetate pH 5,0, as described in Section 2.2.8.

## 4.3. RESULTS

The amount of $|^{35}S|$ sulphate labelled macromolecular material released into the culture medium of aortic smooth muscle cell cultures and that associated with the cell layers and extractable with 4 M guanidine HCl is shown in Fig. 4.1 as a function of time after addition of the radioactive precursor. Secretion of radioactively-labelled macromolecules into the culture medium was linear over the 48 h experimental period. However, this was not the case with incorporation of $|^{35}S|$ sulphate into macromolecules associated with the cell layer. By between 12 and 24 h this incorporation had reached equilibrium and did not increase over the remaining incubation period. This indicated that dynamic equilibrium between labelled macromolecules entering and leaving this compartment had been attained early in the incubation period (12 to 24 h). At the end of the 48 h labelling period, the majority of the $|^{35}S|$ labelled macromolecules were found in the culture medium, 64% of the total material for cells cultured in the presence of ascorbic acid and 85% for cells cultured in its absence. In addition, for cells grown in the absence of ascorbic acid the amount of labelled material found in the extracts of the cell layer was only about one-third of that found in the extracts of cells

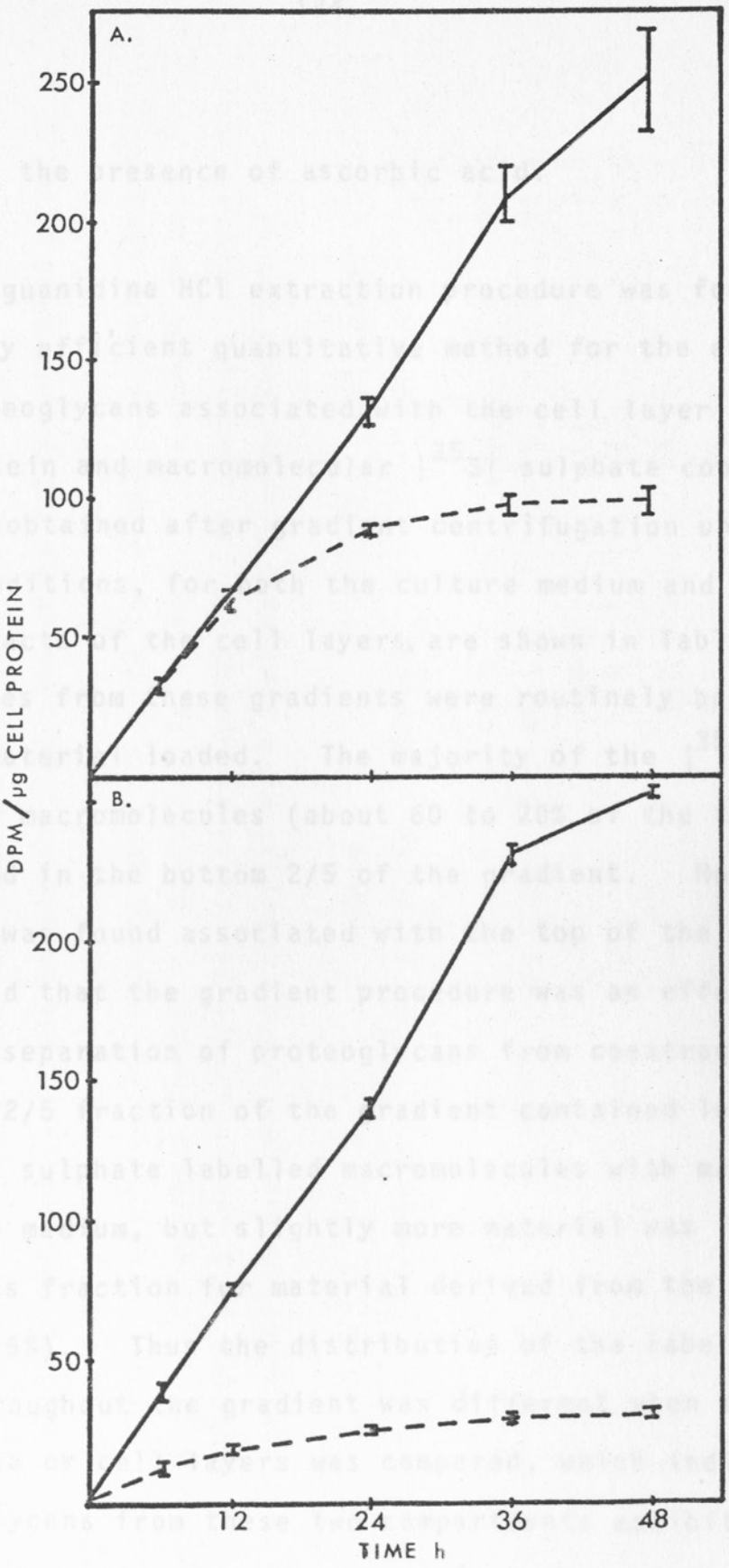



Fig. 4.1. Incorporation of $|^{35}S|$ sulphate into medium or cell layer macromolecular material.

Smooth muscle cells were cultured either in the presence (A) or absence (B) of ascorbic acid and labelled with $|^{35}S|$sulphate (10 µCi/ml). At each time point the medium (——) and guanidine HCl extracts of the cell layer (---) were assayed as described in Section 3.2.6. Each point represents the mean + S.D. of triplicate determinations for 1 of 2 similar experiments.

grown in the presence of ascorbic acid.

The 4 M guanidine HCl extraction procedure was found to be an extremely efficient quantitative method for the extraction of the proteoglycans associated with the cell layer (± 95% of total). The protein and macromolecular $|^{35}S|$ sulphate contents of samples obtained after gradient centrifugation under dissociative conditions, for both the culture medium and guanidine HCl extracts of the cell layers, are shown in Table 4.1. The recoveries from these gradients were routinely better than 90% of the material loaded. The majority of the $|^{35}S|$ sulphate labelled macromolecules (about 60 to 70% of the total) were recovered in the bottom 2/5 of the gradient. Most of the protein was found associated with the top of the gradient which indicated that the gradient procedure was an effective method for the separation of proteoglycans from coextracted proteins. The top 2/5 fraction of the gradient contained less than 17% of $|^{35}S|$ sulphate labelled macromolecules with material derived from the medium, but slightly more material was found associated with this fraction for material derived from the cell layers (about 25%). Thus the distribution of the labelled macromolecules throughout the gradient was different when material from the media or cell layers was compared, which indicated that proteoglycans from these two compartments exhibited different buoyant densities. The presence or absence of ascorbic acid made no difference to the behaviour of the material in the gradients and the profiles obtained from either $A_3$ or $AS_1Cl_6$ cells were almost identical for material

TABLE 4.1

DISTRIBUTION OF PROTEIN AND MACROMOLECULAR $|^{35}S|$ SULPHATE RADIOACTIVITY IN A DISSOCIATIVE CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION

Fractions from the top (D4 and 5) to the bottom (D1) of centrifuge tubes collected after caesium chloride density gradient centrifugation of material extracted from the culture medium or cell layer of smooth muscle cultures were analysed for density, protein content and macromolecular $|^{35}S|$ sulphate content as described in Methods. The amount in each fraction is presented as a percentage of the total recovered off the gradients. Recoveries of $|^{35}S|$ sulphate-labelled macromolecular material were routinely greater than 90% of the material loaded.

| | | | Medium | | Cell Layer | |
|---|---|---|---|---|---|---|
| Fraction | Density (g/ml) | Ascorbate | $\|^{35}S\|$ sulphate (%) | Protein (%) | $\|^{35}S\|$ sulphate (%) | Protein (%) |
| D4,D5 | 1,37 | + | 15,2 | 90,3 | 25,2 | 92,0 |
| | | - | 17,2 | 92,4 | 25,2 | 92,4 |
| D3 | 1,41 | + | 14,0 | 5,9 | 12,8 | 4,8 |
| | | - | 16,8 | 3,8 | 14,5 | 4,5 |
| D2 | 1,48 | + | 26,6 | 2,6 | 24,6 | 2,1 |
| | | - | 22,8 | 2.2 | 21,4 | 2,2 |
| D1 | 1,61 | + | 44,2 | 1,2 | 37,4 | 1,1 |
| | | - | 43,2 | 1,6 | 38,9 | 0,9 |

derived from the same culture compartment (culture medium or cell layer).

Analysis of the various gradient fractions from the medium and cell layer extracts by Sepharose CL-2B column chromatography yielded the profiles shown in Fig. 4.2(a,b). Fractions from $AS_1Cl_6$ or $A_3$ cultures, whether grown in the presence or absence of ascorbic acid, gave very similar profiles on Sepharose CL-2B, so only the results of the material from $A_3$ cells grown in the presence of ascorbic acid are presented. (As indicated, profiles obtained from the other cultures were very similar to these shown). All fractions from both the medium and cell layer contained material that was included on the column. For material derived from the cell layer, the more dense gradient fractions contained material that eluted predominantly in two peaks with $K_{av}$'s of 0,27 and 0,76 (Fig. 4.2b). In the less dense fractions the smaller proteoglycan species was present with no evidence of the larger species (Fig. 4.2b). For material derived from the medium, the less dense gradient fractions also contained a predominantly smaller species of proteoglycan with some evidence of a larger species, whereas the fractions from the bottom (dense area) of the gradient contained two peaks corresponding to $K_{av}$'s of 0,31 and 0,60 (Fig. 4.2a). The bottom 1/5 of each gradient was retained for further analysis.

Thus the proteoglycans extracted from both the culture medium and cell layer of smooth muscle cells grown in either the

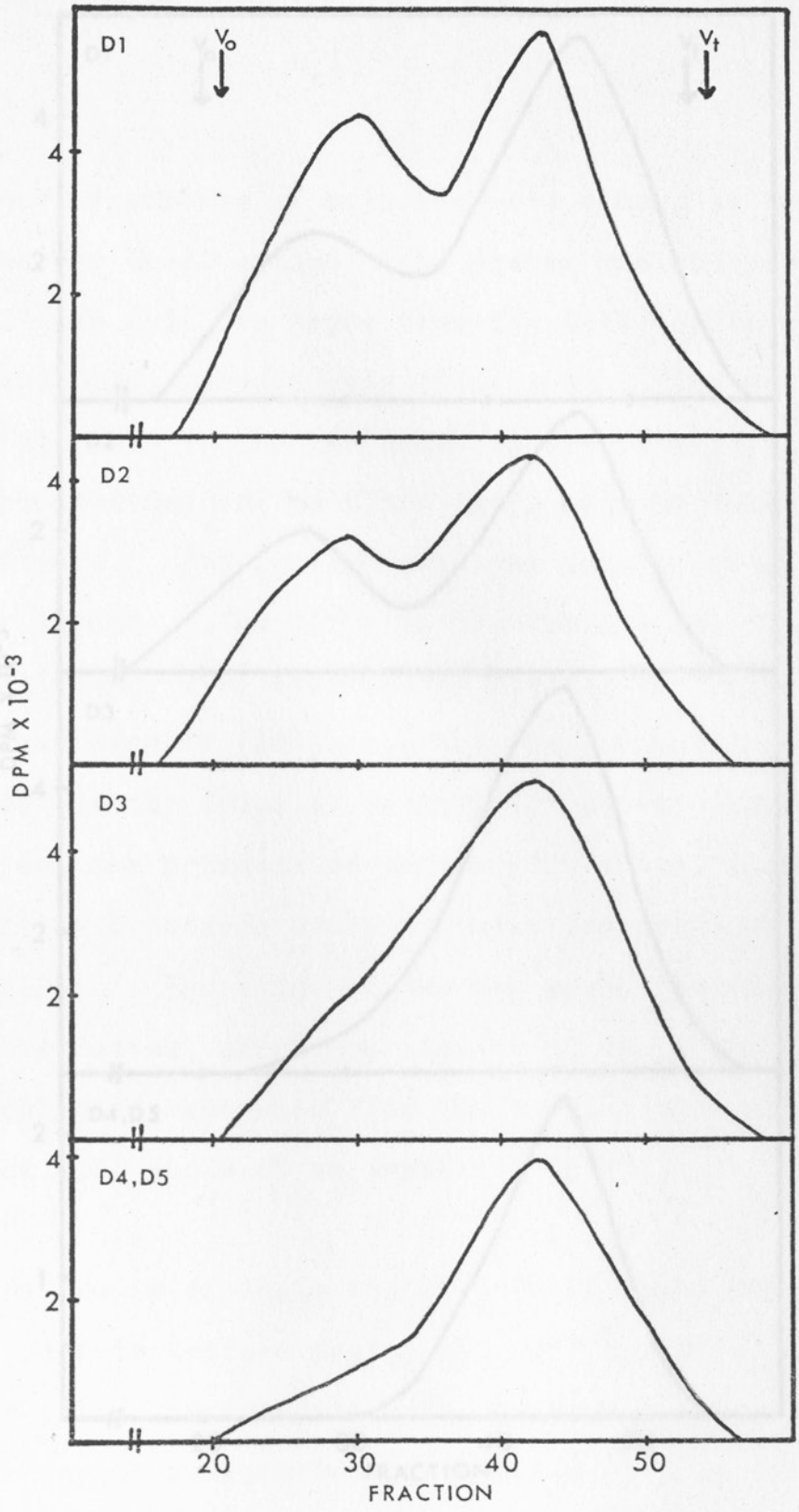



Fig. 4.2a. Sepharose CL-2B chromatography of $|^{35}S|$ sulphate labelled material from the different fractions obtained from caesium chloride density gradient centrifugation of samples isolated from the cultured medium of smooth muscle cells grown in the presence of ascorbic acid.

$|^{35}S|$ Sulphate-labelled macromolecular material from the D1 (bottom) to D4 & D5 (top) fractions of caesium chloride gradients after centrifugation of material extracted from the medium of smooth muscle cells grown in the presence of ascorbic acid was chromatographed on a Sepharose CL-2B column as described in Methods (Section 4.2.4).

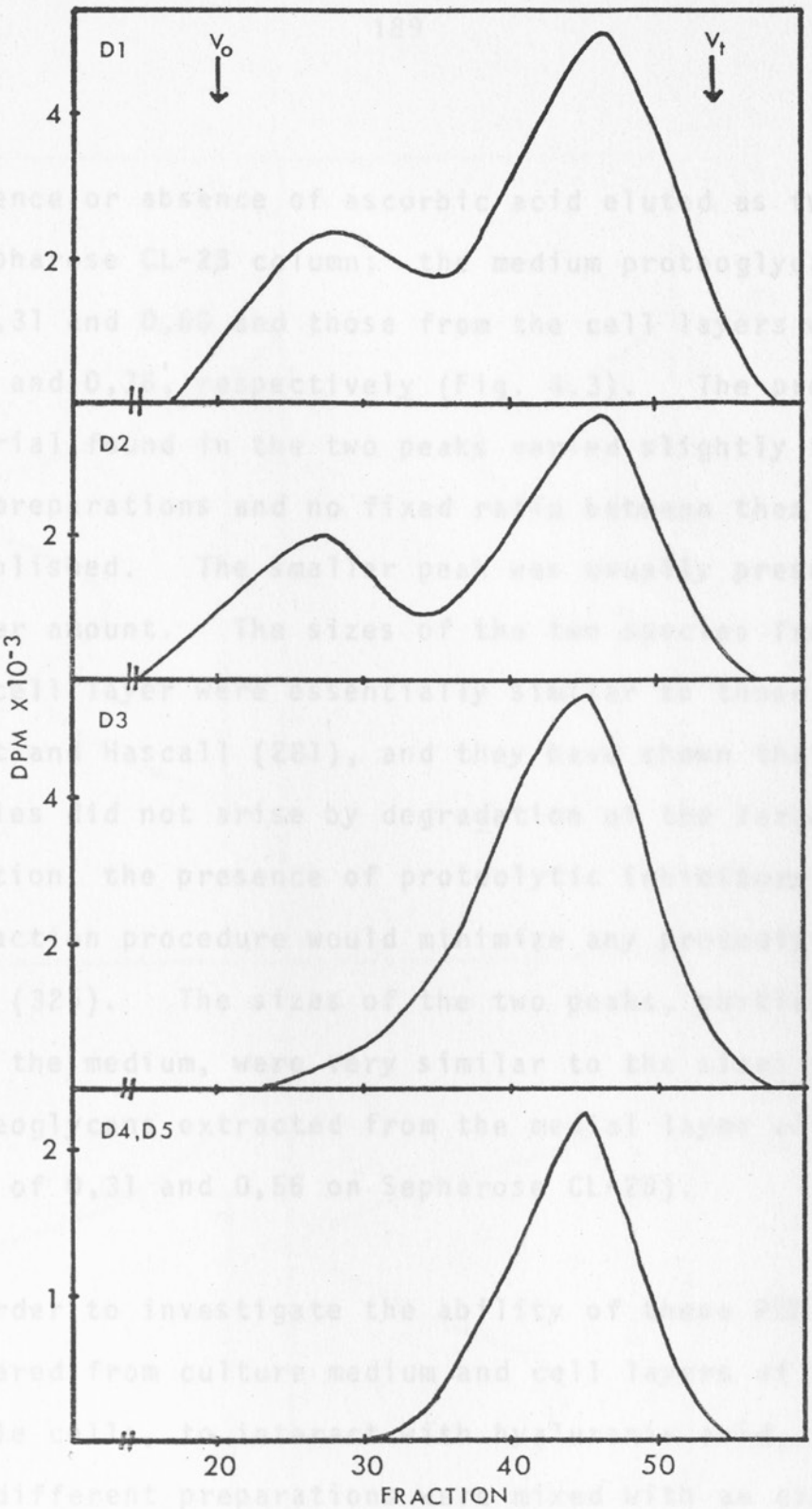



Fig. 4.2b. Sepharose CL-2B chromatography of $|^{35}S|$ sulphate-labelled material from the different fractions obtained from caesium chloride density gradient centrifugation of material isolated from the cell layer of smooth muscle cultures grown in the presence of ascorbic acid.

$|^{35}S|$ Sulphate labelled macromolecular material from the D1 (bottom) to D4 & D5 (top) fractions of caesium chloride gradients after centrifugation of guanidine HCl extracts of the cell layers of smooth muscle cultures grown in the presence of ascorbic acid was chromatographed on a Sepharose CL-2B column as described in Methods (Section 4.2.4).

presence or absence of ascorbic acid eluted as two peaks on a Sepharose CL-2B column; the medium proteoglycans with $K_{av}$'s of 0,31 and 0,60 and those from the cell layers with $K_{av}$'s of 0,27 and 0,76, respectively (Fig. 4.3). The proportions of material found in the two peaks varied slightly from different preparations and no fixed ratio between them could be established. The smaller peak was usually present in the larger amount. The sizes of the two species from the medium and cell layer were essentially similar to those described by Wight and Hascall (281), and they have shown that the smaller species did not arise by degradation of the larger form. In addition, the presence of proteolytic inhibitors during the extraction procedure would minimize any proteolytic degradation (325). The sizes of the two peaks, particularly those from the medium, were very similar to the sizes found for the proteoglycans extracted from the medial layer of bovine foetuses ($K_{av}$ of 0,31 and 0,56 on Sepharose CL-2B).

In order to investigate the ability of these PGM samples, prepared from culture medium and cell layers of aortic smooth muscle cells, to interact with hyaluronic acid, samples from the different preparations were mixed with an excess of high molecular weight hyaluronic acid, dialysed against 0,5 M sodium acetate pH 7,0, and then chromatographed on a Sepharose CL-2B column. Proteoglycans from cultures were not prepared under associative conditions as preliminary experiments had already shown that no hyaluronic acid was synthesized by these cells under culture conditions, and thus true aggregation between

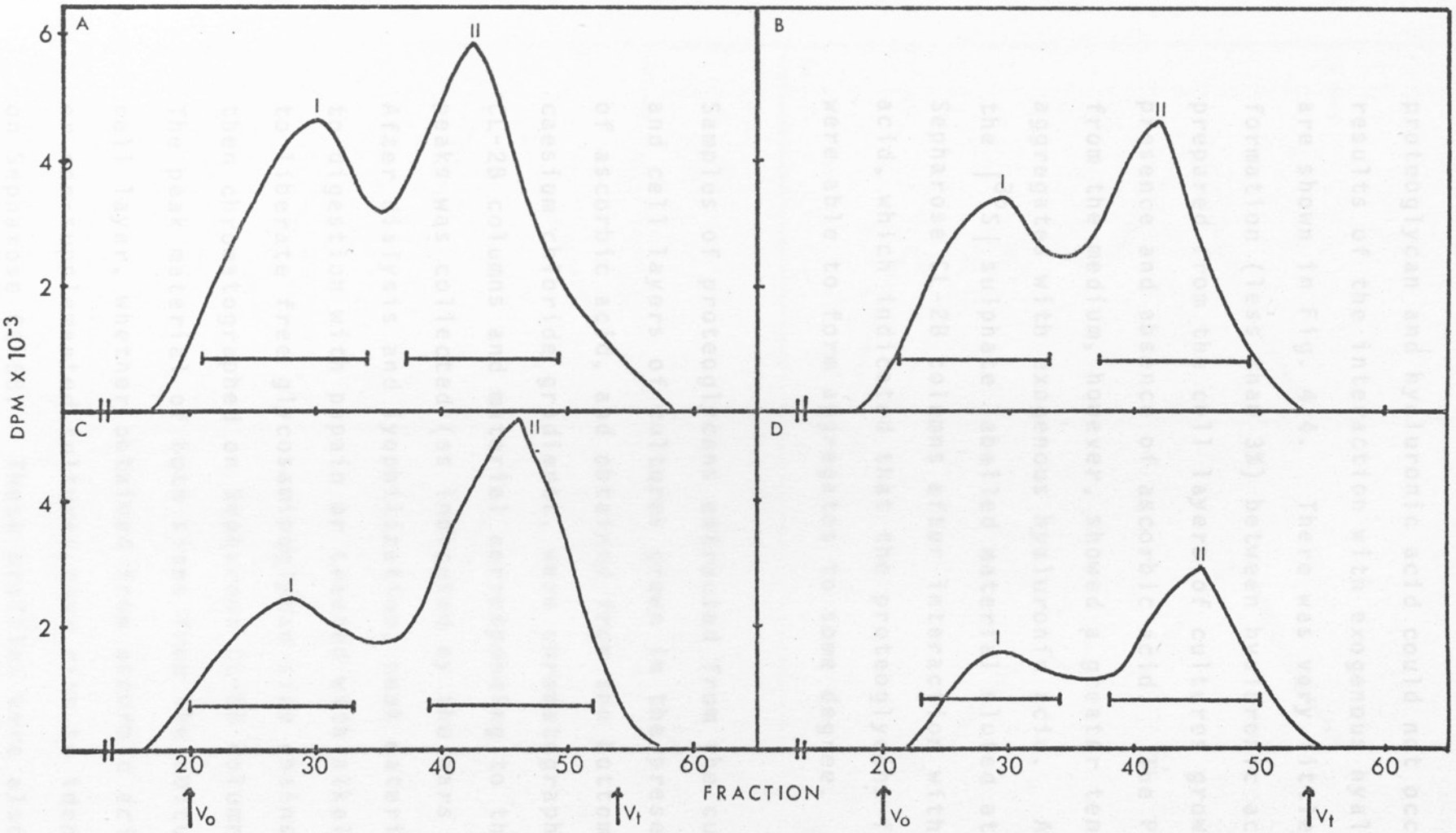



Fig. 4.3. Sepharose CL-2B chromatography of proteoglycans isolated from the culture medium and cell layer of smooth muscle cells grown in the presence and absence of ascorbic acid.

$|^{35}S|$ Sulphate labelled macromolecules from the D1 fraction of the caesium chloride density gradient obtained after centrifugation of culture medium (A,B) and guanidine HCl extracts of cell layers (C,D). Samples were isolated from cultured cells either in the presence of ascorbic acid (A,C) or in its absence (B,D) and were chromatographed on a Sepharose CL-2B column as described in Section 4.4.2. Solid bars indicate the fractions pooled for subsequent glycosaminoglycan analysis.

proteoglycan and hyaluronic acid could not occur. The results of the interaction with exogenous hyaluronic acid are shown in Fig. 4.4. There was very little aggregate formation (less than 3%) between hyaluronic acid and PGM prepared from the cell layers of cultures grown both in the presence and absence of ascorbic acid. The PGM derived from the medium, however, showed a greater tendency to form aggregates with exogenous hyaluronic acid. About 16% of the $|^{35}S|$ sulphate labelled material eluted at the $V_o$ of Sepharose CL-2B columns after interaction with hyaluronic acid, which indicated that the proteoglycans from the medium were able to form aggregates to some degree.

Samples of proteoglycans extracted from the culture medium and cell layers of cultures grown in the presence or absence of ascorbic acid, and obtained from the bottom (D1) of the caesium chloride gradients, were chromatographed on Sepharose CL-2B columns and material corresponding to the different peaks was collected (as indicated by the bars in Fig. 4.3). After dialysis and lyophilization, peak material was subjected to digestion with papain or treated with alkaline borohydride to liberate free glycosaminoglycan side chains. These were then chromatographed on Sepharose CL-6B columns (Fig. 4.5). The peak material of both sizes from the culture medium or cell layer, whether obtained from ascorbic acid-supplemented or non-supplemented cultures, gave rise to identical profiles on Sepharose CL-6B. These profiles were also the same for both papain- or alkaline borohydride-liberated free glycosamino-

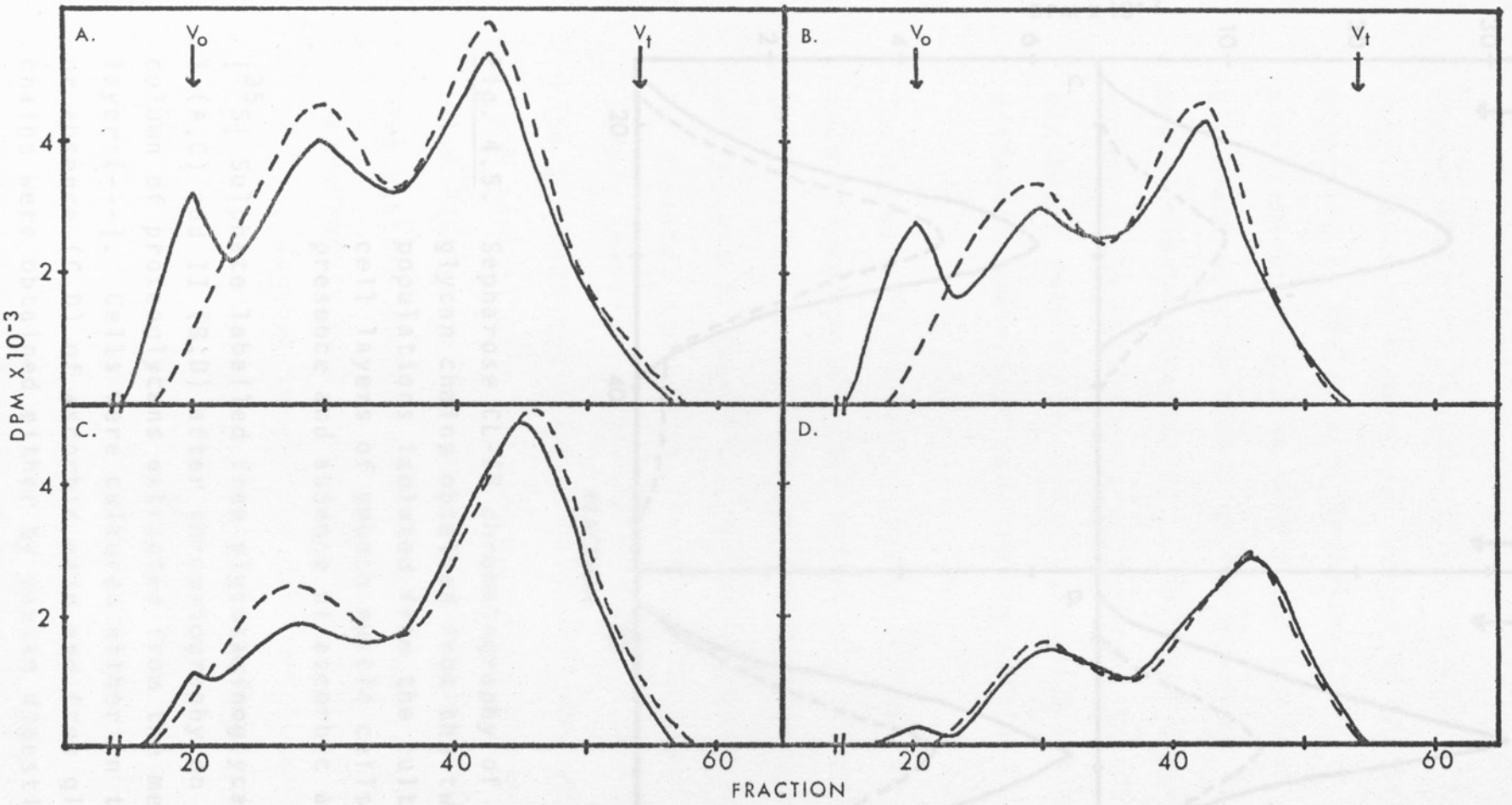



Fig. 4.4. Sepharose CL-2B chromatography of |$^{35}$S| sulphate labelled proteoglycans from the medium and the cell layer of cells in the presence of exogenous hyaluronic acid.

|$^{35}$S| Sulphate labelled proteoglycans obtained from the D1 fractions of caesium chloride density gradients of culture medium (A,B) and guanidine HCl extracts of cell layers (C,D) from cells cultured in the presence (A,C) or absence (C,D) of ascorbic acid. Samples were chromatographed on a Sepharose CL-2B column in the absence (---) or presence (—) of an excess of large molecular weight hyaluronic acid as described in Section 4.2.3.

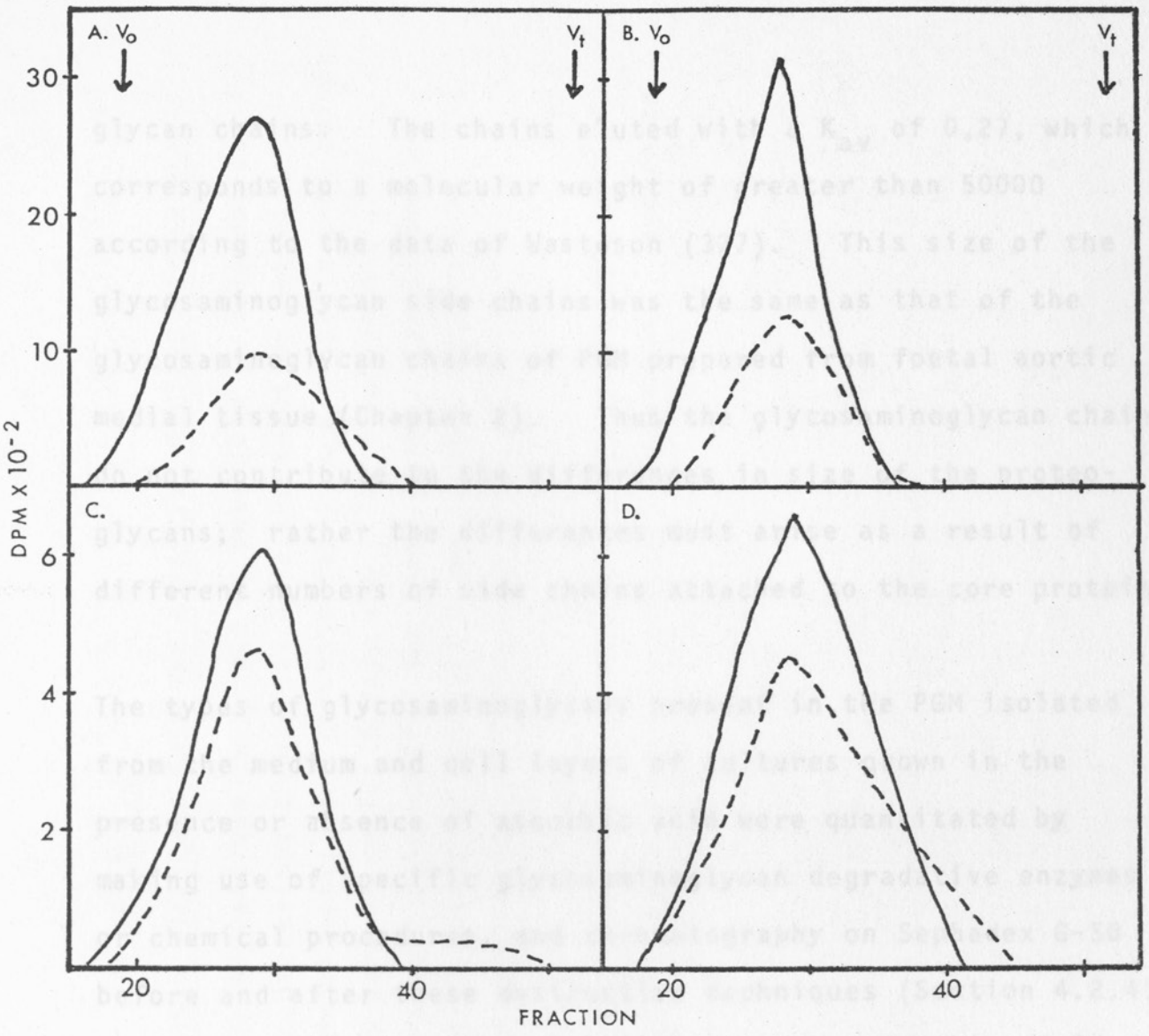



Fig. 4.5. Sepharose CL-6B chromatography of free glycosaminoglycan chains obtained from the two proteoglycan populations isolated from the culture medium and cell layers of smooth muscle cells grown in the presence and absence of ascorbic acid.

|$^{35}$S| Sulphate labelled free glycosaminoglycan chains from peaks I (A,C) and II (B,D) after chromatography on a Sepharose CL-2B column of proteoglycans extracted from the medium (——) and cell layer (---). Cells were cultured either in the presence (A,B) or absence (C,D) of ascorbic acid and free glycosaminoglycan chains were obtained either by papain digestion or treatment with alkaline borohydride as described in Section 4.2.2.

glycan chains. The chains eluted with a $K_{av}$ of 0,27, which corresponds to a molecular weight of greater than 50000 according to the data of Wasteson (327). This size of the glycosaminoglycan side chains was the same as that of the glycosaminoglycan chains of PGM prepared from foetal aortic medial tissue (Chapter 2). Thus the glycosaminoglycan chains do not contribute to the differences in size of the proteoglycans; rather the differences must arise as a result of different numbers of side chains attached to the core protein.

The types of glycosaminoglycans present in the PGM isolated from the medium and cell layers of cultures grown in the presence or absence of ascorbic acid were quantitated by making use of specific glycosaminoglycan degradative enzymes or chemical procedures, and chromatography on Sephadex G-50 before and after these destructive techniques (Section 4.2.4). The glycosaminoglycans from cell layers were further divided into pericellular and intracellular compartments by treatment with Viokase-collagenase, as described under Section 3.2.7. The culture medium, pericellular-matrix and intracellular compartments were digested with papain and then subjected to the degradative treatments referred to above (Section 4.2.2). The results of the analysis of the glycosaminoglycans from the different culture compartments of $A_3$ cells cultured in the presence or absence of ascorbic acid is given in Table 4.2.

The absence of keratan sulphate was indicated, since there was no $|^{35}S|$ sulphate labelled material susceptible to keratanase

TABLE 4.2

COMPOSITIONAL ANALYSIS OF THE GLYCOSAMINOGLYCANS FROM THE DIFFERENT CULTURE COMPARTMENTS

Compositional analysis of glycosaminoglycans was performed as described in Sections 2.2.4 and 4.2.4, using specific enzymatic digestions or nitrous acid treatment of material isolated from the different culture compartments, and chromatography on a Sephadex G-50 column before and after treatment. Cells were grown for 8 days and $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine (when used) were added to the cultures for the final 48 h. The figure in brackets gives the percentage of total sulphated material found in that compartment. The data represent one of three similar experiments.

| Glycosaminoglycan type | Composition (%) | | | | | |
|---|---|---|---|---|---|---|
| | Extracellular | | Pericellular-matrix | | Intracellular | |
| | +Ascorbate (64,2) | -Ascorbate (81) | +Ascorbate (30,5) | -Ascorbate (11,1) | +Ascorbate (5,3) | -Ascorbate (7,9) |
| Chondroitin 4 and 6 sulphate | 61,1 | 70,0 | 30,2 | 39,5 | 11,8 | 32,8 |
| Dermatan sulphate | 22,1 | 22,7 | 36,6 | 11,6 | 76,7 | 48,6 |
| Heparan sulphate/ heparin | 16,8 | 7,3 | 33,2 | 49,3 | 11,5 | 18,6 |
| Keratan sulphate | 0 | 0 | 0 | 0 | 0 | 0 |
| Hyaluronic acid | 0 | 0 | 0 | 0 | 0 | 0 |

digestion. The glycosaminoglycan chains associated with the proteoglycans in the culture medium were predominantly made up of chondroitin sulphate - 61% of the total in cells grown with ascorbic acid and 70% of the total for cells grown without ascorbic acid. Dermatan sulphate made up about 22% of the glycosaminoglycans in the medium for both cells grown with and without ascorbic acid. About twice as much heparan sulphate was found in the medium for cells grown in the presence of ascorbic acid than for cells grown in its absence. The predominant glycosaminoglycan in the pericellular compartment of cells grown in the absence of ascorbic acid was heparan sulphate/heparin, which made up about 50% of the total sulphated glycosaminoglycans in this fraction. Small amounts of dermatan sulphate (about 11%) were found and chondroitin sulphate made up the remainder of the glycosaminoglycans (39%). However, when the glycosaminoglycan composition of the pericellular-matrix compartment of cells grown in the presence of ascorbic acid was analysed, the relative amount of dermatan sulphate increased at least 3-fold to make up over one-third of the total sulphated glycosaminoglycans, whereas the relative amount of heparan sulphate/heparin decreased to about 67% of the amount found in the absence of ascorbic acid, making up about 33% of the total glycosaminoglycan content. The relative amount of chondroitin sulphate present decreased by 23% to make up some 30% of the total sulphated glycosaminoglycan content of the pericellular-matrix compartment. However, as the amount of macromolecular material in the pericellular-matrix compartment from cells grown in the presence of ascorbic acid is about four

times that found in this compartment from cells grown in the absence of the vitamin, increased amounts of chondroitin-4- and -6-sulphate, and heparan sulphate/heparin, were actually found, but the most marked effect of ascorbic acid was to increase incorporation of labelled precursors into dermatan sulphate in this compartment. When glycosaminoglycans were analysed from the intracellular compartment, it could be seen that the major glycosaminoglycan found was dermatan sulphate, particularly in cells grown in the presence of ascorbic acid. Here dermatan sulphate made up nearly 80% of the total sulphated glycosaminoglycans. The remainder consisted of chondroitin sulphate (12%) and heparan sulphate/heparin (11%). In cells grown without ascorbic acid, dermatan sulphate was still the major glycosaminoglycan present in the intracellular compartment, but its amount had decreased to about 63% of that found in the presence of ascorbic acid and thus it made up about 49% of the total glycosaminoglycans. Chondroitin-4- and -6-sulphate increased 2,8-fold, making up 33% of the total sulphated glycosaminoglycans, and the amount of heparan sulphate/heparin increased 1,6 times, making up 18,6% of the total in the intracellular compartment in the absence of ascorbic acid.

In order to assess the size of the dermatan sulphate chains associated with proteoglycans derived from the culture medium and cell layers, samples were treated with either chondroitinase ABC or AC prior to papain digestion and chromatography on Sepharose CL-6B (Fig. 4.6). The chondroitinase AC-resistant dermatan sulphate did not elute as a single peak but as a

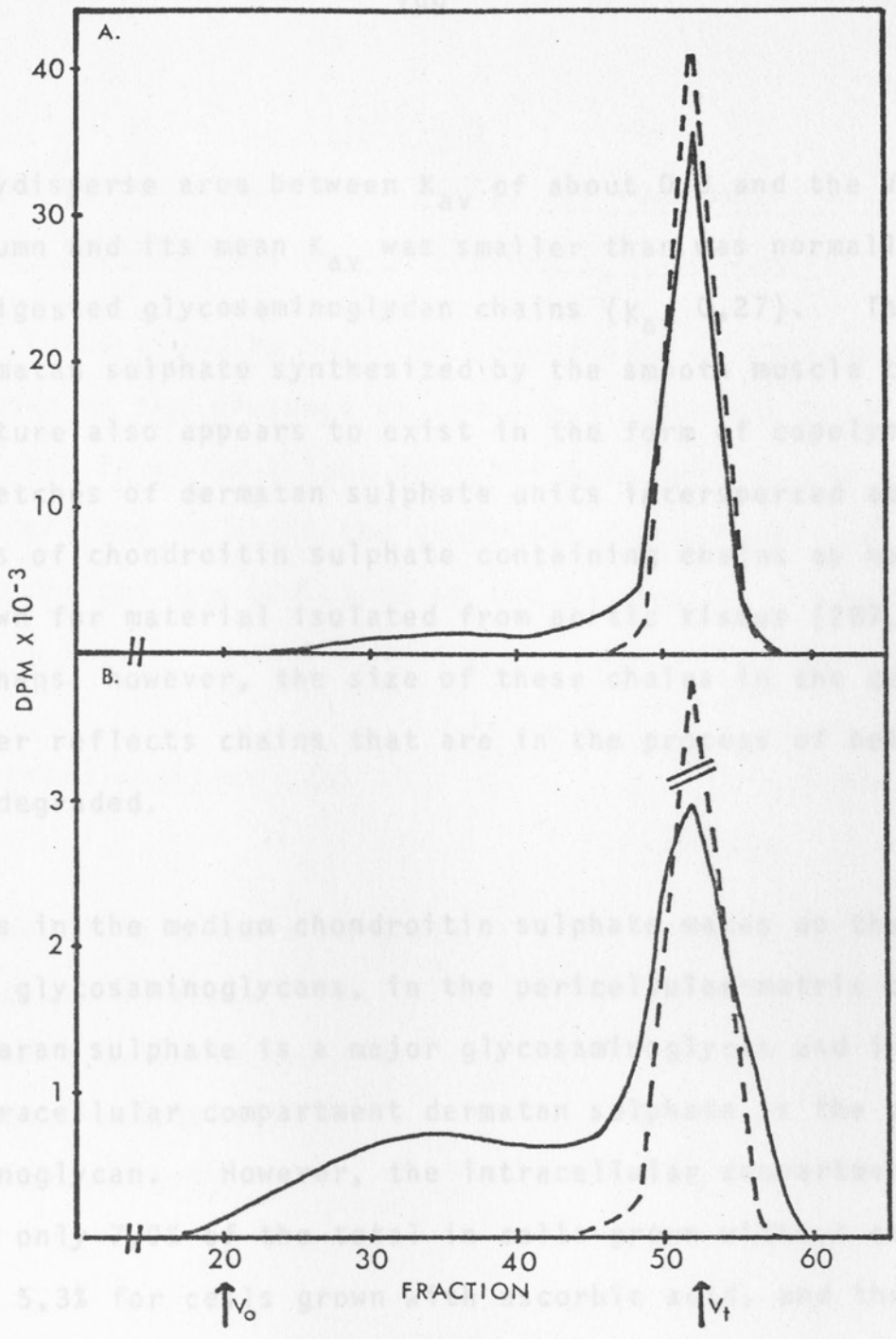



Fig. 4.6. Sepharose CL-6B chromatography of chondroitinase ABC and AC digests of material from the culture medium and cell layer of cells grown in the presence of ascorbic acid.

|$^{35}$S| Sulphate labelled proteoglycans were obtained from the culture medium (A) and guanidine HCl extract of cell layers (B) after fractionation by caesium chloride density gradient centrifugation. Cells were cultured in the presence of ascorbic acid and glycosaminoglycan chains and were digested with either chondroitinase ABC (---) or chondroitinase AC (—) as described in Section 2.2.4 prior to chromatography on a Sepharose CL-6B column.

polydisperse area between $K_{av}$ of about 0,3 and the $V_t$ of the column and its mean $K_{av}$ was smaller than was normally seen for undigested glycosaminoglycan chains ($K_{av}$ 0,27). Thus the dermatan sulphate synthesized by the smooth muscle cells in culture also appears to exist in the form of copolymers with stretches of dermatan sulphate units interspersed among stretches of chondroitin sulphate containing chains as has been shown for material isolated from aortic tissue (287,290). Perhaps, however, the size of these chains in the cell-matrix layer reflects chains that are in the process of being modified or degraded.

Thus in the medium chondroitin sulphate makes up the bulk of the glycosaminoglycans, in the pericellular-matrix compartment heparan sulphate is a major glycosaminoglycan and in the intracellular compartment dermatan sulphate is the major glycosaminoglycan. However, the intracellular compartment accounted for only 7,9% of the total in cells grown without ascorbic acid and 5,3% for cells grown with ascorbic acid, and the pericellular-matrix compartment accounted for 11,1% and 30,5%, respectively, for cells grown without and with ascorbic acid. If the proportions in which the different compartments occurred are taken into account, the total glycosaminoglycan composition for the medium, pericellular-matrix and intracellular compartments for cells grown either in the absence or presence of ascorbic acid is as shown in Table 4.3. For both cultures grown with or without added ascorbic acid, chondroitin sulphate was the major glycosaminoglycan, especially for those grown in the

TABLE 4.3

TOTAL GLYCOSAMINOGLYCAN COMPOSITION FOR SMOOTH MUSCLE CELLS GROWN IN THE PRESENCE AND ABSENCE OF ASCORBIC ACID

The total glycosaminoglycan composition of cultures grown either in the presence or absence of ascorbic acid was calculated from the values in Table 4.2, taking into account the proportions in which the different compartments occur. The proportions are indicated by the figures in brackets in Table 4.2.

| Glycosaminoglycan type | Composition (%) | |
|---|---|---|
| | + Ascorbate | - Ascorbate |
| Chondroitin 4 and 6 sulphate | 49,0 | 63,6 |
| Dermatan sulphate | 29,5 | 23,5 |
| Heparan sulphate/heparin | 21,5 | 12,9 |
| Keratan sulphate | 0 | 0 |
| Hyaluronic acid | 0 | 0 |

absence of the vitamin. The relative amount of dermatan sulphate increased about 1,3 times in cultures grown in the presence of ascorbic acid and the relative amount of heparan sulphate/heparin increased 1,7-fold. In addition, the total amount of material synthesized by the cells in the presence of ascorbic acid was routinely about 1,7 to 1,8 times the amount synthesized in its absence. Thus the amounts of $|^{35}S|$ sulphate in dermatan sulphate and heparan sulphate/heparin found in the presence of ascorbic acid were actually 2 to 3 times the amounts found in the absence of the vitamin and the amount in chondroitin-4- and -6-sulphate was about 1,3 times that found in the absence of ascorbic acid.

To check for the presence of hyaluronic acid in the cultures they were labelled with both $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine (5 µCi/ml), and the glycosaminoglycans in the medium, pericellular-matrix and intracellular compartments were treated with hyaluronidase. None of the macromolecular sulphated material was digested by the enzyme, indicating that it was not contaminated with chondroitinase activity. However, with all three compartments macromolecular $|^{3}H|$ glucosamine digested by the enzyme was less than 1% of the total. Thus no hyaluronic acid is synthesized by the aortic smooth muscle cells in culture, an observation that has been reported previously.

## 4.4. SUMMARY AND DISCUSSION

Aortic smooth muscle cells in culture were shown to synthesize and secrete $|^{35}S|$ labelled macromolecules. After a 48 h labelling period the majority of the sulphated macromolecules were found in the medium but for cells grown in the presence of ascorbic acid a somewhat higher percentage was found associated with the cell layer (36% as against 15% for cells grown in the absence of ascorbic acid). 4 M Guanidine HCl was shown to efficiently extract over 95% of the macromolecules from the cell layer. Caesium chloride density gradient centrifugation of extracted PGM gave clear evidence of differences in buoyant density between material obtained from the culture medium and the cell layer, and this was underlined from varying proportions of material found in the different gradient fractions from the two samples (Fig. 4,2). The presence or absence of ascorbic acid during culture made no difference to this distribution throughout the gradient.

The smooth muscle cells were shown to synthesize at least two different size classes of proteoglycans. The proteoglycans from both the medium and cell layer contained a larger species, usually present in a smaller amount, which eluted on a Sepharose CL-2B column with a $K_{av}$ of 0,31 and 0,27 for the culture medium and cell layer proteoglycans, respectively. In addition, both the medium and the cell layer contained a smaller proteoglycan species which eluted with a $K_{av}$ of 0,6 and 0,76 for the culture medium and cell layer proteoglycans, respectively. These sizes are in very close agreement with

those found by Wight and Hascall for primate arterial smooth muscle cells but they showed that the larger proteoglycan was the predominant one which increased with time of culture relative to the smaller one (281). In addition, the $K_{av}$'s noted on Sepharose CL-2B for the culture medium proteoglycans, which made up the bulk of the material, were similar to those found on chromatography of PGM isolated from foetal aorta (Chapter 2).

The proteoglycans isolated from the cell layer of cultures grown both in the absence and presence of ascorbic acid, using dissociative salt concentrations, were unable to interact with exogenous, large molecular weight hyaluronic acid, but those isolated from the medium were able to form a small amount of aggregated material. These data were to some extent consistent with results obtained in this regard with PGM from bovine foetal aorta, namely that these molecules are unable to interact with hyaluronic acid to any significant extent. In contrast to this, Wight and Hascall found that although the majority of the proteoglycans synthesized and secreted by cultured cells and extracted with associative solvents were not already bound in aggregate structures, the larger proteoglycan species was able to form aggregates after it had been incubated with rat chondrosarcoma material prepared under associative conditions (281). They showed that 90% of their larger proteoglycan species consisted of chondroitin sulphate and the smaller species contained 20 to 30% dermatan sulphate. This agreed with the findings of several authors, in relation to aortic proteoglycans, who have described a large chondroitin sulphate proteoglycan able to interact with hyaluronic acid,

and a smaller proteoglycan made up of a high content of dermatan sulphate which was unable to form aggregates (54, 299,303). It is likely that purification steps involving caesium chloride centrifugation resulted in the loss of some component essential for aggregation. Proteoglycans were not isolated from the smooth muscle cells under associative conditions since, as already mentioned, preliminary studies had shown that the cultures were unable to synthesize hyaluronic acid.

Analysis of the glycosaminoglycan side chains from the larger and smaller proteoglycan species from both the medium and cell layer from cells grown in the presence or absence of ascorbic acid showed these chains to be exactly the same size, with an estimated molecular weight of greater than 50000. The size of these chains was identical to those found in the PGM isolated from foetal aortic media.

Analysis of the glycosaminoglycan types found in the different culture compartments showed that the smooth muscle cells did not synthesize hyaluronic acid or keratan sulphate. The absence of hyaluronic acid supported earlier studies by Wight and Ross (310) and by Gamse *et al*. (306). This was a surprising finding in view of the presence of large amounts of hyaluronic acid normally found in aortic medial tissue (Table 2.2). Gamse *et al*. found that arterial smooth muscle cells synthesize small amounts of keratan sulphate (306), but we were unable to detect any keratanase-sensitive $|^{35}S|$ sulphate

material produced by the smooth muscle cells. The quantitation of glycosaminoglycans from the extracellular, pericellular-matrix and intracellular compartments supported the observation that the glycosaminoglycans are distributed to the different compartments in a distinct manner (153,306). The glycosaminoglycans found in the medium (extracellular) were largely chondroitin sulphate. In the pericellular-matrix compartment heparan sulphate/heparin was an important glycosaminoglycan, and in the presence of ascorbic acid the proportion of dermatan sulphate increased in this pool. The overall results supported those of other workers who have shown that heparan sulphate is associated with the cell surface, and in some cases it has been identified as an integral membrane component and may play important roles in cell-cell interactions (242). The glycosaminoglycans found in the intracellular compartment were largely dermatan sulphate, and this agreed with studies showing that relatively small amounts of chondroitin sulphate are found inside cells (306). In addition, the dermatan sulphate synthesized by the smooth muscle cells was shown to occur in copolymeric form with chondroitin sulphate in agreement with previous reports on the structural organization of dermatan sulphate in aortic tissue (287,290).

When the proportions of glycosaminoglycans that were found in the different compartments were taken into account and the results calculated accordingly, it could be seen that chondroitin sulphate made up the bulk of the glycosaminoglycans

synthesized by the smooth muscle cells (Table 4.3). With cells cultured in the presence of ascorbic acid the relative amounts of dermatan sulphate and heparan sulphate increased and that of chondroitin sulphate decreased. The glycosaminoglycan composition of cells cultured in the absence of vitamin C was similar to that of foetal aortic medial PGM in that chondroitin sulphate made up the bulk of the glycosaminoglycans and heparan sulphate accounted for a small percentage. However, dermatan sulphate was not found in PGM isolated from foetal bovine aortas, whereas significant amounts were synthesized by the smooth muscle cells in culture. This presence of significant amounts of dermatan sulphate in glycosaminoglycans secreted by smooth muscle cells in culture was noted by Wight and Ross (310). They suggested this perhaps to be a response by the cells to some form of mechanical or chemical stress or to reflect some particular state of development of the cells.

Thus it has been shown that the proteoglycans synthesized by smooth muscle cells in culture were similar to those isolated from the intact foetal medial tissue in terms of their size on Sepharose CL-2B, glycosaminoglycan chain size and ability to interact with hyaluronic acid. Likewise the composition of glycosaminoglycans isolated from intact tissue or cell cultures was very similar.
However the absence of any hyaluronic acid in the cultures may indicate that the aggregation between hyaluronic acid and PGM in aorta may not be important physiologically.

The sizes and nature of the proteoglycans synthesized by both the $AS_1Cl_6$ and $A_3$ cell lines were identical and indicated that the different smooth muscle cell lines retained their differentiated function with respect to proteoglycan synthesis *in vitro*. The presence of ascorbic acid during growth had no effect on the sizes of the proteoglycans formed or on their ability to interact with hyaluronic acid but affected the glycosaminoglycan composition to some extent, resulting in increased synthesis of dermatan sulphate and heparan sulphate. It was thus reassuring that the proteoglycans produced by cultured smooth muscle cells were representative of those produced by foetal tissue.

# CHAPTER 5

# THE EFFECT OF ASCORBIC ACID ON GLYCOSAMINOGLYCAN SYNTHESIS BY CULTURED SMOOTH MUSCLE CELLS

## 5.1. INTRODUCTION

Supplementation of aortic smooth muscle cells in culture with ascorbic acid led to increased cell numbers (Chapter 3). This finding has been reported by other laboratories (335) and may be a result of a reduction in cell-doubling time. In addition, cultures supplemented with ascorbic acid produced greatly increased amounts of extracellular matrix material containing a high collagen content (Table 3.1). Concomitant with this increased matrix production there was increased incorporation of $|^{35}S|$ sulphate into macromolecular material (Table 3.2). This was shown to be a net increase in the amount of $|^{35}S|$ sulphate incorporated per µg cellular protein and not merely as a result of an increased metabolic activity brought about by vitamin C. The most marked effect was noted in the pericellular-matrix compartment.

The role of ascorbic acid in the biosynthesis of connective tissue proteins, especially collagen, has been well-documented by numerous laboratories (108-111). In general the effect of ascorbic acid on collagen biosynthesis has been thought to be mediated through its effect on hydroxylation. Ascorbic acid is a cofactor for the enzymes prolyl hydroxylase and lysyl hydroxylase, which are responsible for the posttranslational conversions of proline and lysine residues in the procollagen

α-chains to hydroxyproline and hydroxylysine (338). The formation of hydroxyproline stabilizes the triple helix of collagen (339) and the absence of ascorbic acid has been shown to result in structurally unstable collagen (119,340) which is not secreted from the cell at a normal rate (108). In addition, hydroxylysine residues are the sites for glycosylation of collagen, and they also promote intermolecular cross-link formation in the molecule (120). From time to time additional roles for the vitamin in collagen biosynthesis and normal growth have been indicated apart from its known role in hydroxylation (112-114). Most recently Murad et al. have postulated a direct effect of ascorbic acid in the control of collagen synthesis, perhaps at the level of the protein synthetic machinery (341). They reported that a comparison of the short-term and long-term effects of ascorbic acid in cultured human skin fibroblasts showed that collagen synthesis continued to increase even after prolyl hydroxylation had been maximally stimulated. Thus the cofactor function of the vitamin for the enzymes involved in the hydroxylation of proline and lysine residues may be just one of many related functions. Collagen polypeptide synthesis, posttranslational hydroxylation and the activities of prolyl and lysyl hydroxylases may be independently regulated by ascorbic acid.

In addition to its roles in the biosynthesis of the extracellular matrix proteins, there have been suggestions in the literature that the sulphated derivative of ascorbic acid (ascorbic acid 2-sulphate) plays a direct role in the process of biological sulphate transfer, including the sulphation

process which occurs in proteoglycan synthesis (see Section 1.2.3.3) (115,116,342). However, using radiolabelled ascorbic acid 2-|$^{35}$S| sulphate, Shapiro and Poon provided evidence that ascorbic acid 2-sulphate is not the direct sulphate donor in the sulphation process of glycosaminoglycans (118). They showed that any labelled sulphate associated with the proteoglycans synthesized in their systems arose from degradation of the ascorbic acid-2-sulphate and the incorporation of the resultant inorganic labelled sulphate into proteoglycans after activation in the manner established by Gregory and Lipman (343). However, their results were disputed by Hatanaka and Egami, who showed that radioactivity from ascorbic acid 2-|$^{35}$S| sulphate was incorporated into chondroitin sulphate by embryonic chick cartilage epiphyses and suggested that ascorbic acid 2-sulphate may act as a long-term storage form associated with the ascorbic acid pool of the body (117). Ascorbic acid has also been shown to promote the assembly of microtubules by maintaining cytoplasmic tubulin in a polymerizable form and thus may also have a role in the efficient secretion of molecules from cells (114,344).

The data mentioned in the previous chapters prompted a further investigation of the effect of ascorbic acid on the biosynthesis of proteoglycans/glycosaminoglycans by cultured aortic smooth muscle cells. It was important to establish whether the increased radioactive sulphate incorporation into pericellular/matrix material by the bovine cells was a result of the increased collagen present and thus due to a collagen-

related phenomenon. Interactions between proteoglycans and the extracellular matrix proteins have been shown to occur in particular between collagen and dermatan sulphate and between elastin and heparan sulphate (Section 1.4.1). The extracellular matrix is thought to be important in determining cell behaviour and differentiative state of cells. Alternatively, the increased sulphate incorporation may be unrelated to the increased secretion of collagen into the matrix and its subsequent interaction with proteoglycans and may be a direct result of the presence of ascorbic acid. The nature of the increased $|^{35}S|$ sulphate incorporation was also investigated, i.e. whether it reflected a general increase in proteoglycan synthesis in the presence of ascorbic acid or whether it reflected an increased level of sulphation of the glycosaminoglycan chains.

## 5.2. METHODS

### 5.2.1. Cell culture

Bovine smooth muscle cells were prepared from medial layers of foetal bovine descending thoracic aortas and cultured as described in Section 3.2.1. For cultures supplemented with ascorbic acid (50 µg/ml culture medium), fresh vitamin additions were made daily as it is rapidly destroyed under tissue culture conditions.

### 5.2.2. Incorporation of radioactive isotopes

Incorporation studies were carried out on cultures grown in

35 mm Petri dishes which were initiated at a seeding density of $10^5$ cells/dish. Cells were pulsed with |$^{35}$S| sulphate (10 µCi/ml) or D-|6-$^3$H| glucosamine (5 µCi/ml) for 24 h and thereafter the extracellular, pericellular-matrix and intracellular compartments were fractionated and collected as described in Section 3.2.7. The amount of radioactivity incorporated into macromolecular material was determined by subjecting aliquots of these fractions to descending paper chromatography on Whatman 3MM paper, as described in Section 3.2.7.

5.2.3. Estimation of collagen synthesis

The estimation of synthesis, deposition and cross-linking of collagen into the extracellular matrix compartment of the culture system was carried out, after labelling matrix proteins with |$^3$H| proline, essentially as described in Section 3.2.4. Dried matrices were prepared and the collagen content was assayed using pure collagenase dissolved in 0,02 M Tris/HCl pH 7,4, containing 2,5 mM N-ethylmaleimide and 10 mM calcium chloride.

5.2.4. Estimation of protein synthesis

The estimation of protein synthesis was carried out after labelling cultures with |$^3$H| leucine (2,5 µCi/ml), |$^{14}$C| phenylalanine (2 µCi/ml) or |$^{14}$C| amino acid mixture (2,5 µCi/ml). At times 0 and 24 h after labelling, the culture medium was removed and retained, cell layers washed 5 times with 2 ml of PBS and cells lysed with 1 ml of 1% SDS. 100 µl aliquots of

the medium samples and 500 µl aliquots of cell lysates were treated with an equal volume of 20% trichloroacetic acid (TCA) at 5°C overnight. Samples were heated at 90°C for 30 min prior to cooling and collection on GFC filters. Filters were washed 5 times with 10 ml of 5% TCA, air-dried and counted in scintillation cocktail as described in Section 3.2.4. The 24 h incorporation into TCA precipitable material was computed by subtraction of the background radioactivity for the zero time samples.

### 5.2.5. Ion-exchange chromatography

Samples of the pericellular-matrix fractions obtained from the cultures were digested with papain (0,5 mg/ml) at 65°C for 8 to 10 h. The enzyme was destroyed by boiling digests for 3 min and samples were clarified by centrifugation in a Beckman microfuge at 11000 rpm for 3 min. The supernatants were dialysed against 5 ℓ of distilled deionized water at 5°C for 24 h with 2 changes. Samples, each in a total volume of 1 ml, were then applied to columns (1,0 x 6 cm) of DEAE-cellulose equilibrated with 0,05 M sodium acetate pH 4,0. Columns were eluted initially (10 fractions) with equilibration buffer and then subsequently with a linear gradient of lithium chloride (0,2 M to 1,5 M) in 0,05 M sodium acetate pH 4,0, as described in Section 2.2.7. Fractions were counted in the scintillation mixture described in Section 3.2.4.

### 5.2.6. Quantitation of glycosaminoglycan chains

Glycosaminoglycan analysis was carried out on samples of the

radiolabelled culture compartments which had been digested with papain, dialysed and freeze-dried. Samples were dissolved in appropriate volumes of 0,01 M Tris/acetate pH 7,3, and then subjected to degradation using various specific glycosaminoglycan degradative procedures, as described in Section 2.2.4. The amount of material digested was assayed by chromatography on a column (1,0 x 120 cm) of Sephadex G-50 (medium) equilibrated with 0,2 M pyridine acetate pH 5,0, as described in Section 2.2.8.

### 5.2.7. Analytical procedures

Protein determinations were carried out using the method of Lowry *et al.* (323), with bovine serum albumin as standard. When carried out on material solubilized by 1% SDS BSA standards were dissolved in the same solution. Uronic acid was estimated by the procedure of Bitter and Muir which had been adapted for use with the Technicon Autoanalyser (317).

## 5.3. RESULTS

As already seen in Chapter 3, the supplementation of aortic smooth muscle cell cultures with ascorbic acid had no gross effects on their morphology, but routinely resulted in increased cell numbers. In addition, a profuse extracellular matrix was laid down in the presence of ascorbic acid, which consisted largely of collagen (Table 3.1). In contrast, the extracellular matrix laid down in the absence of the vitamin consisted to a very large degree of material that was

susceptible to trypsin treatment, indicating the presence of glycoproteins and some proteoglycans (Table 3.1). Other workers have shown that in the absence of ascorbic acid, collagen is still secreted by aortic smooth muscle cells, but, as it is not cross-linked, it is not deposited as a component of the extracellular matrix (335). In the presence of ascorbic acid, collagen synthesis started early in culture and increased steadily until about 10 days after plating (Fig. 3.2). Preliminary experiments also showed that there was a net increase in the amount of $|^{35}S|$ sulphate incorporated into macromolecular material in the presence of ascorbic acid, the most pronounced increase being associated with the pericellular-matrix compartment (Table 3.2). This is not unexpected, bearing in mind the large increase undergone by this compartment with vitamin supplementation (Table 3.1). These results for the cell lines are presented again here in Table 5.1. In another experiment, the pericellular-matrix fraction was further divided into two subfractions. After labelling cells with $|^{35}S|$ sulphate for 24 h the medium was retained and the cell layer was initially treated with pure collagenase and then with trypsin, essentially as previously described. The results are shown in Table 5.2. The biggest increase in $|^{35}S|$ sulphate incorporation (3-fold) was found for that portion of the pericellular-matrix fraction digested by collagenase, although that portion digested with trypsin also increased about 1,5 times.

The ascorbate dose response of the smooth muscle cells with respect to the production of collagen, cell number or cellular

TABLE 5.1

THE EFFECTS OF ASCORBIC ACID ON THE INCORPORATION OF $|^{35}S|$ SULPHATE INTO MACROMOLECULAR MATERIAL ASSOCIATED WITH THE DIFFERENT CULTURE COMPARTMENTS OF SMOOTH MUSCLE CELLS

Cells were grown for 8 days in the presence or absence of ascorbic acid. $|^{35}S|$ sulphate (10 µCi/ml) was present during the final 24 h of culture. The assessment of incorporation of $|^{35}S|$ sulphate into macromolecular material in the extracellular, pericellular-matrix and intracellular compartments was carried out as described in Section 3.2.7. The data represent the mean of triplicate determinations in one of several similar experiments.

| Smooth muscle cell line | Ascorbate | Incorporation of $|^{35}S|$ sulphate (dpm/µg cell protein) | | | |
|---|---|---|---|---|---|
| | | Total | Extracellular | Pericellular-Matrix | Intracellular |
| $AS_1Cl_6$ | + | 515,8±19,8 | 352,6±21,2 | 115,7±5,4 | 47,5±1,4 |
| | - | 300,0±12,4 | 238,7±9,3 | 33,6±2,3 | 27,7±1,1 |
| $A_3$ | + | 465,3±21,2 | 289,4±15,4 | 154,9+6,5 | 21,0±2,8 |
| | - | 368,6±17,5 | 311,5±15,7 | 37,6±2,6 | 19,4±1,6 |

TABLE 5.2

THE EFFECTS OF ASCORBIC ACID ON THE INCORPORATION OF $|^{35}S|$ SULPHATE INTO MACROMOLECULAR MATERIAL ASSOCIATED WITH THE DIFFERENT CULTURE COMPARTMENTS OF SMOOTH MUSCLE CELLS

Cells were grown for 8 days in the presence or absence of ascorbic acid. $|^{35}S|$ sulphate (10 µCi/ml) was present for the final 24 h of culture. The assessment of incorporation of $|^{35}S|$ sulphate into macromolecular material in the extracellular, pericellular-matrix and intracellular compartments was carried out as described in Section 3.2.7. The pericellular-matrix compartment represented macromolecular $|^{35}S|$ sulphate labelled material released by collagenase digestion followed by trypsin digestion and was performed essentially as described in Section 3.2.5. The data represent the means of triplicate determinations in one of several similar experiments.

| | Incorporation of $|^{35}S|$ sulphate (dpm/µg cell protein) | | | | |
|---|---|---|---|---|---|
| Ascorbate | Total | Extracellular | Pericellular-Matrix | | Intracellular |
| | | | Collagenase | Trypsin | |
| + | 448,3±31,4 | 329,6±21,2 | 53,9+2,1 | 32,9+2,0 | 31,9+2,6 |
| - | 363,6±32,7 | 302,3±23,4 | 18,5±1,1 | 21,2±1,4 | 20,6±1,4 |

protein content, and $|^{35}S|$ sulphate incorporation into the different culture compartments, is shown in Fig. 5.1. As may be seen, the production of collagen was very sensitive to the presence of ascorbic acid and increased rapidly, even with the addition of relatively low doses of ascorbic acid (Fig. 5.1a). Collagen production reached a plateau at a dose of about 10 µg/ml and then stayed fairly constant until doses of 100 µg/ml or higher, at which it declined. Cell numbers or the related total amount of cellular protein increased with increasing doses of ascorbic acid until a level of about 10 µg/ml. The very high doses tested (100 - 200 µg/ml) were found to be toxic to the cells and the cell numbers declined. The amount of $|^{35}S|$ sulphate incorporated into macromolecules in the different culture compartments is shown in Fig. 5.1b. At low doses (0-1 µg/ml) the amount of macromolecular $|^{35}S|$ sulphated material associated with the pericellular-matrix compartment remained unchanged, but thereafter increased sharply to reach a plateau at 5 µg/ml. The amount of $|^{35}S|$ sulphate labelled macromolecular material in the medium remained fairly constant at the lower doses but decreased at doses of 25 µg/ml or above, presumably as more material was being trapped in the matrix. The macromolecular material found in the intracellular compartment also remained fairly constant at the different doses of ascorbic acid but decreased as the vitamin reached toxic levels.

The large increase in $|^{35}S|$ sulphate incorporation found in the presence of ascorbic acid, particularly in the pericellular-matrix compartment, was in keeping with the current theories

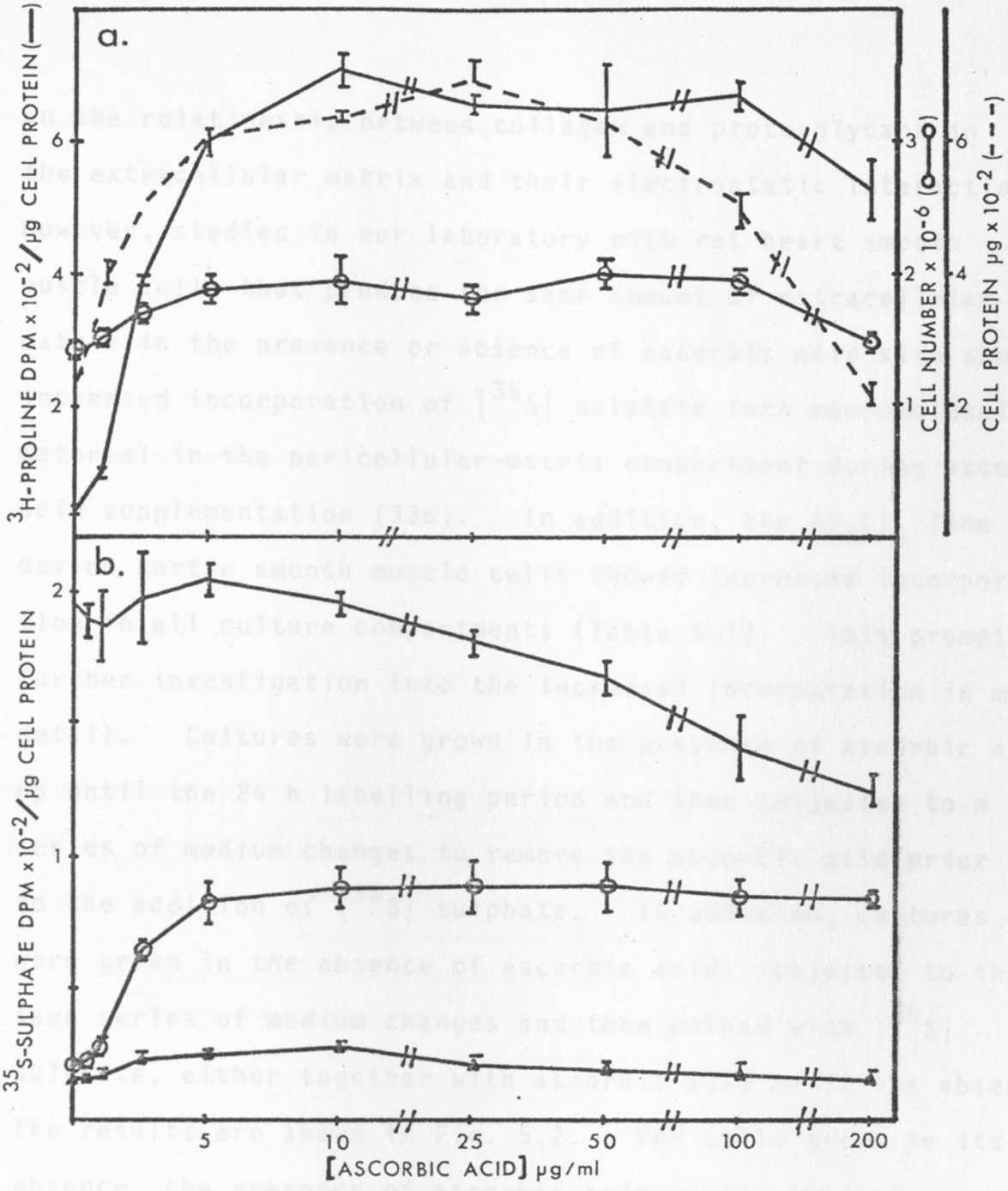



Fig. 5.1. The effect of different concentrations of ascorbic acid on cell number, incorporation of |$^{3}$H| proline and |$^{35}$S| sulphate incorporation into the different culture compartments.

Cells were cultured for 8 days in the presence of the ascorbic acid concentrations indicated.

(a) The cell number (o-o) and concentration of cellular protein (---) were determined as described previously (3.2.2 and 3.2.3). The incorporation of |$^{3}$H| proline into collagenase sensitive matrix components (—) was assessed as described in Section 3.2.4.

(b) |$^{35}$S| Sulphate incorporation into macromolecular material in the extracellular (—), pericellular-matrix (o-o) and intracellular (▲-▲) compartments was assessed during the final 24 h of culture as described in Section 3.2.7.

The data represent one of several similar experiments.

on the relationship between collagen and proteoglycans in the extracellular matrix and their electrostatic interactions. However, studies in our laboratory with rat heart smooth muscle cells that produce the same amount of extracellular matrix in the presence or absence of ascorbic acid also showed increased incorporation of $|^{35}S|$ sulphate into macromolecular material in the pericellular-matrix compartment during ascorbic acid supplementation (336). In addition, the $AS_1Cl_6$ line of bovine aortic smooth muscle cells showed increased incorporation in all culture compartments (Table 5.1). This prompted further investigation into the increased incorporation in more detail. Cultures were grown in the presence of ascorbic acid up until the 24 h labelling period and then subjected to a series of medium changes to remove the ascorbic acid prior to the addition of $|^{35}S|$ sulphate. In addition, cultures were grown in the absence of ascorbic acid, subjected to the same series of medium changes and then pulsed with $|^{35}S|$ sulphate, either together with ascorbic acid or in its absence. The results are shown in Fig. 5.2. For cells grown in its absence, the presence of ascorbic acid during the pulse resulted in marked increases in the amount of $|^{35}S|$ sulphate incorporation in both the extracellular and pericellular-matrix compartments. For cells normally grown in the presence of ascorbic acid the removal of the vitamin during the 24 h labelling period resulted in a significant reduction in $|^{35}S|$ sulphate incorporation into the pericellular-matrix compartment compared to those pulsed in the presence of ascorbic acid. The other compartments were not affected to any significant extent. Thus cultures incubated with $|^{35}S|$ sulphate in the

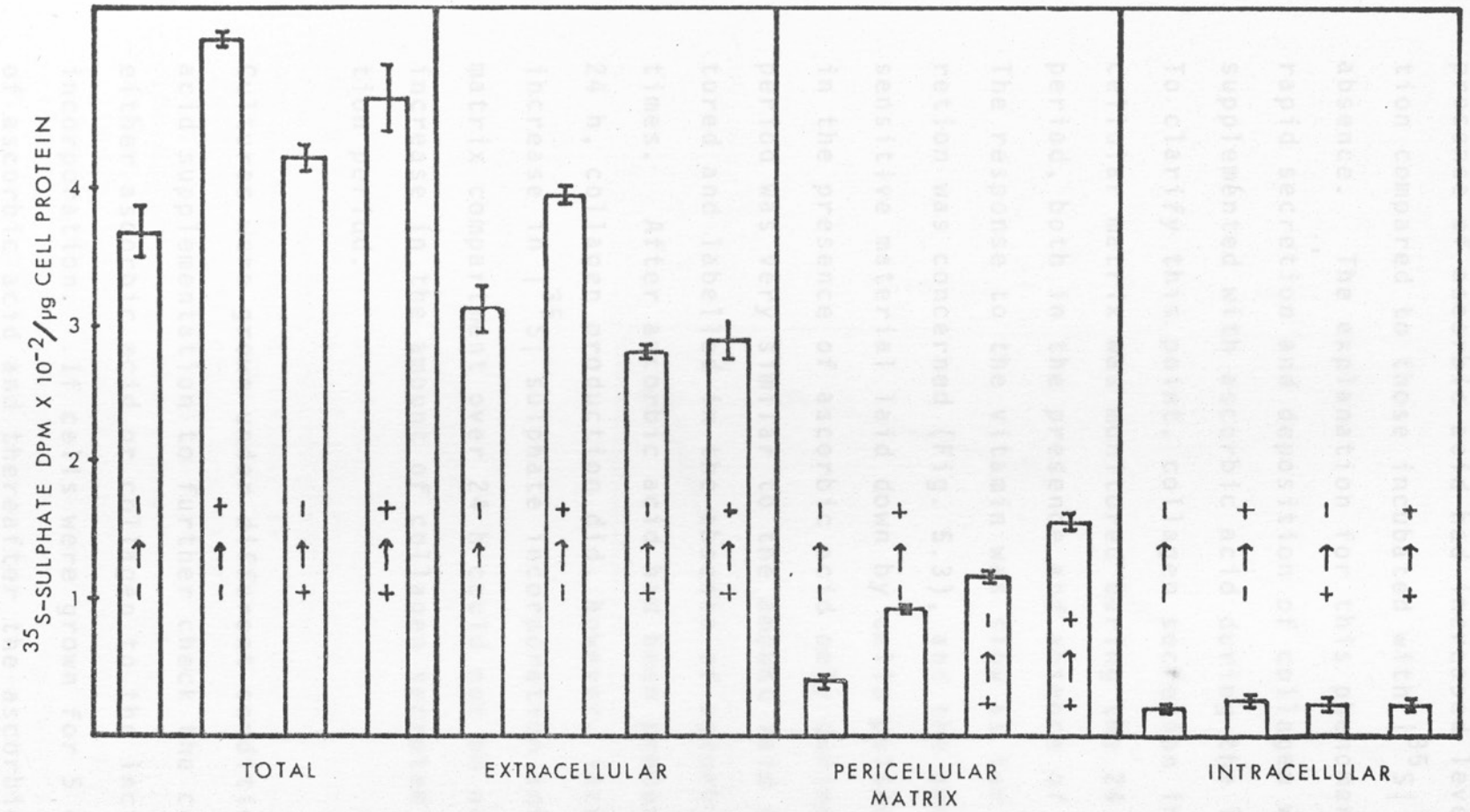



Fig. 5.2. The incorporation of |$^{35}$S| sulphate into macromolecular material associated with the different culture compartments of smooth muscle cells pulsed in the presence or absence of ascorbic acid.

Cells were grown for 8 days under conditions of ascorbic acid-supplementation (+) or -deprivation (-) as indicated in the respective histograms. After changes of medium, cells were cultured for 24 h in the presence of 10 µCi/ml |$^{35}$S| sulphate either together with ascorbic acid (→+) or after its deliberate removal (→-). |$^{35}$S| Sulphate incorporation into macromolecular material in the extracellular, pericellular-matrix and intracellular culture compartments was determined as described in Section 3.2.7. The data represent the mean ± S.D. of quadruplicate determinations for one of several similar experiments.

presence of ascorbic acid had increased levels of incorporation compared to those incubated with $|^{35}S|$ sulphate in its absence. The explanation for this phenomenon could be the rapid secretion and deposition of collagen when cells were supplemented with ascorbic acid during the labelling period. To clarify this point, collagen secretion into the extracellular matrix was monitored during the 24 h labelling period, both in the presence and absence of ascorbic acid. The response to the vitamin was slow as far as collagen secretion was concerned (Fig. 5.3), and the amount of collagenase-sensitive material laid down by cells pulsed with $|^{3}H|$ proline in the presence of ascorbic acid only during the labelling period was very similar to the amount laid down by cells cultured and labelled in the absence of ascorbic acid at all times. After ascorbic acid had been present for longer than 24 h, collagen production did, however, increase. Thus the increase in $|^{35}S|$ sulphate incorporation into the pericellular-matrix compartment over 24 h could not be accounted for by an increase in the amount of collagen secreted during this incubation period.

Cultures were grown under different conditions of ascorbic acid supplementation to further check the contribution by either ascorbic acid or collagen to the increased $|^{35}S|$ sulphate incorporation. If cells were grown for 5 days in the presence of ascorbic acid and thereafter the ascorbic acid was removed by a series of medium changes and the cells maintained for a further 3 days in the absence of the vitamin, although cultures had laid down a large quantity of extracellular matrix consisting

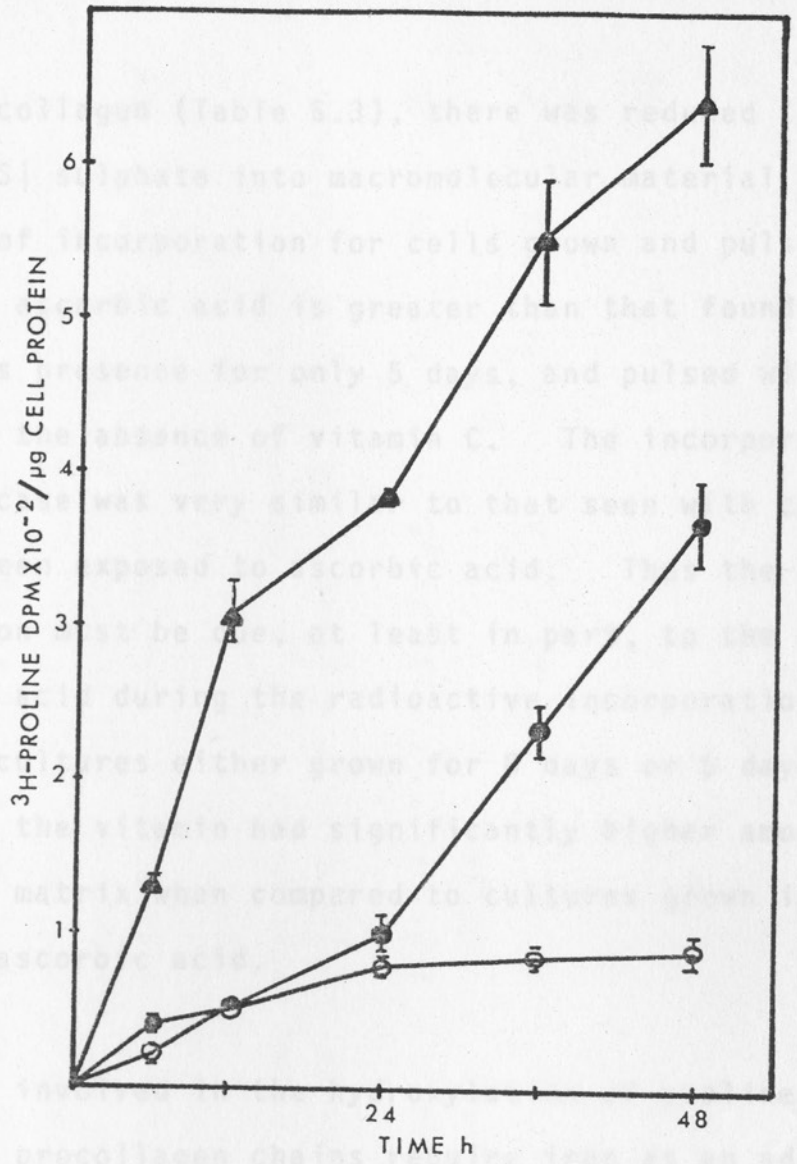



Fig. 5.3. The rate of incorporation of |$^3$H| proline into the collagenase-sensitive component of the extracellular matrix of smooth muscle cells.

Cells were cultured for 8 days in the absence (o-o, ●-●) or presence (▲-▲) of ascorbic acid and then, following medium changes, ascorbic acid was added to some cultures (▲-▲, ●-●) for a further 24 h in the presence of 5 µCi/ml |$^3$H| proline. The symbols thus represent the following culture conditions: ▲-▲ growth for 8 days in the presence of ascorbic acid and pulsed for 24 h with |$^3$H| proline in the presence of the vitamin; ●-● growth for 8 days in the absence of ascorbic acid but with the addition of the vitamin during the 24 h pulse period; and o-o growth and pulse in the absence of ascorbic acid. The incorporation of label into collagenase-sensitive material in the extracellular matrix was determined as described in Section 3.2.4. The data represent the mean ± S.D. for triplicate determinations for one of two similar experiments.

largely of collagen (Table 5.3), there was reduced incorporation of $|^{35}S|$ sulphate into macromolecular material (Fig. 5.4). The amount of incorporation for cells grown and pulsed in the presence of ascorbic acid is greater than that found for cells grown in its presence for only 5 days, and pulsed with $|^{35}S|$ sulphate in the absence of vitamin C. The incorporation in the latter case was very similar to that seen with cells that had never been exposed to ascorbic acid. Thus the increased incorporation must be due, at least in part, to the presence of ascorbic acid during the radioactive incorporation period, since both cultures either grown for 8 days or 5 days in the presence of the vitamin had significantly higher amounts of collagenous matrix when compared to cultures grown in the absence of ascorbic acid.

The enzymes involved in the hydroxylation of proline and lysine residues in procollagen chains require iron as an additional cofactor for their activity, apart from vitamin C (345,346). The compound αα-dipyridyl, when added to culture medium, chelates available iron, and has been used to prevent hydroxylation of prolyl residues and thus impair collagen synthesis (347-349). We used this compound to further test the above effects of ascorbic acid. Initially, to check the response of the smooth muscle cells to the compound, the amount of collagen produced by cells in the presence of both ascorbic acid and different doses of αα-dipyridyl were tested. As is shown in Fig. 5.5, even at the lowest dose tested (50 μM) the amount of collagen deposited by the cells into the extracellular matrix was markedly reduced (10% of the controls), and at higher

TABLE 5.3

PROTEIN COMPOSITION OF EXTRACELLULAR MATRIX MATERIAL LAID DOWN BY SMOOTH MUSCLE CELLS UNDER DIFFERENT CONDITIONS OF ASCORBIC ACID SUPPLEMENTATION

Cells were grown for 8 days in the presence (++) or absence (--) of ascorbic acid or for 5 days in its presence and thereafter, after changes of medium, for a further 3 days in the absence of vitamin (+-). The cells were cultured in the presence of $|^{3}H|$ proline (5 µCi/ml). Compositional analysis was carried out on sequential enzymatic digestions as described in Section 3.2.5. The data represent the mean ± S.D. from 8 determinations.

| Ascorbate | Total Radioactivity (dpm) | Radioactivity (dpm) released by Trypsin | Elastase | Collagenase | Composition (%) Glycoproteins | Elastin | Collagen |
|---|---|---|---|---|---|---|---|
| ++ | 3770298 ± 98854 | 1192177± 56596 | 56859± 8615 | 2521262± 50224 | 31,7+1.5 | 1,5±0 | 66,8+1,5 |
| +- | 1721631 ± 143291 | 746261± 72140 | 29778± 2329 | 945592 ± 63790 | 43,6±2,2 | 1,7±0,1 | 54,7±2,3 |
| -- | 347642 ± 31648 | 339254± 34747 | 5728± 236 | 2660± 235 | 97,6±0,2 | 1,6±0,1 | 0,8±0,06 |

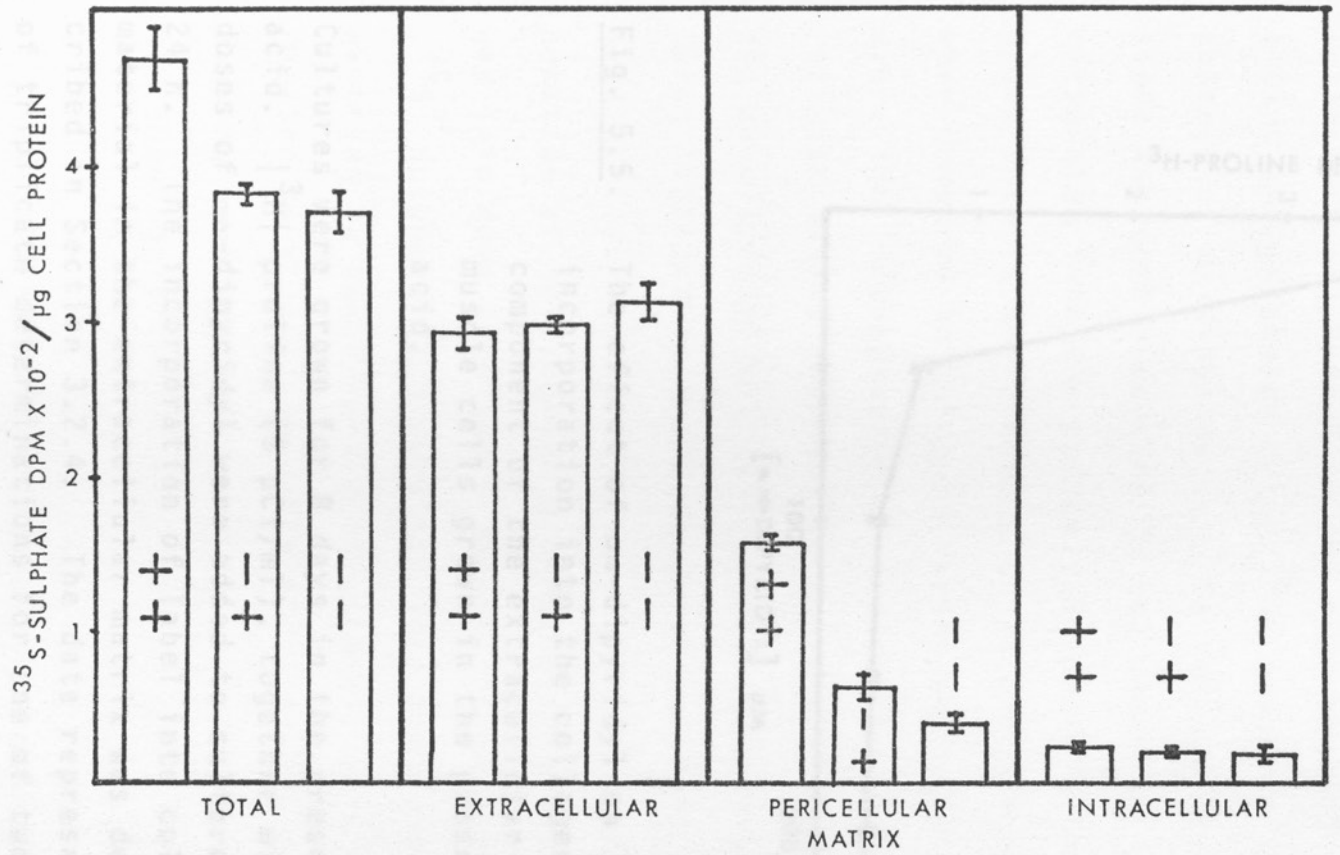



Fig. 5.4. The effect of a collagenous matrix on |$^{35}$S| sulphate incorporation into the different culture compartments of smooth muscle cells.

Cells were cultured for 8 days in the presence of ascorbic acid (++) or in its absence (--) or for 5 days in the presence of ascorbic acid and thereafter the vitamin was removed and the cells maintained for a further 3 days in its absence (+-). 10 µCi/ml |$^{35}$S| sulphate was present during the final 24 h of culture. Incorporation into macromolecular material in the extracellular, pericellular-matrix and intracellular culture compartments was determined as described in Section 3.2.7. The data represent the mean ± S.D. of quadruplicate determinations for one of several similar experiments.

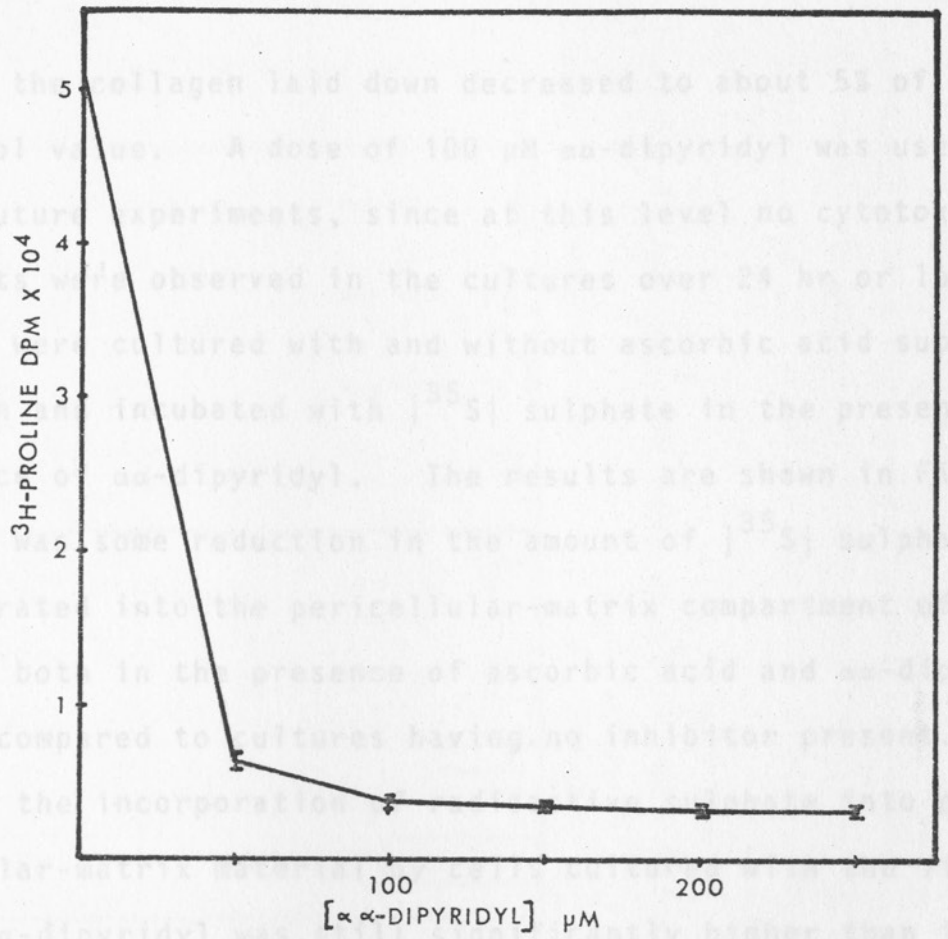



Fig. 5.5. The effect of αα-dipyridyl on |$^3$H| proline incorporation into the collagenase-sensitive component of the extracellular matrix of smooth muscle cells grown in the presence of ascorbic acid.

Cultures were grown for 8 days in the presence of ascorbic acid. |$^3$H| proline (5 μCi/ml), together with the different doses of αα-dipyridyl were added to cultures for the final 24 h. The incorporation of label into collagenase-sensitive material in the extracellular matrix was determined as described in Section 3.2.4. The data represent the mean ± S.D. of triplicate determinations for one of two similar experiments.

doses the collagen laid down decreased to about 5% of the control value. A dose of 100 μM αα-dipyridyl was used for all future experiments, since at this level no cytotoxic effects were observed in the cultures over 24 hr or longer. Cells were cultured with and without ascorbic acid supplementation and incubated with $|^{35}S|$ sulphate in the presence or absence of αα-dipyridyl. The results are shown in Fig. 5.6. There was some reduction in the amount of $|^{35}S|$ sulphate incorporated into the pericellular-matrix compartment of cultures grown both in the presence of ascorbic acid and αα-dipyridyl when compared to cultures having no inhibitor present. However, the incorporation of radioactive sulphate into pericellular-matrix material by cells cultured with the vitamin and αα-dipyridyl was still significantly higher than in cultures labelled in the absence of ascorbic acid. As expected, αα-dipyridyl had no effect on the radioactive sulphate incorporation of cells grown without ascorbic acid, since they anyway laid down very small amounts of collagen. The increased $|^{35}S|$ sulphate incorporation into pericellular-matrix material observed in the presence of ascorbic acid was thus not merely related to a "collagen effect". Ascorbic acid is therefore playing some specific role in the biosynthesis of sulphated glycosaminoglycans.

To check whether the increased incorporation of $|^{35}S|$ sulphate in the presence of ascorbic acid merely resulted from a general increase in metabolic activity of the cell in the presence of the vitamin, the incorporation of labelled amino acids into trichloroacetic acid-precipitable material was

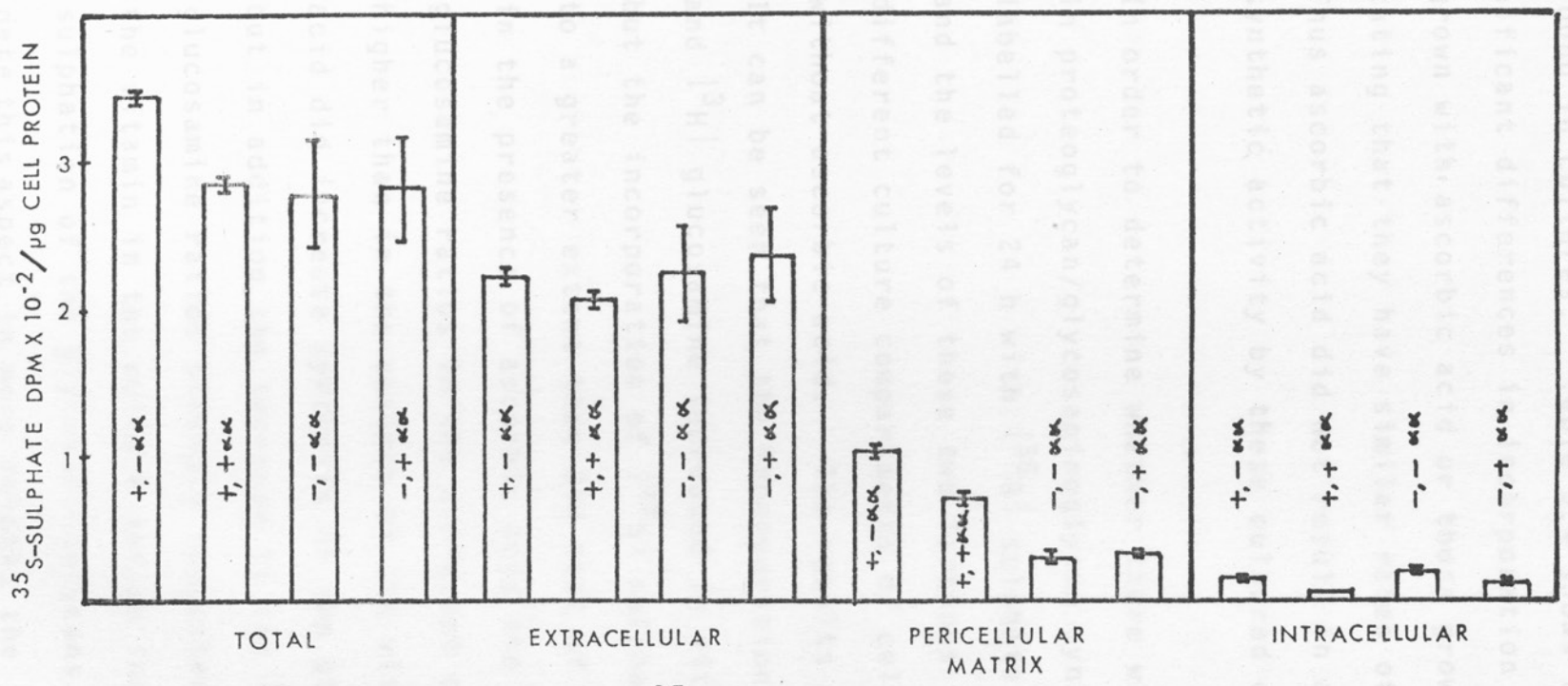



Fig. 5.6. The effect of αα-dipyridyl on |$^{35}$S| sulphate incorporation into macromolecular material in the different culture compartments of smooth muscle cells.

Cells were grown for 8 days under conditions of ascorbic acid supplementation (+) or deprivation (-). |$^{35}$S| sulphate (10 µCi/ml) was added for the final 24 h either together with 100 µM αα-dipyridyl (+,+αα; -,+αα) or in its absence (+,-αα; -,-αα). Incorporation of label into macromolecular material in the extracellular, pericellular-matrix and intracellular culture compartments was determined as described in Section 3.2.7. The data represent the mean ± S.D. for quadruplicate determinations for one of three similar experiments.

studied and related to the total amount of cellular protein found in cultures. Table 5.4 shows that there were no significant differences in incorporation over 24 h between cells grown with ascorbic acid or those grown in its absence, indicating that they have similar rates of protein synthesis. Thus ascorbic acid did not result in a general increase in synthetic activity by these cultured cells.

In order to determine whether there was an overall increase in proteoglycan/glycosaminoglycan synthesis, cultures were labelled for 24 h with $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine and the levels of these two isotopes were checked in the different culture compartments of cells grown either with or without ascorbic acid. The results are shown in Table 5.5. It can be seen that the incorporation of both $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine increased in vitamin-supplemented cultures but the incorporation of $|^{35}S|$ sulphate routinely increased to a greater extent than did that of $|^{3}H|$ glucosamine so that in the presence of ascorbic acid the $|^{35}S|$ sulphate to $|^{3}H|$ glucosamine ratios in the different compartments were always higher than in the absence of the vitamin. Thus ascorbic acid did increase synthesis of the glycosaminoglycan chains, but in addition, the increase in the $|^{35}S|$ sulphate to $|^{3}H|$ glucosamine ratios strongly suggested that the presence of the vitamin in the culture medium increased the degree of sulphation of the glycosaminoglycans. In order to investigate this aspect in more detail, the $|^{35}S|$ sulphate to uronic acid ratios of the glycosaminoglycan molecules were examined in the various culture compartments under conditions of

TABLE 5.4

EFFECT OF ASCORBIC ACID ON THE INCORPORATION OF AMINO ACID PRECURSORS BY SMOOTH MUSCLE CELLS

The incorporation of labelled amino acid precursors into TCA precipitable material in the medium or SDS lysates of the cell layer was carried out as described in Section 5.2.4. The data represent the mean ± S.D. of quadruplicate determinations from one of two similar experiments.

| Precursor | Ascorbate | Radioactivity Incorporated (dpm/µg cell protein) | |
|---|---|---|---|
| | | Medium | Intracellular |
| [$^{3}$H]Leucine | + | 57,8±7,1 | 60,2±5,8 |
| | - | 70,7+3,4 | 65,6±5,3 |
| [$^{14}$C]Phenylalanine | + | 51,1±2,2 | 51,7±0,4 |
| | - | 45,8±0,1 | 51,2±0,2 |
| [$^{14}$C]Amino acid mixture | + | 530,1±17,5 | 282,2±14,5 |
| | - | 503,3±12.9 | 308,0±10,3 |

TABLE 5.5

THE EFFECT OF ASCORBIC ACID ON THE INCORPORATION OF $|^{35}S|$ SULPHATE AND $|^{3}H|$ GLUCOSAMINE INTO THE DIFFERENT CULTURE COMPARTMENTS OF SMOOTH MUSCLE CELLS

Cells were cultured for 8 days in the presence or absence of ascorbic acid. $|^{35}S|$ sulphate (10 µCi/ml) and $|^{3}H|$ glucosamine (5 µCi/ml) were present for the final 24 h of culture. The assessment of incorporation of $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine into macromolecular material in the extracellular, pericellular-matrix and intracellular compartments was carried out as described in Section 3.2.7. The data represent the mean ± S.D. of quadruplicate determinations from one of three similar experiments.

| | Ascorbate | Radioactivity incorporated (dpm/µg cell protein) | | $|^{35}S|$Sulphate : $|^{3}H|$Glucosamine |
|---|---|---|---|---|
| | | $|^{35}S|$Sulphate | $|^{3}H|$ Glucosamine | |
| Total | + | 230144±15239 | 203449±15339 | 1,13 ± 0,06 |
| | - | 117964±4337 | 140704±7160 | 0,84 ± 0,04 |
| Extracellular | + | 129771±12224 | 127066±8707 | 1,03 ± 0,01 |
| | - | 94713±3925 | 110248±7101 | 0,86 ± 0,05 |
| Pericellular-Matrix | + | 75913±5332 | 51540±4577 | 1,5 ± 0,02 |
| | - | 13760±729 | 13603±699 | 1,02 ± 0,10 |
| Intracellular | + | 24460±1795 | 24843±1936 | 0,98 ± 0,06 |
| | - | 9488±273 | 16853±821 | 0,56 ± 0,03 |

ascorbic acid-deprivation and supplementation. These determinations were carried out on cultures that had accumulated $|^{35}S|$ sulphate labelled material for 8 days in order to permit accurate determination of the uronic acid contents of the different samples. At each medium change $|^{35}S|$ sulphate was added to the fresh medium; all media were retained and pooled. Media samples were dialysed extensively before analysis, as failure to do this resulted in erroneous uronic acid determinations due to the colour of the medium. Medium which had never been exposed to cells was routinely treated in exactly the same way to determine the levels of uronic acid normally present in the medium. From the data shown in Table 5.6 it was clear that there was an increase in the $|^{35}S|$ sulphate to uronic acid ratios in all compartments in the presence of ascorbic acid, providing further evidence for an increase in the sulphation levels of the glycosaminoglycans in the presence of the vitamin.

It was shown in Chapter 4 that the glycosaminoglycan chains derived from proteoglycans from the medium and cell layer for cultures grown both in the presence or in the absence of ascorbic acid were all the same size. Thus, if the chains synthesized in the presence of ascorbic acid were the same size but had a greater degree of sulphation and hence a greater total negative charge, they would be expected to bind more strongly to DEAE-cellulose at low ionic strength and require higher levels of ions to displace them compared to less sulphated species. To test this hypothesis, samples from both ascorbic acid-supplemented or-deprived cultures

TABLE 5.6

THE EFFECT OF ASCORBIC ACID ON THE RATIOS OF MACROMOLECULAR $|^{35}S|$ SULPHATE TO URONIC ACID IN THE DIFFERENT CULTURE COMPARTMENTS OF SMOOTH MUSCLE CELLS

Cells were cultured for 8 days in the presence or absence of ascorbic acid and with $|^{35}S|$ sulphate (10 μCi/ml). At each medium change during this period $|^{35}S|$ sulphate was added to the new medium; all media were retained and pooled. At the end of the culture period these samples were exhaustively dialysed against running water at 5°C. The media, pericellular-matrix and intracellular compartments were analysed for total macromolecular $|^{35}S|$ sulphate and uronic acid contents. The data represent one of three similar experiments.

| | Ascorbate | Total Radioactivity (dpm) | Total Uronic Acid (μg) | Specific Radioactivity (dpm/μg UA) |
|---|---|---|---|---|
| Extracellular | + | 9735243 | 2970 | 3278 |
| | - | 4344325 | 2880 | 1058 |
| Pericellular-matrix | + | 54577 | 65 | 842 |
| | - | 24447 | 59 | 412 |
| Intracellular | + | 58003 | 72 | 809 |
| | - | 14630 | 63 | 232 |

were run on the same DEAE-cellulose column. Cultures were incubated for 24 h in the presence of ascorbic acid and $|^{3}H|$ glucosamine, or in the absence of the vitamin and $|^{14}C|$ glucosamine. Pericellular-matrix material from the cultures was prepared and processed as described in Section 5.2.5. Samples were mixed at approximately equivalent radioactivity levels and an aliquot was analysed by DEAE-cellulose chromatography as described previously (Fig. 5.7). The majority of the glycosaminoglycan chains from the pericellular-matrix compartment of cells grown in the presence of ascorbic acid ($|^{3}H|$ glucosamine labelled) eluted from the column at a higher salt concentration than did the chains synthesized by cultures grown in the absence of ascorbic acid ($|^{14}C|$ glucosamine labelled) (Fig. 5.7a). In the material from the supplemented cells the biggest change was a pronounced peak of radioactivity (peak IV) eluting at approximately the ionic strength required to elute commercial chondroitin-4-sulphate, whereas the glycosaminoglycans from ascorbic acid-deprived cultures mostly eluted at lower ionic strengths. Digestion of equal aliquots of the mixed samples with chondroitinase ABC (Fig. 5.7b) or nitrous acid (Fig. 5.7c) revealed that peaks I and IV were chondroitinase ABC-sensitive, peak III was sensitive to nitrous acid treatment and peak II contained material degraded by both chondroitinase ABC and nitrous acid. The differences in elution profiles on DEAE-cellulose for material from the pericellular-matrix compartments of cultures grown either in the presence or absence of ascorbic acid supported the observations that the vitamin caused increased levels of sulphation of the glycosaminoglycans.

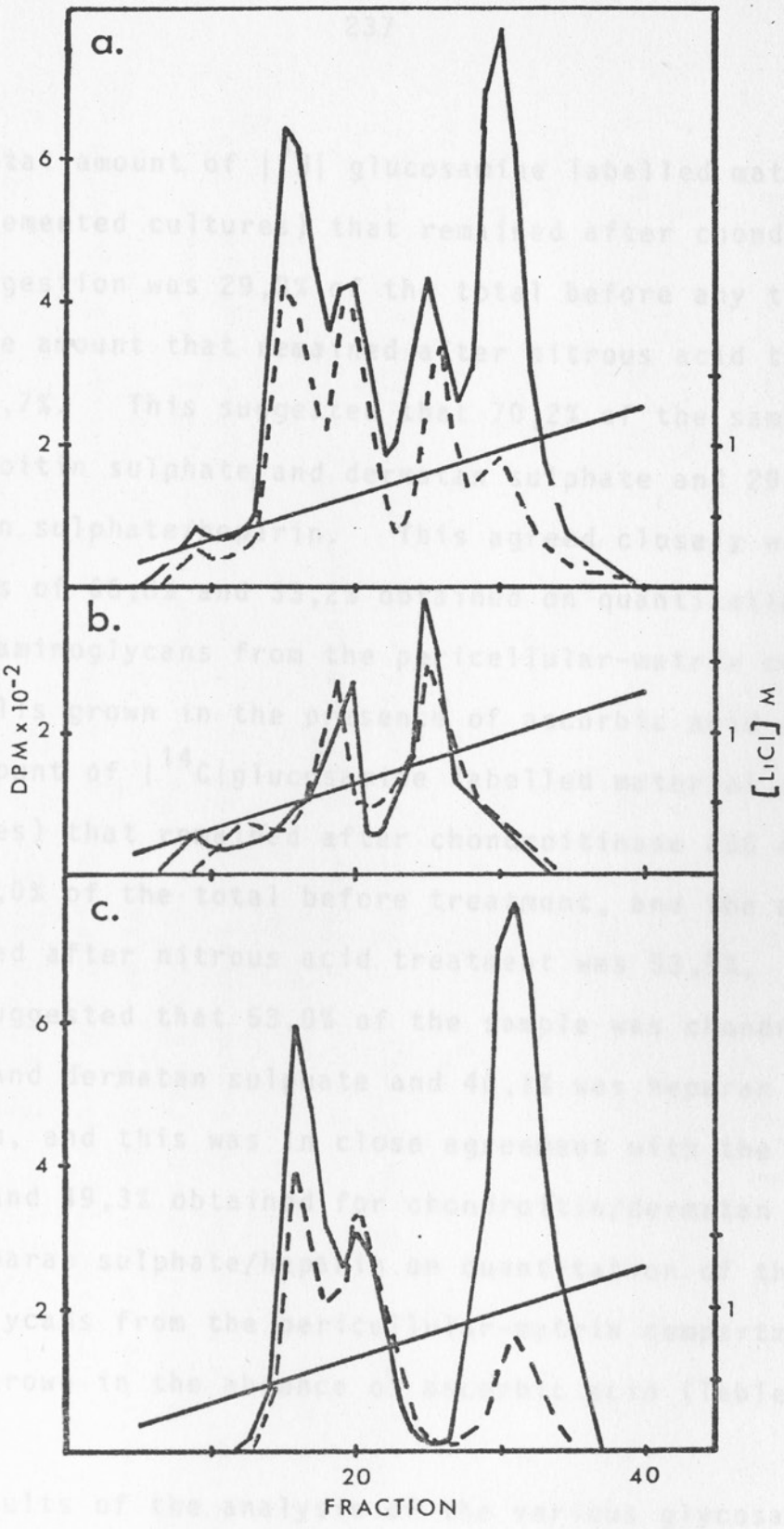



Fig. 5.7. Elution profiles of radioactively-labelled glycosaminoglycan chains analysed by DEAE-cellulose column chromatography.
Cells were cultured for 8 days under conditions of ascorbic acid supplementation or deprivation. During the final 24 h [$^{14}$C]glucosamine (--) was added to those cultures grown in the absence of ascorbic acid and [$^{3}$H]glucosamine (—) was added to those cultures grown in the presence of the vitamin. After labelling the pericellular-matrix, material from both ascorbic acid-supplemented and -deprived cultures was mixed and applied to DEAE-cellulose columns before (a) and after digestion with chondroitinase ABC (b) or treatment with nitrous acid (c). Recovery of radioactivity from the columns was better than 95%.

The total amount of $|^{3}H|$ glucosamine labelled material (supplemented cultures) that remained after chondroitinase ABC digestion was 29,8% of the total before any treatment, and the amount that remained after nitrous acid treatment was 70,7%. This suggested that 70,2% of the sample was chondroitin sulphate and dermatan sulphate and 29,3% was heparan sulphate/heparin. This agreed closely with previous figures of 66,8% and 33,2% obtained on quantitation of the glycosaminoglycans from the pericellular-matrix compartment for cells grown in the presence of ascorbic acid (Table 4.2). The amount of $|^{14}C|$glucosamine labelled material (deprived cultures) that remained after chondroitinase ABC digestion was 47,0% of the total before treatment, and the amount that remained after nitrous acid treatment was 53,9%. This likewise suggested that 53,0% of the sample was chondroitin sulphate and dermatan sulphate and 46,1% was heparan sulphate/heparin, and this was in close agreement with the values of 50,7% and 49,3% obtained for chondroitin/dermatan sulphate and heparan sulphate/heparin on quantitation of the glycosaminoglycans from the pericellular-matrix compartment of cells grown in the absence of ascorbic acid (Table 4.2).

The results of the analysis of the various glycosaminoglycans from the different culture compartments from cells grown in the presence or absence of ascorbic acid was discussed fully in Chapter 4. These results are summarized here in Table 5.7. Briefly, the presence of ascorbic acid caused an overall increase in the relative amounts of dermatan sulphate and heparan sulphate/heparin, and a decrease in the relative amount

TABLE 5.7

COMPOSITIONAL ANALYSIS OF THE SULPHATED GLYCOSAMINOGLYCANS FROM THE DIFFERENT CULTURE COMPARTMENTS OF SMOOTH MUSCLE CELLS

Compositional analysis of glycosaminoglycans was performed as described in Section 5.2.6, using specific enzymatic digestions or nitrous-acid treatment of material isolated from the different culture compartments. Cells were grown for 8 days and $|^{35}S|$ sulphate (10 µCi/ml) was added to the cultures for the last 48 h. The data represent the results from one of three similar experiments.

| Glycosaminoglycan Type | Ascorbate | Glycosaminoglycan Content (%) | | | |
|---|---|---|---|---|---|
| | | Extracellular | Pericellular-Matrix | Intracellular | Total |
| Chondroitin-4- and -6-sulphate | + | 61,1% | 30,2% | 11,8% | 49,0 |
| | - | 70,0% | 39,1% | 32,8% | 63,6 |
| Dermatan sulphate | + | 22,1% | 36,6% | 76,7% | 29,5 |
| | - | 22,7% | 11,6% | 48,6% | 23,5 |
| Heparan sulphate/ heparin | + | 16,8% | 33,2% | 11,5% | 21,5 |
| | - | 7,3% | 49,3% | 18,6% | 12,9 |

of chondroitin sulphate synthesized and secreted by the cells. In the pericellular-matrix and intracellular compartments there was a marked increase in dermatan sulphate in the presence of ascorbic acid and in the intracellular compartment this was concomitant with a decrease in the amounts of chondroitin sulphate and heparan sulphate/heparin. In the extracellular compartment the amount of heparan sulphate/heparin increased in the presence of the vitamin but that of dermatan sulphate remained constant.

Thus the presence of ascorbic acid in the culture medium of bovine aortic smooth muscle cells resulted in increased levels of proteoglycan synthesis, increased levels of sulphation of the glycosaminoglycan chains of these proteoglycans and resulted in the increased synthesis of dermatan sulphate and heparan sulphate/heparin at the expense of chondroitin sulphate. These effects appeared to be due to the direct influence of ascorbic acid and were not only mediated via the vitamin's effects on collagen biosynthesis. However, the large increase in radioactive sulphate associated with the pericellular-matrix compartment may be due to some extent to an increased trapping of the proteoglycans by the much-increased extensive collagen matrix.

## 5.4. DISCUSSION

Until recently the role of ascorbic acid in collagen synthesis had been accepted as an exclusive involvement in the hydroxylation of proline and lysine residues. However, alternative roles for ascorbic acid in the control of collagen biosynthesis

have now been reported (341).

Other roles for ascorbic acid have also been suggested, for example, as a promotor for microtubule assembly and thence the process of secretion (114,344). Studies in our laboratory with rat heart smooth muscle cells in culture showed that bethanecol, which has been used to mimic the effect of ascorbic acid on this process, did not cause enhanced secretion of sulphated proteoglycans into the pericellular-matrix compartment.

The results with bovine aortic smooth muscle cells showed that the presence of ascorbic acid resulted in an increased incorporation of $|^{35}S|$ sulphate into macromolecular material, particularly in the pericellular-matrix compartment. In the presence of ascorbic acid a greater proportion of the total sulphated macromolecules was found in the pericellular-matrix compartment (30% compared to 11% in the absence of the vitamin). Presumably, the proteoglycan molecules are trapped in the extensive extracellular matrix by electrostatic interactions between their glycosaminoglycan chains and the matrix proteins. The results showed that the increased incorporation of $|^{35}S|$ sulphate into macromolecular material in the presence of ascorbic acid was in part due to an increased synthesis of proteoglycans and in part due to an increase in the sulphation level of the glycosaminoglycans. The increased levels of macromolecular radioactive sulphate would seem to be due not only to the increased amount of collagen found in the extracellular matrix of ascorbic acid-supplemented cultures but

also as a direct result of the addition of the vitamin. Although Shapiro and Poon have negated the role of ascorbic acid 2-sulphate as a sulphate donor (118), other workers have suggested that this sulphate intermediate of ascorbic acid does have a role to play in biological sulphate transfer (117). The findings presented here do not support either contention but clearly show that the vitamin plays an important role in the biosynthetic pathway leading to secretion and deposition of the sulphated macromolecules.

There is increasing awareness of the presence of desulphatase enzymes associated with the external surface of some cultured cells (169). The presence of these enzymes has been postulated to be involved in the desulphation of external proteoglycans which can then be internalized by receptor-mediated endocytosis, prior to being degraded intracellularly (see Section 1.3.1) (167-169). Ascorbic acid may inhibit these ecto-sulphatases and this may result in a higher sulphate content for the macromolecules associated with the pericellular-matrix compartment in the presence of the vitamin.

The process of sulphation is a complex one and involves the formation of both sulphamino groups and O-sulphates in heparan sulphate (93,350). Under tissue culture conditions in the presence of ascorbic acid there may be over-sulphation of certain glycosaminoglycan species - dermatan sulphate and heparan sulphate. Sulphate levels have been shown to vary from tissue to tissue, for example, chondroitin 4 and 6 sulphate may have between 0,1 and 1,3 sulphate residues per

disaccharide unit (351). Studies in our laboratory showed that the sulphate to uronic acid ratio of glycosaminoglycans synthesized by rat heart smooth muscle cells under scorbutic conditions is low; this is based on direct analysis for inorganic sulphate. Thus in the absence of ascorbic acid, glycosaminoglycans synthesized in tissue culture may be undersulphated and the supplementation of the culture with ascorbic acid may result in an increased incorporation of sulphate at the many sites that are still available for sulphation. The undersulphation of glycosaminoglycans in tissue culture is supported by the fact that, although the pericellular-matrix compartment of vitamin deprived cells contains about 50% chondroitin and dermatan sulphate, the glycosaminoglycans eluted from a DEAE-cellulose column at a much lower ionic strength than that required for the elution of commercial chondroitin sulphate of shark origin.

The interaction of connective tissue macromolecules and their coordinated synthesis has been under study for some time (352). Collagenous matrices have been shown to alter the growth of normal and transformed cultures. Thus some of the effects reported here, such as the increased cell number, may be due to the increased collagen production in the presence of ascorbic acid. The changes in the types of glycosaminoglycan chains synthesized by cells cultured in the presence of the vitamin that were observed, may be due to such phenomena, since a certain degree of specificity of interaction between dermatan sulphate and collagen on the one hand, and between elastin and heparan sulphate on the other, has been proposed

(187). Ascorbic acid-supplementation caused an increase in the amount of dermatan sulphate in the pericellular-matrix compartment and a decrease in the amount of heparan sulphate/heparin, possibly as a result of an increased amount of collagen. This is at variance with the data reported by Schwartz et al. (335). Using antiserum to heparan sulphate, they have reported an increase in this glycosaminoglycan associated with the cell layers of ascorbic acid-supplemented bovine calf aortic smooth muscle cells in culture. The overall pattern in our culture system, however, revealed increases in both dermatan sulphate and heparan sulphate/heparin in the presence of ascorbic acid at the expense of chondroitin sulphate. The data that have been presented relating to the inhibition of matrix protein deposition caused by αα-dipyridyl showed that the changes in glycosaminoglycan content may nevertheless occur in the absence of the interacting protein components.

Thus supplementation of aortic smooth muscle cells with ascorbic acid led to both increased synthesis of sulphated macromolecules and increased levels of sulphation of these molecules. In addition, the glycosaminoglycan patterns in the presence of the vitamin were altered with increasing amounts of dermatan sulphate and heparan sulphate/heparin being synthesized at the expense of chondroitin sulphate. These changes may occur as a result of increased collagen production as well as by some direct effect mediated by the addition of the vitamin. As yet the mechanism whereby ascorbic acid exerts this direct effect is unknown.

# CHAPTER 6

# THE EFFECT OF β-D-XYLOSIDES ON GLYCOSAMINOGLYCAN SYNTHESIS BY CULTURED SMOOTH MUSCLE CELLS

## 6.1. INTRODUCTION

Five of the the seven known types of glycosaminoglycan chain, namely chondroitin-4- or -6-sulphate, dermatan sulphate, heparan sulphate and heparin have been shown to be initiated on peptidyl serine residues on the proteoglycan core protein via a unique linkage region involving a specific tetrasaccharide (Section 1.2):

- GlcUA - Gal - Gal - Xyl - Ser (97)

The enzyme xylosyltransferase transfers the initial xylose residue from UDP-xylose to the hydroxyl group of the serine and the covalent attachment that ensues gives rise to an acceptor for the transfer of the first galactose residue by galactosyltransferase 1. Other monosaccharides are added sequentially from their respective UDP-activated derivatives by distinct, specific transferases (353-355). Thus it is not surprising that this metabolic process can be mimicked and short-circuited by the introduction of a group of compounds, the β-D-xylosides. The use of these compounds with tissue culture cells has resulted in the synthesis of glycosaminoglycan chains initiated on the exogenously supplied acceptor. Thus the requirement for core protein may be circumvented and glycosaminoglycan synthesis is no longer regulated by the activity of xylosyltransferase.

Several workers have made use of β-D-xylosides as tools for dissecting the pathways of proteoglycan synthesis. In general these artificial acceptors have been shown to prime the synthesis of glycosaminoglycans, specifically chondroitin sulphate, more efficiently than does the core protein-xyloside, and their addition to cells in culture results in a marked stimulation of incorporation of radioactive precursors into free glycosaminoglycan chains which are initiated on xyloside acceptors (98,100-103,356). The β-conformation of the acceptor is essential because of the specificity of the first galactosyltransferase for β orientation of the hydroxyl group on the anomeric carbon (100,102,103). D-xylose has been shown to be able to initiate chondroitin sulphate chains but only at a very high concentration, and then only to a limited extent (100). Thus, presumably, galactosyltransferase I exhibits preference for a xyloside linkage. The aglycone moiety of the β-D-xylosides also influences the stimulation of glycosaminoglycan synthesis; non-polar residues such as n-butyl, phenyl and benzyl groups are most effective (100, 102,103). This may result from differences in the rate of transport of the different xylosides through the plasmalemma or from differences in affinity for the xylosides exhibited by UDP-galactosyltransferase I. Furthermore, it has been shown that other sugar derivates further along in the sequence, such as β-D-galactosides and β-D-N-acetylgalactosamine, cannot initiate glycosaminoglycan synthesis since clearly only UDP-galactosyltransferase I recognises a monosaccharide as an acceptor (100,103). At variance with this finding, however, is the result of Robinson et al., who showed

that β-D-galactosides could act as artificial initiators of chondroitin sulphate synthesis (102).

It is generally agreed that the glycosaminoglycan chains formed on xyloside acceptors are shorter than those synthesized on core protein in the absence of xyloside (100,103, 357,358), although there have been one or two reports in which no differences were found in glycosaminoglycan chain length from control or xyloside-treated cultures (100). These studies have given rise to the proposal that the core protein plays a role in the normal regulation of chain length. In addition, chains initiated on β-D-xyloside acceptors have been shown to be more soluble and were rapidly secreted from cells so that in the presence of β-D-xylosides a much greater proportion of the glycosaminoglycan material was found in the culture medium, and correspondingly less was associated with the extracellular matrix (100,358-360). The response to the addition of β-D-xylosides is rapid and has been shown to reach a maximum after 3 to 6 h of exposure (100).

Several workers have reported a marked stimulation of radioactive $|^{35}S|$ sulphate incorporation into macromolecular material in the presence of β-D-xylosides (96,100,103,356). However, this increase is almost always attribued to an increased synthesis of glycosaminoglycans. Only in a few instances have the levels of sulphation of the synthesized glycosaminoglycans been examined. Some laboratories have found no change in sulphation level of glycosaminoglycans initiated on β-D-xylosides compared to those formed on the

core protein in the absence of the xyloside (360). Yet again others have reported a decrease in sulphation of chondroitin sulphate chains from xyloside-treated cultures, whether they are primed on the acceptor or on core protein (358,361). Sudhakaran et al. were able to induce heparan sulphate synthesis on xyloside if synthesis of core protein was inhibited by cycloheximide (362). They reported that the heparan sulphate initiated on the artificial acceptor was more sulphated, as well as being shorter than heparan sulphate synthesized in the absence of β-D-xylosides.

It has been observed that addition of β-D-xylosides to cultures enhances the synthesis of chondroitinase ABC-sensitive material. Greater proportions of chondroitin sulphate are synthesized in the presence of β-D-xylosides than in its absence, even by non-connective tissue cell types that normally synthesize very small amounts of this glycosaminoglycan (100,107). Stevens and Austen have shown that rat mast cells which normally synthesize heparin proteoglycan almost exclusively (greater than 90% of total proteoglycan) can be stimulated by β-D-xylosides to synthesize free glycosaminoglycan chains which were almost entirely degradable by chondroitinase ABC (107). In their system, as well as in that of other authors (103), it has been shown that relatively few, if any, heparin chains were initiated on the artificial acceptors, which suggested that, in spite of having the same tetrasaccharide linkage sequence to core protein, more specific initiation requirements than those offered by the β-D-xyloside may be required for heparin biosynthesis. Alternatively, the cell may have the capacity

to synthesize a greater amount of chondroitin sulphate than is normally expressed in the presence of core protein, but the synthesis of heparin may be at a maximal level and thus cannot be stimulated by addition of xyloside. Thus in heparin proteoglycan synthesis the elongation enzymes may be the limiting factor.

Whether the increase in chondroitinase ABC-sensitive material is a general increase in chondroitin synthesis or a specific increase in one form of chondroitin sulphate, i.e. chondroitin-4-sulphate, chondroitin-6-sulphate, or dermatan sulphate has not been established. Lohmander et al. found that a somewhat larger proportion of 6-sulphated disaccharide was isolated from xyloside-treated chondrocyte cultures than from control cultures, and comparisons of digestions with chondroitinases ABC and AC showed that dermatan sulphate was not synthesized in these cultures (359).

In addition to greatly stimulating the synthesis of free glycosaminoglycan chains, the presence of β-D-xyloside was also shown to markedly decrease the amount of glycosaminoglycan chain polymerized on core protein (101,131,360). However, by the use of a specific radioimmunoassay, the concentration of core protein/cell was shown to be the same for both control and xyloside-treated chondrocyte cultures (101). Furthermore, glycosaminoglycans that were synthesized on core protein in xyloside-treated cultures were shown to be of a smaller size as compared to those synthesized on core protein in the absence of xyloside (354,358). By analysis of

$|^{3}H|$ glucose to $|^{35}S|$ sulphate ratios and relative amounts of unsulphated to sulphated disaccharides, glycosaminoglycans initiated on core protein in the presence of β-D-xyloside have been shown to be undersulphated (357,358). In addition, it has been shown that the number of chondroitin sulphate chains initiated on core protein from xyloside-treated chick limb bud cultures decreased but the number of keratan sulphate chains initiated remained the same (359). This may be a reflection of their different linkage trisaccharide requirements. However, the length of both chondroitin sulphate and keratan sulphate chains was shown to decrease, and this has been explained by the fact that the synthesis of both chains utilizes common precursor pools, and the high stimulation of synthesis of free chondroitin sulphate chains on the artificial xyloside acceptor has the effect of lowering the concentration of available precursors (359).

Preliminary experiments with p-nitrophenyl-β-D-xyloside resulted in a marked increase in $|^{35}S|$ sulphate incorporation into macromolecular material in the medium of aortic medial smooth muscle cells. The effects of the β-D-xyloside on glycosaminoglycans synthesized both on core protein and on the artificial acceptors were therefore investigated with a view to the characterization of the glycosaminoglycan length, degree of sulphation and composition.

## 6.2. METHODS

### 6.2.1. Preparation of cell cultures

Bovine foetal aortic smooth muscle cells ($A_3$) were isolated and cultured as described in Section 3.2.1 and were grown in either the presence or absence of daily additions of ascorbic acid (50 µg/ml).

### 6.2.2. Incorporation of radioactive isotopes

Incorporation studies were usually carried out on cultures grown in 35 mm Petri dishes which were initiated at $10^5$ cells/dish. Cells were grown for 8 days and $|^{35}S|$ sulphate (10 µCi/ml) and/or $|^{3}H|$ glucosamine (5 µCi/ml) were added for the final 24 h (unless otherwise stated), in the presence or absence of the requisite dosage of either p-nitrophenyl-β-D-xylopyranoside or 4-methylumbelliferyl-β-D-xyloside dissolved in dimethylsulphoxide (DMSO). Control cultures were treated with a volume of DMSO equal to that used to administer the β-D-xyloside. At the end of the incubation period the medium (extracellular compartment) was removed and retained for analysis, and the pericellular-matrix and intracellular compartments were isolated after treating the cell layers with a solution of Viokase and collagenase as described in Section 3.2.7. Incorporation of radioactive precursors into macromolecular material was assessed after descending paper chromatography to remove unincorporated isotope, as described in Section 3.2.7.

6.2.3. Molecular exclusion chromatography

Columns of Sepharose CL-6B (0,6 x 110 cm) equilibrated at room temperature with 0,05 M sodium acetate pH 6,8, or Sephadex G100 (medium) equilibrated at room temperature with PBS, were used to compare the sizes of glycosaminoglycan chains synthesized by β-D-xyloside-treated or control cultures. Glycosaminoglycans attached to either the core protein or to the artificial acceptors, in cultures treated with β-D-xyloside, were prepared after chromatography of the sample on a preparative Sepharose CL-6B column (1,6 x 80 cm). Columns (1,2 x 28 cm) of Biogel P-2 (100-200 mesh), equilibrated and eluted with distilled-deionized water, were used to desalt samples. Columns were calibrated with respect to their void volumes ($V_0$) and total volumes ($V_t$), using either proteoglycans purified from bovine nasal septum or blue dextran and $|^{35}S|$ sulphate, respectively.

6.2.4. DEAE-cellulose chromatography

When radioactive culture media were to be analysed by DEAE-cellulose chromatography, they were first digested with papain (1 mg/ml) at 65°C for 8 to 10 h and then dialysed against 5 litres of cold distilled-deionized water at 5°C for 48 h with five changes of water, after which they were freeze-dried. Freeze-dried samples, dissolved in appropriate volumes of 0.05 M sodium acetate pH 4,0 were applied to columns (1,0 x 6,0 cm) of DEAE-cellulose equilibrated with the same buffer. Columns were eluted initially (five

fractions) with equilibration buffer and subsequently with a linear gradient of lithium chloride (0,2 M to 1,5 M) in 0,05 M sodium acetate pH 4,0, as described in Section 2.2.7.

6.2.5. Quantitation of glycosaminoglycan chains

Glycosaminoglycan analysis was carried out on radiolabelled, papain-digested, freeze-dried samples prepared from culture media as described above, but isolated from cultures initiated in 75 $cm^3$ tissue culture flasks at $10^6$ cells/flask. Lyophilized samples were dissolved in the appropriate volume of 0,01 M Tris acetate pH 7,3, and then subjected to digestion with chondroitinase ABC, chondroitinase AC, hyaluronidase or treatment with nitrous acid as described in Section 2.2.4. Samples were chromatographed on a column (1,0 x 120 cm) of Sephadex G-50 (medium) equilibrated with 0,2 M pyridine acetate pH 5,0, before and after digestion, as described in Section 2.2.8. The amount of excluded material before and after digestion was used to assess the content of a specific glycosaminoglycan type.

6.2.6. Thin layer chromatography

Freeze-dried samples were dissolved in the appropriate volume of 0,01 M Tris acetate pH 7,3 and then subjected to digestions with either chondroitinase ABC or chondroitinase AC. The digests were filtered through sterile Millipore filters (0,45 µM pore size). Carrier disaccharides were added to the digests and aliquots were streaked onto cellulose-coated thin layer plates and subjected to ascending chromatography in

glacial acetic acid : 1-butanol : 1 N ammonium hydroxide 6 : 4 : 3 (v/v/v) (332), at room temperature for approximately 6 h. Thin layer plates were air-dried and stained in 0.1%(w/v) toluidine blue in absolute ethanol. The bands corresponding to the various disaccharides were collected by scraping, and counted as described in Section 3.2.4.

### 6.2.7. Analytical procedures

Determinations of the concentration of cellular protein were carried out on washed cell layers after lysis of the cells by a solution of 1% (w/v) SDS, using a modification of the method of Lowry et al. (323) as described in Section 3.2.3.

## 6.3. RESULTS

### 6.3.1. Comparison of glycosaminoglycans from control and β-D-xyloside-treated aortic smooth muscle cell cultures

The $A_3$ line of bovine aortic smooth muscle cells showed a marked stimulation of $|^{35}S|$ sulphate incorporation into macromolecular material when cultured in the presence of various levels of p-nitrophenol-β-D-xyloside (Table 6.1) or 4-methylumbelliferyl-β-D-xyloside (Table 6.2). The effect was most marked for cultures exposed to the xyloside for 24 h at a final concentration of 0,5 mM. At the higher doses tested, the incorporation decreased slightly, although at all concentrations it was still significantly higher than that of control cultures. The highest doses of β-D-xyloside tested were toxic to the cells and resulted in cell death, and thus

TABLE 6.1

THE EFFECT OF p-NITROPHENYL-β-D-XYLOSIDE ON |$^{35}$S| SULPHATE INCORPORATION INTO MACROMOLECULAR MATERIAL IN THE DIFFERENT CULTURE COMPARTMENTS OF SMOOTH MUSCLE CELLS

Cells were cultured for 8 days in either the presence or absence of ascorbic acid. |$^{35}$S| Sulphate (10 μCi/ml) was present during the final 24 h of culture, together with the different doses of p-nitrophenyl-β-D-xyloside described in Section 6.2.2. The amounts of macromolecular |$^{35}$S| sulphate in the extracellular, pericellular-matrix and intracellular culture compartments were determined as described in Section 3.2.7. The data represent the mean ± S.D. for triplicate determinations for one of two similar experiments. The figure in brackets gives the percentage of the total radioactivity found in that compartment.

| Ascorbate | Dose (mM) | Cellular Protein (μg) | Radioactivity Incorporated (dpm/μg cellular protein) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total | Extracellular | | Pericellular-Matrix | | Intracellular | | |
| + | 0 | 740 | 347,3±5,0 | 211,5±6,5 | (61) | 117±6,8 | (33) | 19,3±1,3 | (6) | |
| | DMSO | 732 | 331,3±11,0 | 204,3±6,1 | (62) | 109±4,7 | (33) | 17,5±0,5 | (5) | |
| | 0,1M | 744 | 2054,5±30,4 | 1936,9±30,7 | (94) | 94±1,8 | (5) | 23,7±1,1 | (1) | |
| | 0,5 | 699 | 2357,0±27,0 | 2240,0±25,6 | (95) | 89±2,0 | (4) | 23,8±3,8 | (1) | |
| | 1,0 | 664 | 1967,2±239 | 1865,0±23,0 | (94) | 81,0±5,4 | (4) | 21,7±3,7 | (1) | |
| | 2,5 | 646 | 1535,0±50,0 | 1442,3±49,0 | (94) | 72,3±2,4 | (5) | 20,5±0,6 | (1) | |
| | 5,0 | 545 | 1361,6±19,6 | 1280,0±22,3 | (94) | 64,0±4,6 | (5) | 18,3±1,7 | (1) | |
| | 10,0 | 530 | 1176,1±245,3 | 1116,0±22,4 | (95) | 48,0±18,2 | (4) | 13,1±3,2 | (1) | |
| - | 0 | 519 | 303,9±12,0 | 252,4±10 | (83) | 27,5±2,7 | (9) | 24,1±1,9 | (8) | |
| | DMSO | 525 | 288,0±5,6 | 231,3±6 | (83) | 26,0±0,9 | (9) | 20,9±1,1 | (8) | |
| | 0,1 | 511 | 1711,3±135,6 | 1653,2±136,5 | (97) | 27,9±1,0 | (2) | 30,3±2,7 | (1) | |
| | 0,5 | 517 | 1838,5±7,8 | 1786,1±77,2 | (97) | 25,5±0,9 | (1) | 26,9±0,5 | (2) | |
| | 1,0 | 491 | 1649,1±47,0 | 1599±469,0 | (97) | 25,0±0,7 | (2) | 24,9±0,8 | (1) | |
| | 5,0 | 424 | 1201,4±23,0 | 1164,3±22,2 | (97) | 15,7±1,2 | (1) | 21,4±0,1 | (2) | |
| | 10,0 | 400 | 764,5±93,2 | 739±90,3 | (97) | 12,0±0,8 | (2) | 13,0±2,1 | (1) | |

TABLE 6.2

THE EFFECT OF 4-METHYLUMBELLIFERYL-β-D-XYLOSIDE ON $|^{35}S|$ INCORPORATION INTO MACROMOLECULAR MATERIAL IN THE DIFFERENT CULTURE COMPARTMENTS OF SMOOTH MUSCLE CELLS

Cells were cultured for 8 days in either the presence or absence of ascorbic acid. $|^{35}S|$ Sulphate (10 μCi/ml) was present during the final 24 h of culture, together with the different doses of 4-methylumbelliferyl β-D-xyloside as described in Section 6.2.2. The amounts of macromolecular $|^{35}S|$ sulphate in the extracellular, pericellular-matrix and intracellular culture compartments were determined as described in Section 3.2.7. The data represent the mean ± S.D. for triplicate determinations for one of two similar experiments. The figure in brackets gives the percentage of the total radioactivity found in that compartment.

| Ascorbate | Dose (mM) | Cellular Protein (μg) | Radioactivity Incorporated (dpm/μg cellular protein) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Total | Extracellular | | Pericellular-Matrix | | Intracellular | |
| + | 0 | 668 | 277±8 | 184,8±13,1 | (67) | 79,1±21,0 | (29) | 12,7±1,5 | (4) |
| | 0,1 | 644 | 1595±163 | 1507,2±143,1 | (95) | 70,3±0,8 | (4) | 17,1±0,8 | (1) |
| | 0,5 | 596 | 1851±90 | 1747,1±92,0 | (94) | 85,4±2,9 | (5) | 17,8±1,0 | (1) |
| | 1,0 | 568 | 1376±192 | 1290,2±112,5 | (94) | 68,2±2,8 | (5) | 17,0±1,4 | (1) |
| | 2,5 | 576 | 1430±104 | 1361,3±106,7 | (95) | 56,8±6,0 | (4) | 13,5±2,1 | (1) |
| - | 0 | 480 | 224±21 | 194,2±19,2 | (87) | 22,1±1,0 | (10) | 8,3±0,5 | (3) |
| | 0,1 | 492 | 1496±156 | 1461,5±153,1 | (98) | 24,2±2,3 | (16) | 10,5±0,6 | (0,4) |
| | 0,5 | 472 | 1543±31 | 1512,7±31,3 | (98) | 18,1±0,7 | (1) | 12,0±0 | (1) |
| | 1,0 | 464 | 1403±146 | 1372,1±118,2 | (98) | 20,0±1,4 | (1) | 11,0±0 | (1) |
| | 2,5 | 254 | 1283±72 | 1261,6±72,1 | (98) | 13,6±1,4 | (1) | 9,5±0,7 | (1) |

decreased amounts of cellular protein in these cultures. Therefore, in all future experiments a dose of 0,5 mM p-nitrophenyl-β-D-xyloside was used. Stock solutions of β-D-xyloside were made up in DMSO and dilutions were made in order that the amount of DMSO added to the cultures was never greater than 5 µl/ml of medium. This level of DMSO was shown to have no effects on the amount of cellular protein or on the amount of $|^{35}S|$ sulphate incorporated into macromolecular material (Table 6.1). At a dose of 0,5 mM β-D-xyloside there was a 6- to 7-fold increase in the amount of $|^{35}S|$ sulphate incorporated into macromolecular material/µg cellular protein for both cultures grown in the presence and absence of ascorbic acid. Thus the presence of the vitamin did not enhance the effect of the xyloside to any significant degree.

Apart from the effect of β-D-xyloside on total incorporation of $|^{35}S|$ sulphate, there were marked changes in the distribution of the xyloside-initiated material within the culture system. More than 94% of the macromolecular $|^{35}S|$ sulphate was associated with the culture medium in the presence of xyloside, whereas in its absence only some 60% of the total macromolecular material was found in the culture medium after 24 h when cultures were grown in the presence of ascorbic acid. The amount of radioactivity found in the pericellular-matrix and intracellular compartments decreased correspondingly in the presence of β-D-xyloside. The increase in the relative amount of macromolecular radioactive material associated with the culture medium has been noted by other workers (100,359-361),

and implies that the glycosaminoglycan chains initiated on β-D-xyloside acceptors were more soluble and were secreted more readily into the medium. The timing of macromolecular radioactive sulphate appearance in the culture medium for β-D-xyloside-treated or control cultures grown in either the presence or absence of ascorbic acid supported this implication (Fig. 6.1). Smooth muscle cells were preincubated either in the presence of 0,5 mM β-D-xyloside (treated) or in the presence of equivalent amounts of DMSO alone (control) for 3 h. At time zero $|^{35}S|$ sulphate was added to the culture medium and the amount of macromolecular material in the medium was assayed at various times after the introduction of the radioisotope. It may be clearly seen that for cultures incubated in the presence of β-D-xyloside, the appearance of macromolecular material in the medium was much more rapid than in control cultures. In treated cultures a significant amount of macromolecular material had already been secreted into the culture medium even after only 1 h of incubation.

It had previously been noted that the addition of ascorbic acid to bovine smooth muscle cell cultures caused an increase in $|^{35}S|$ sulphate incorporation into macromolecular material by these cultures (Chapter 5, 363). This prompted further investigation into the effects of β-D-xyloside on the incorporation of radioactive sulphate into macromolecular material in order to determine whether this simply reflected an increased synthesis of glycosaminoglycans or, alternatively, whether the xyloside actually stimulated the sulphation of the glycosaminoglycan chains. Cultures were consequently

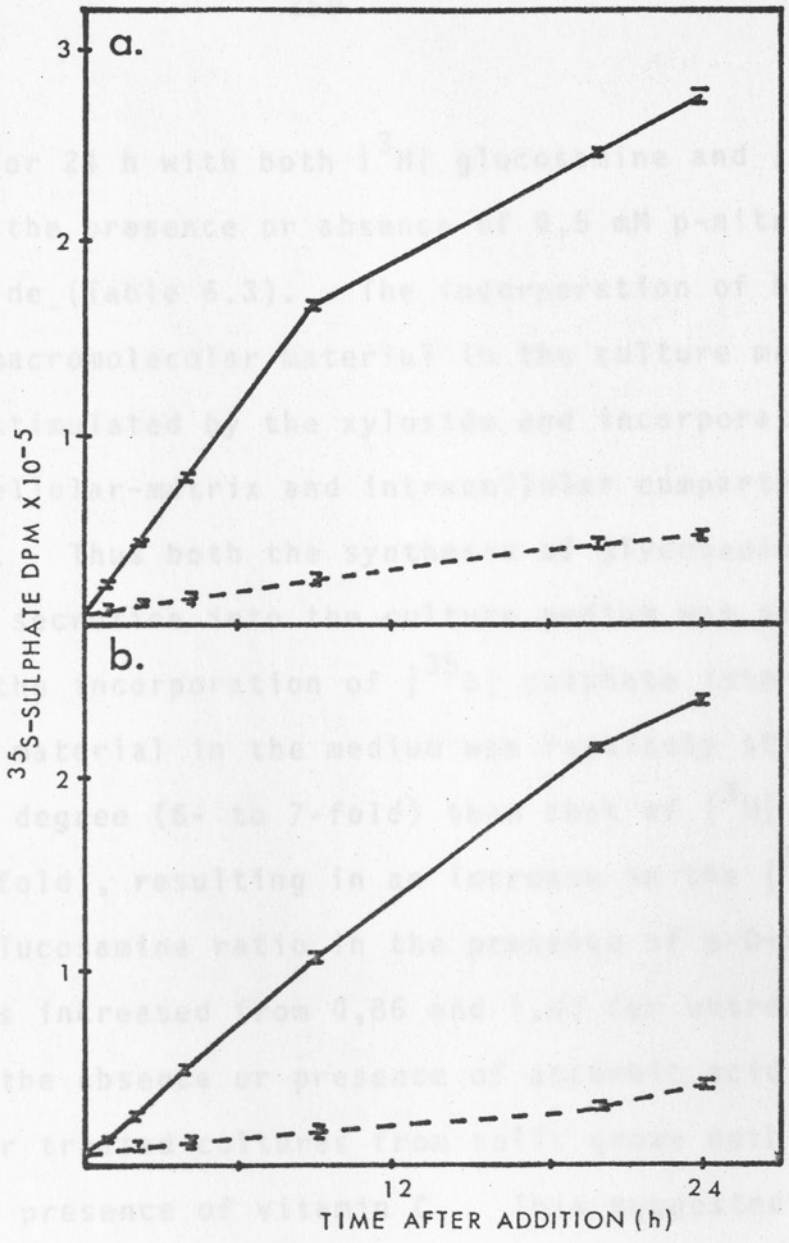



Fig. 6.1. Kinetics of secretion of $|^{35}S|$ sulphate labelled glycosaminoglycans into the culture medium of smooth muscle cells either in the presence of absence of p-nitrophenyl-β-D-xyloside.

Cells were cultured for 8 days either in the presence (a) or absence (b) of ascorbic acid. Dishes were pretreated with 10 µl DMSO (---) or 10 µl p-nitrophenyl-β-D-xyloside in DMSO to give a final concentration of 0,5 mM (—). After 3 h both control and treated cultures were incubated with 10 µCi/ml $|^{35}S|$ sulphate for the times shown in the figure. At each time point media were removed from triplicate dishes and processed to obtain the radioactivity present in macromolecular species (Section 3.2.7). The data represent the mean ± S.D. of triplicate determinations for one of two similar experiments.

labelled for 24 h with both |$^{3}$H| glucosamine and |$^{35}$S| sulphate in either the presence or absence of 0,5 mM p-nitrophenyl-β-D-xyloside (Table 6.3). The incorporation of both isotopes into the macromolecular material in the culture medium was markedly stimulated by the xyloside and incorporation into the pericellular-matrix and intracellular compartments was depressed. Thus both the synthesis of glycosaminoglycans and their secretion into the culture medium was stimulated. However, the incorporation of |$^{35}$S| sulphate into macromolecular material in the medium was routinely stimulated to a greater degree (6- to 7-fold) than that of |$^{3}$H| glucosamine (about 4-fold), resulting in an increase in the |$^{35}$S| sulphate to |$^{3}$H| glucosamine ratio in the presence of β-D-xyloside. The ratios increased from 0,86 and 1,03 for untreated cultures grown in the absence or presence of ascorbic acid, respectively, to 1,7 for treated cultures from cells grown both in the absence and presence of vitamin C. This suggested that β-D-xyloside might be causing an increased sulphation of glycosaminoglycan chains. Although less labelled macromolecular material was found in the pericellular-matrix and intracellular compartments, the |$^{35}$S| sulphate to |$^{3}$H| glucosamine ratios of material from these compartments of cultures grown in the presence of β-D-xyloside were also higher than those from corresponding compartments of cultures grown in the absence of xyloside.

In order to further check the effect of β-D-xyloside on sulphation, glycosaminoglycans from control and treated cultures were analysed by DEAE-cellulose ion-exchange chromatography.

TABLE 6.3

THE EFFECT OF β-D-XYLOSIDE ON THE INCORPORATION OF |$^{35}$S| SULPHATE AND |$^{3}$H| GLUCOSAMINE INTO THE DIFFERENT CULTURE COMPARTMENTS OF SMOOTH MUSCLE CELLS

Cells were cultured for 8 days in either the presence or absence of ascorbic acid. |$^{35}$S| Sulphate (10 μCi/ml) and |$^{3}$H| glucosamine (5 μCi/ml) were added for the final 24 h of culture either in the presence or absence of 0,5 mM p-nitrophenyl-β-D-xyloside in DMSO. The assessment of incorporation of |$^{35}$S| sulphate and |$^{3}$H| glucosamine into macromolecular material in the extracellular, pericellular-matrix and intracellular compartments was carried out as described in Section 3.2.7. The data represent the mean ± S.D. of quadruplicate determinations from one of three similar experiments.

| | Ascorbate | Xyloside | Radioactivity Incorporated (dpm/μg cellular protein) | | |$^{35}$S| Sulphate / |$^{3}$H| Glucosamine |
|---|---|---|---|---|---|
| | | | |$^{35}$S| Sulphate | |$^{3}$H| Glucosamine | |
| Total | + | + | 999072±26438 | 583171±2199 | 1,69±0,07 |
| | | - | 230144±15239 | 203449±15339 | 1,13±0,06 |
| Extracellular | + | + | 929555±25362 | 542621±22983 | 1,70±0,09 |
| | | - | 129771±12224 | 127066±8707 | 1,03±0,01 |
| Pericellular-matrix | + | + | 45988±2224 | 25973±3550 | 1,79±0,12 |
| | | - | 75913±5332 | 51540±4577 | 1,50±0,02 |
| Intracellular | + | + | 23350±1372 | 15460±2799 | 1,54±0,23 |
| | | - | 24460±1795 | 24843±1936 | 0,98±0,06 |
| Total | - | + | 674647±6005 | 399733±28341 | 1,70±0,25 |
| | | - | 117964±4337 | 140704±7160 | 0,84±0,04 |
| Extracellular | - | + | 654570±59026 | 387063±24808 | 1,70±0,04 |
| | | - | 94713±3925 | 110248±7101 | 0,86±0,05 |
| Pericellular-matrix | - | + | 9238±751 | 6123±329 | 1,51±0,16 |
| | | - | 13760±729 | 13603±699 | 1,02±0,1 |
| Intracellular | - | + | 10840±578 | 8048±729 | 1,36±0,16 |
| | | - | 9488±273 | 16853±821 | 0,56±0,03 |

Polyanions bind more tightly to these columns when their total negative-charge density is increased. However, it was necessary to know the relative length of the chains being applied to these columns, as longer chains would bind more tightly as a result of having more negatively-charged groups, but this would not necessarily mean that the charge/unit length was increased. Most workers who have studied the effects of β-D-xylosides on glycosaminoglycan synthesis in other systems have reported that the chains initiated on xyloside acceptors were shorter than their natural counterparts initiated on core protein (102,103,357,352). These findings have been confirmed with bovine aortic smooth muscle cells. Glycosaminoglycan chains from the culture medium of control cultures eluted with a $K_{av}$ of 0,28 when analysed on Sepharose CL-6B (Fig. 6.2a), whereas those from the medium of cultures treated with 0,5 mM β-D-xyloside eluted with a $K_{av}$ of 0,59 to 0,60 (Fig. 6.2b). The position of elution from Sepharose CL-6B columns was unaffected by the presence of ascorbic acid during culture. According to the data of Wasteson (327), these $K_{av}$ values correspond to molecular weights of approximately 50000 for glycosaminoglycans from control cultures and 12500 for those from xyloside-treated cultures. Similar values were obtained from the data supplied by Churms (364). In a separate experiment, control cultures were labelled with $|^{14}C|$ glucosamine and treated cultures with $|^{3}H|$ glucosamine, and the glycosaminoglycans prepared from the media of these two cultures were chromatographed on the same Sepharose CL-6B column (Fig. 6.3), confirming the difference in chain length. At doses of

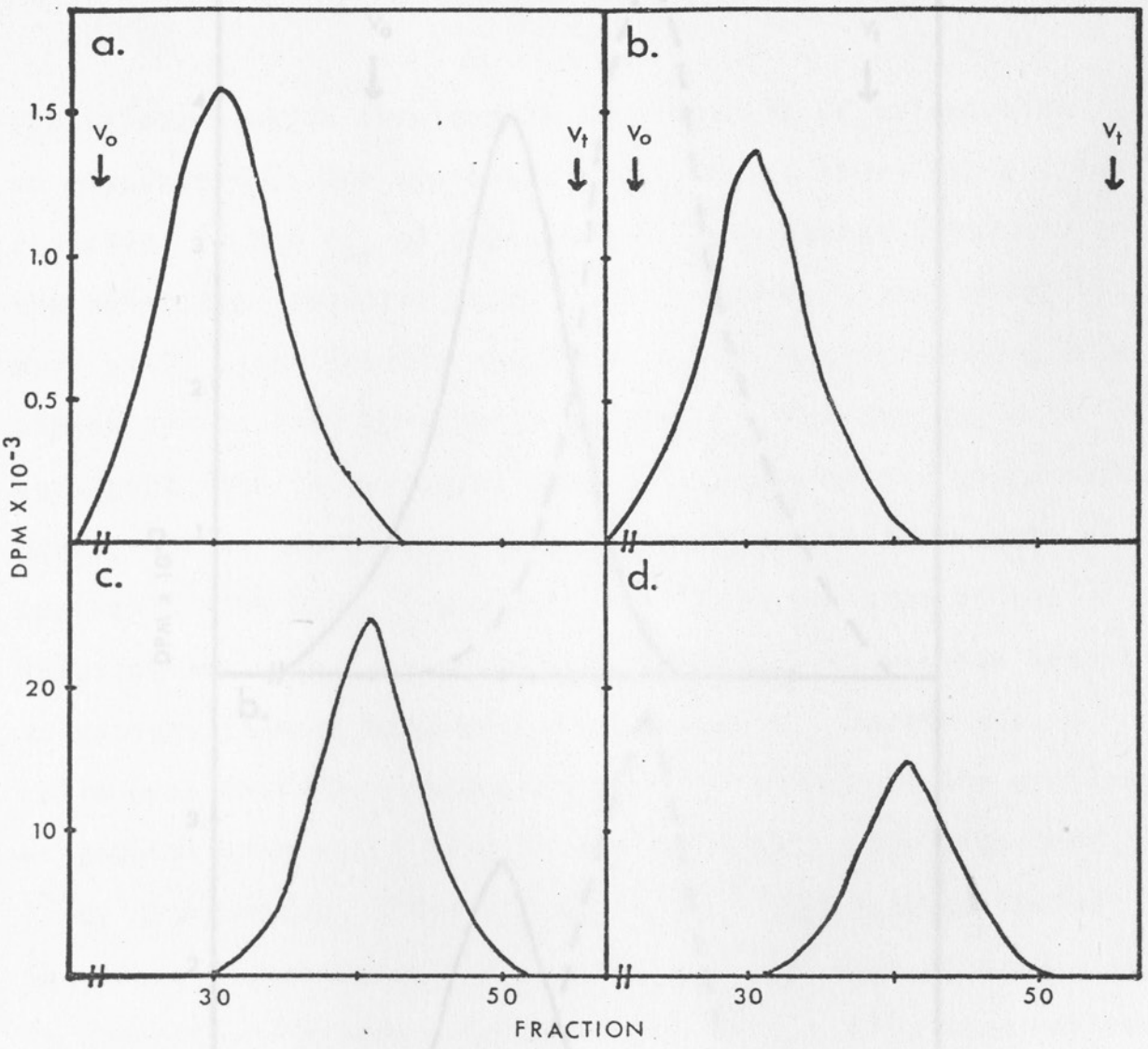



Fig. 6.2. Sepharose CL-6B chromatography of glycosaminoglycans from the medium of control or β-D-xyloside-treated smooth muscle cell cultures.

Cells were cultured for 8 days in the presence (a,c) or absence (b,d) of ascorbic acid. $|^{35}S|$ Sulphate (10 µCi/ml) was present during the final 24 h of culture, either in the presence of DMSO alone (a,b) or in the presence of 0,5 mM p-nitrophenyl-β-D-xyloside dissolved in DMSO (c,d). The glycosaminoglycans were isolated from culture media as described in Section 6.2.4 and chromatographed on a Sepharose CL-6B column.

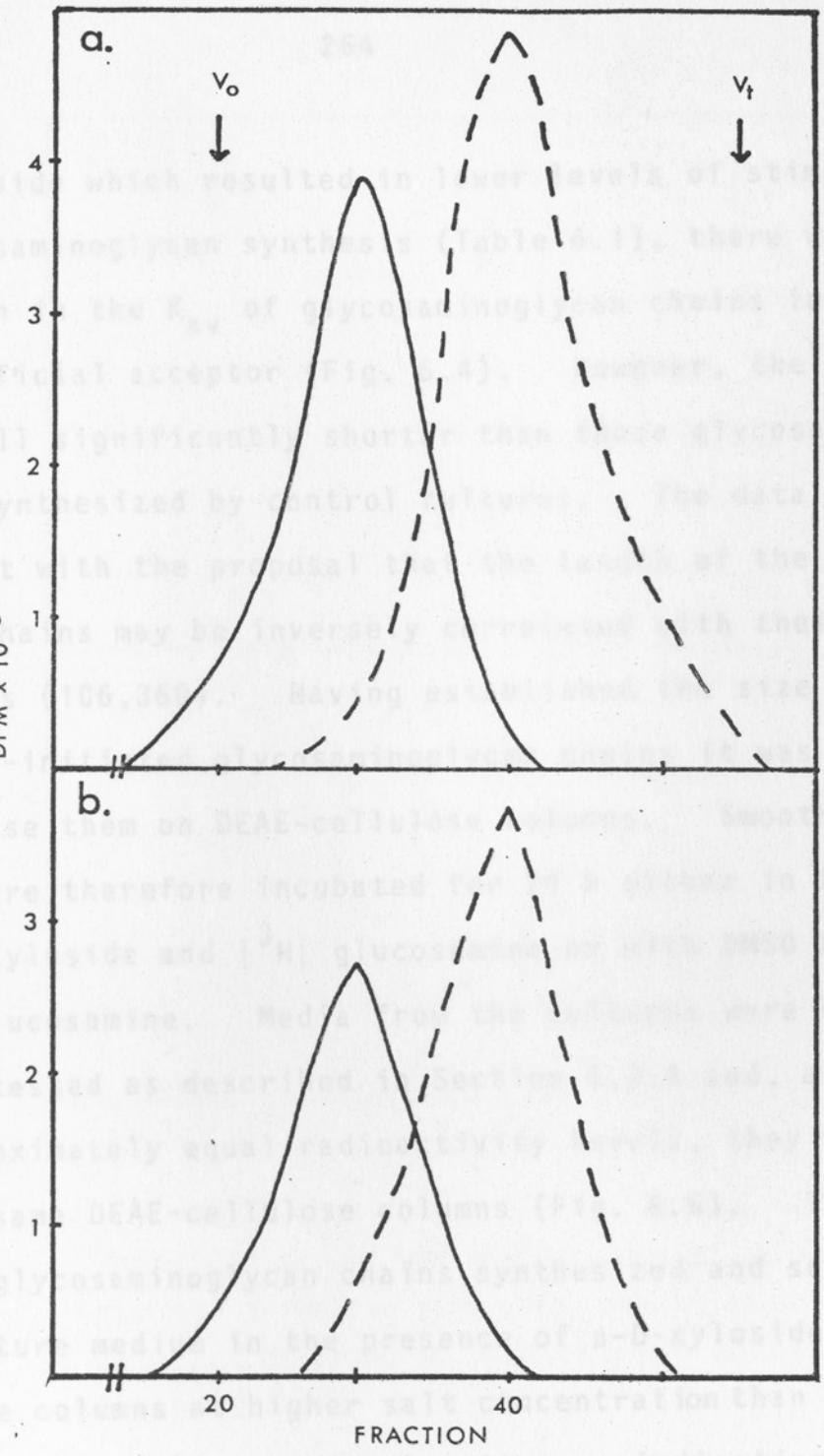



Fig. 6.3. Sepharose CL-6B chromatography of glycosaminoglycans from the medium of control or β-D-xyloside-treated smooth muscle cell cultures.

Cells were cultured for 8 days in the presence (a) or absence (b) of ascorbic acid. For the final 24 h in culture they were treated with 0,5 mM p-nitrophenyl-β-D-xyloside in DMSO in the presence of $|^{3}H|$ glucosamine (---) or with DMSO alone in the presence of $|^{14}C|$ glucosamine (—). Isotopes were used at a level of 5 μCi/ml culture medium. At the end of the 24 h labelling period the glycosaminoglycans were isolated from the media as described in Section 6.2.4 and samples from both control and xyloside-treated cultures were chromatographed on the same Sepharose CL-6B column.

β-D-xyloside which resulted in lower levels of stimulation of glycosaminoglycan synthesis (Table 6.1), there was a slight reduction in the $K_{av}$ of glycosaminoglycan chains initiated on the artificial acceptor (Fig. 6.4). However, the latter were still significantly shorter than those glycosaminoglycan chains synthesized by control cultures. The data were in agreement with the proposal that the length of the glycosaminoglycan chains may be inversely correlated with their rate of synthesis (106,360). Having established the size of the xyloside-initiated glycosaminoglycan chains it was now cogent to analyse them on DEAE-cellulose columns. Smooth muscle cells were therefore incubated for 24 h either in the presence of β-D-xyloside and |$^{3}$H| glucosamine or with DMSO alone and |$^{14}$C| glucosamine. Media from the cultures were collected and processed as described in Section 6.2.4 and, after mixing at approximately equal radioactivity levels, they were analysed on the same DEAE-cellulose columns (Fig. 6.5). The majority of the glycosaminoglycan chains synthesized and secreted into the culture medium in the presence of β-D-xyloside eluted from the columns at higher salt concentration than did the chains synthesized by control cultures, indicating that they had a higher total negative charge. Since these chains from treated cultures have been shown to be shorter than those from control cultures (Fig. 6.2, 6.3), they must, of necessity, contain more negative charges per unit length. With material from treated cultures, there was a pronounced peak of radioactivity (peak IV), eluting at about the ionic strength required to elute commercial chondroitin-4-sulphate, whereas the |$^{14}$C| glucosamine labelled chains from control cultures eluted

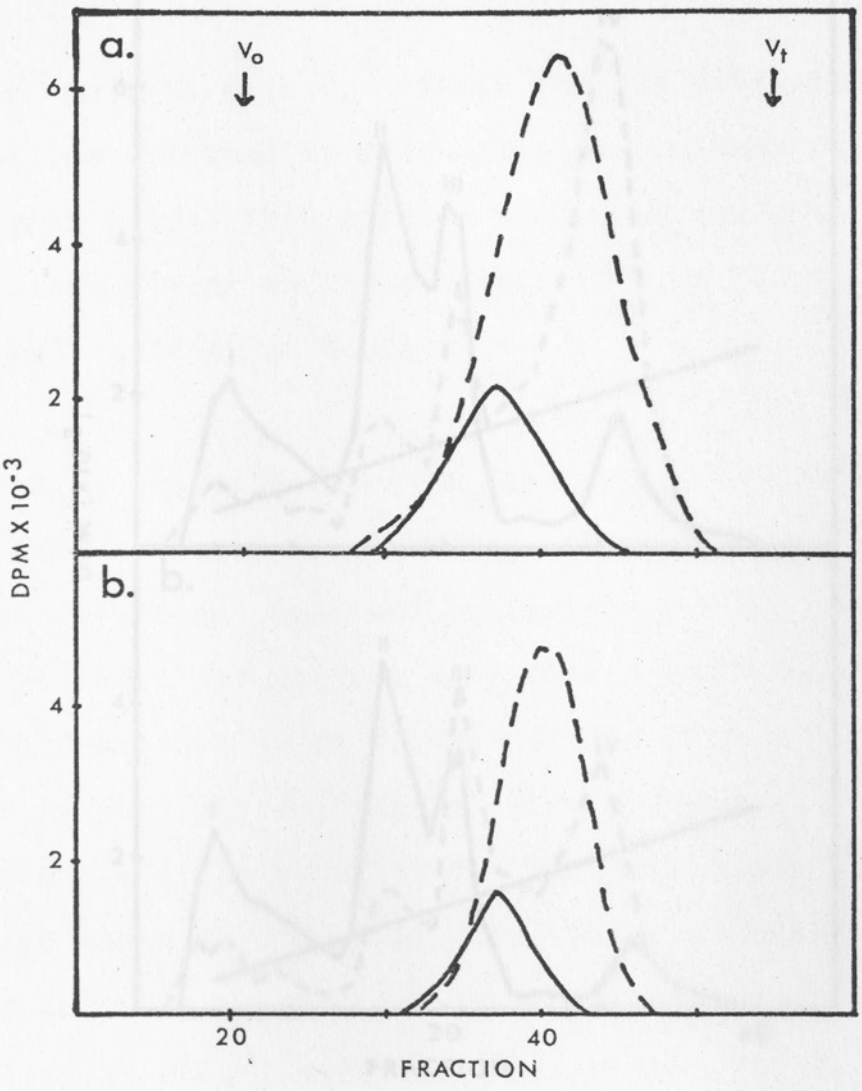



Fig. 6.4. Sepharose CL-6B chromatography of glycosaminoglycans from the media of smooth muscle cell cultures treated with different doses of β-D-xyloside.

Cells were cultured for 8 days in the presence (a) or absence (b) of ascorbic acid. For the final 24 h of culture they were treated with 0,5 mM p-nitrophenyl-β-D-xyloside in DMSO in the presence of $|^{3}H|$ glucosamine (---) or with 5,0 mM p-nitrophenyl-β-D-xyloside in DMSO in the presence of $|^{14}C|$ glucosamine (—). Isotopes were used at a level of 5 μCi/ml culture medium. At the end of the 24 h labelling period, the glycosaminoglycans were isolated from the media as described in Section 6.2.4 and samples from both 0,5 mM and 5,0 mM xyloside-treated cultures were chromatographed on the same Sepharose CL-6B column.

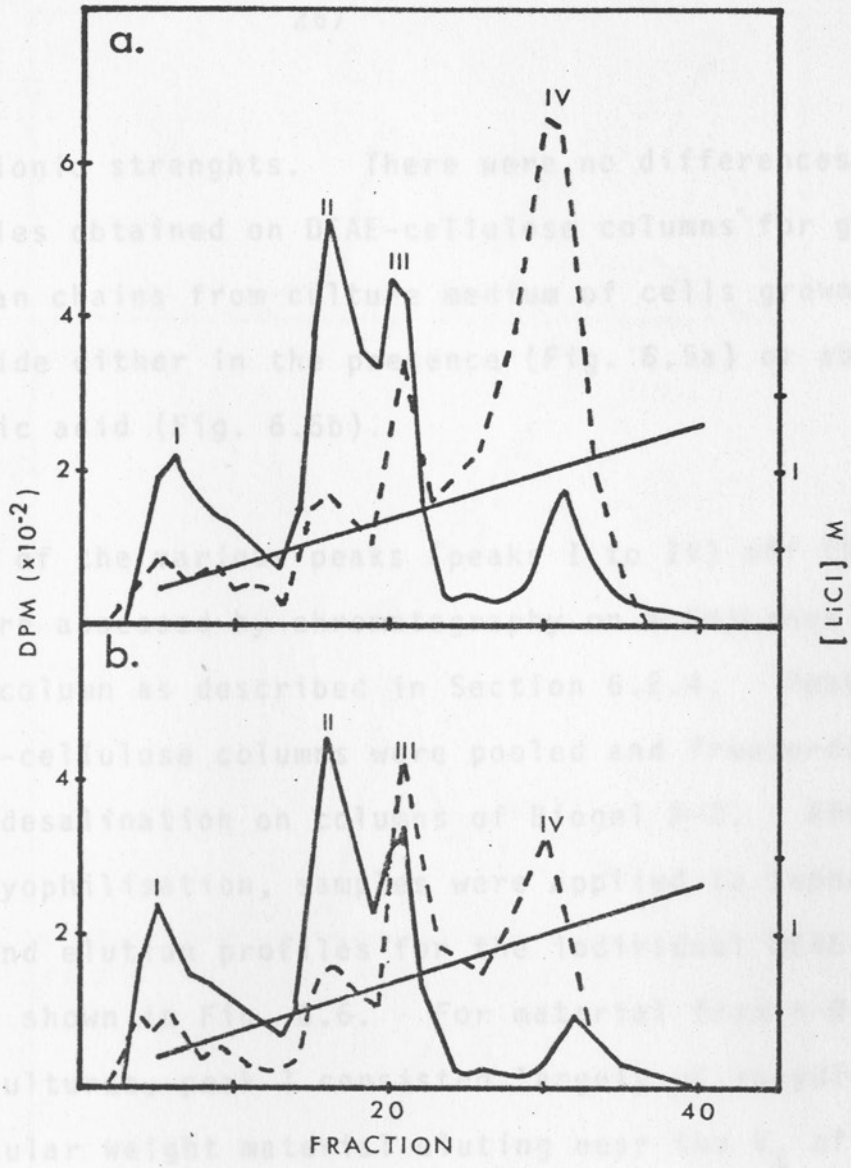



Fig. 6.5. DEAE-cellulose chromatography of glycosaminoglycans from the medium of control or β-D-xyloside-treated smooth muscle cell cultures.

Cells were cultured for 8 days in the presence (a) or absence (b) of ascorbic acid. For the final 24 h they were treated with 0,5 mM p-nitrophenyl-β-D-xyloside in DMSO in the presence of $|^{3}H|$ glucosamine (---) or with DMSO alone in the presence of $|^{14}C|$ glucosamine (—). Isotopes were used at a level of 5 μCi/ml culture medium. At the end of the labelling period the glycosaminoglycans were isolated from the media as described in Section 6.2.4 and samples from treated and control cultures were mixed at approximately equal levels of radioactivity and applied to the same DEAE-cellulose column. Profiles were reproducibly obtained many times.

at lower ionic strenghts. There were no differences between the profiles obtained on DEAE-cellulose columns for glycosaminoglycan chains from culture medium of cells grown with β-D-xyloside either in the presence (Fig. 6.5a) or absence of ascorbic acid (Fig. 6.5b).

The sizes of the various peaks (peaks I to IV) off the DEAE column were assessed by chromatography on a Sephadex G100 (medium) column as described in Section 6.2.4. Peaks I to IV from DEAE-cellulose columns were pooled and freeze-dried, prior to desalination on columns of Biogel P-2. After further lyophilisation, samples were applied to Sephadex G100 columns and elution profiles for the individual DEAE-cellulose peaks are shown in Fig. 6.6. For material from β-D-xyloside-treated cultures, peak I consisted largely of polydisperse low molecular weight material eluting near the $V_t$ of the column, whereas peak I material from control cultures contained mainly large molecular weight material. "DEAE" peaks II and III from both xyloside-treated and control cultures eluted in broad peaks, indicating that they were polydisperse in size, but they were of large molecular weight, with the material from control cultures on the whole eluting earlier than that from treated cultures. "DEAE" peak IV, the predominant peak obtained from treated cultures, eluted as a single broad peak with a $K_{av}$ of 0,29. The fact that the peaks were polydisperse in size, and yet eluted as single sharp peaks on DEAE-cellulose, indicated that it was their charged nature only that accounted for their elution profiles on ion-exchange chromatography. The presence of ascorbic acid (Fig. 6.6a),

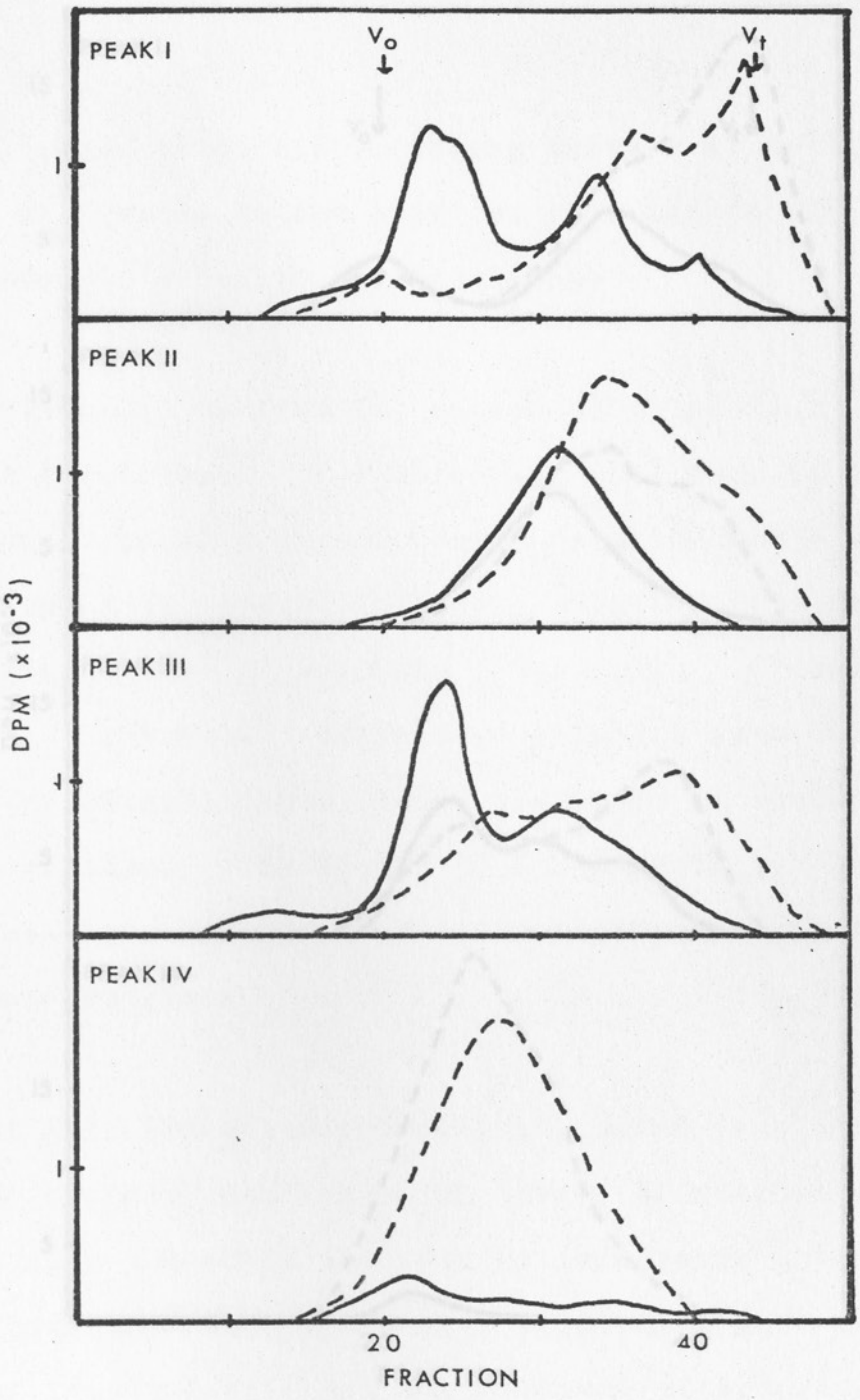



Fig. 6.6a. Sephadex G100 chromatography of peaks I to IV obtained after DEAE-cellulose chromatography of glycosaminoglycans from the medium of control and β-D-xyloside-treated smooth muscle cells cultured in the presence of ascorbic acid.

Cells were cultured for 8 days in the presence of ascorbic acid. For the final 24 h they were treated with 0,5 mM p-nitrophenyl-β-D-xyloside in DMSO together with |$^{3}$H| glucosamine (---) or DMSO alone, together with |$^{14}$C| glucosamine (—). Media from control and treated cultures were processed as described in Section 6.2.4, mixed and the glycosaminoglycans analysed by DEAE-cellulose chromatography (Fig. 6.5a). Material corresponding to peaks I to IV were pooled and rechromatographed on a Sephadex G-100 column as described in Section 5.2.3.

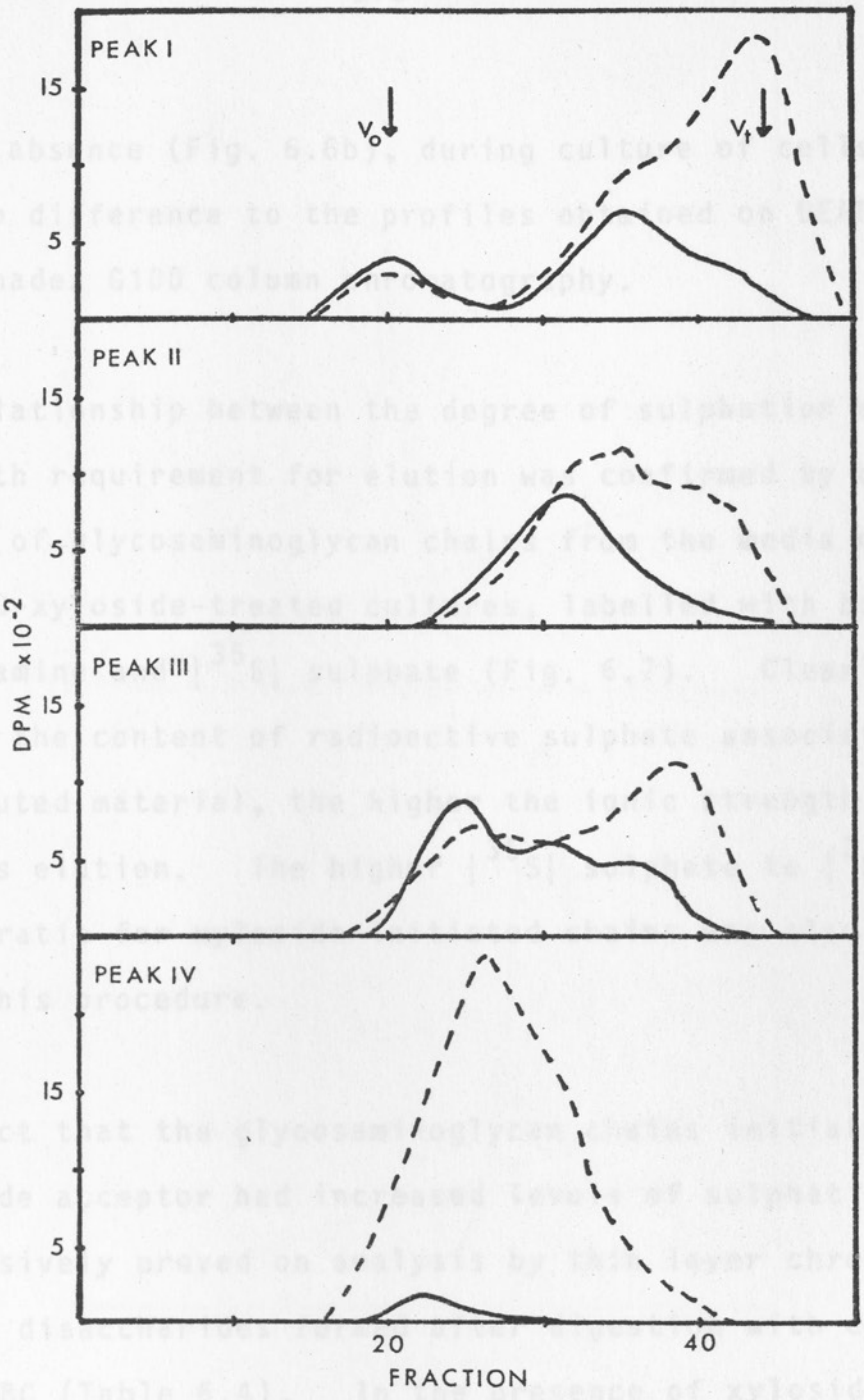



Fig. 6.6b. Sephadex G-100 chromatography of peaks I to IV obtained after DEAE-cellulose chromatography of glycosaminoglycans from the medium of control and β-D-xyloside-treated smooth muscle cells cultured in the absence of ascorbic acid.

Cells were cultured for 8 days in the absence of ascorbic acid. For the final 24 h they were treated with 0,5 mM p-nitrophenyl-β-D-xyloside in DMSO, together with |$^{3}$H| glucosamine (---), or DMSO alone together with |$^{14}$C| glucosamine (—). Media from control and treated cultures were processed as described in Section 6.2.4, mixed and the glycosaminoglycans analysed by DEAE-cellulose chromatography (Fig. 6.5b). Material corresponding to peaks I to IV were pooled and rechromatographed on a Sephadex G-100 column as described in Section 6.2.3.

or its absence (Fig. 6.6b), during culture of cells again made no difference to the profiles obtained on DEAE cellulose or Sephadex G100 column chromatography.

The relationship between the degree of sulphation and ionic strength requirement for elution was confirmed by chromatography of glycosaminoglycan chains from the media of control and β-D-xyloside-treated cultures, labelled with both $|^{3}H|$ glucosamine and $|^{35}S|$ sulphate (Fig. 6.7). Clearly, the higher the content of radioactive sulphate associated with the eluted material, the higher the ionic strength required for its elution. The higher $|^{35}S|$ sulphate to $|^{3}H|$ glucosamine ratio for xyloside-initiated chains was also observed with this procedure.

The fact that the glycosaminoglycan chains initiated on the xyloside acceptor had increased levels of sulphation was conclusively proved on analysis by thin layer chromatography of the disaccharides formed after digestion with chondroitinases ABC (Table 6.4). In the presence of xyloside there was a decrease in the proportion of $|^{3}H|$ glucosamine associated with material corresponding to unsulphated disaccharides, and a corresponding increase in sulphated species. The marked increases in the amount of material that migrated as ΔDi-6S was most probably a result of contamination by the ΔDi-diS (disulphated) disaccharide species that migrated only slightly behind the single sulphated species. This disulphated disaccharide is generally associated with dermatan sulphate chains and arises after chondroitinase ABC digestion.

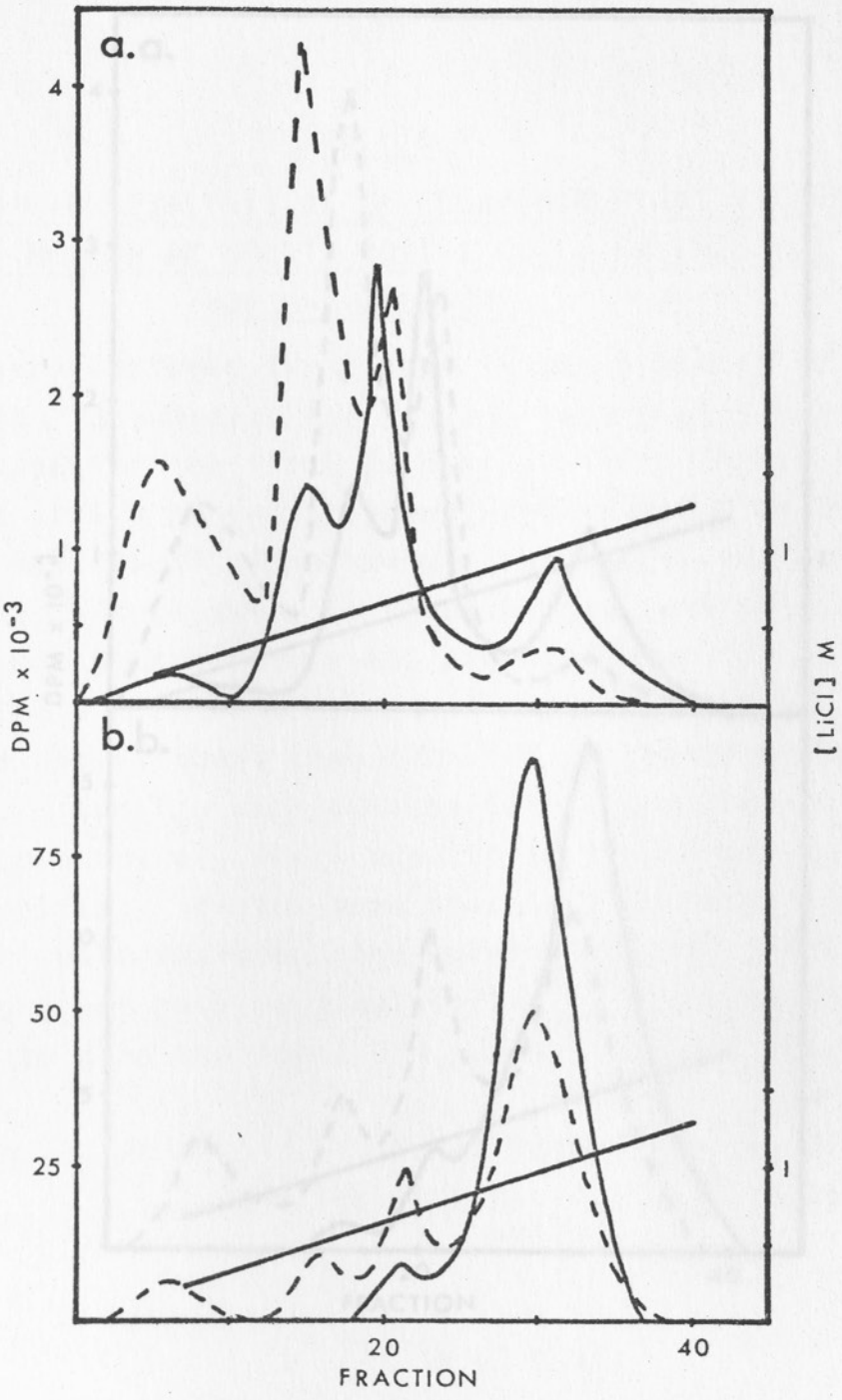



Fig. 6.7a. DEAE-cellulose chromatography of $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine labelled glycosaminoglycans from the medium of control and β-D-xyloside-treated smooth muscle cells cultured in the presence of ascorbic acid.

Cells were cultured for 8 days in the presence of ascorbic acid. For the final 24 h they were treated with DMSO alone (a) or with 0,5 mM p-nitrophenyl-β-D-xyloside in DMSO (b) together with 10 µCi/ml $|^{35}S|$ sulphate (—) and 5 µCi/ml $|^{3}H|$ glucosamine(--). At the end of the labelling period the glycosaminoglycans were isolated from the media as described in Section 6.2.4 and samples were applied to DEAE-cellulose columns.

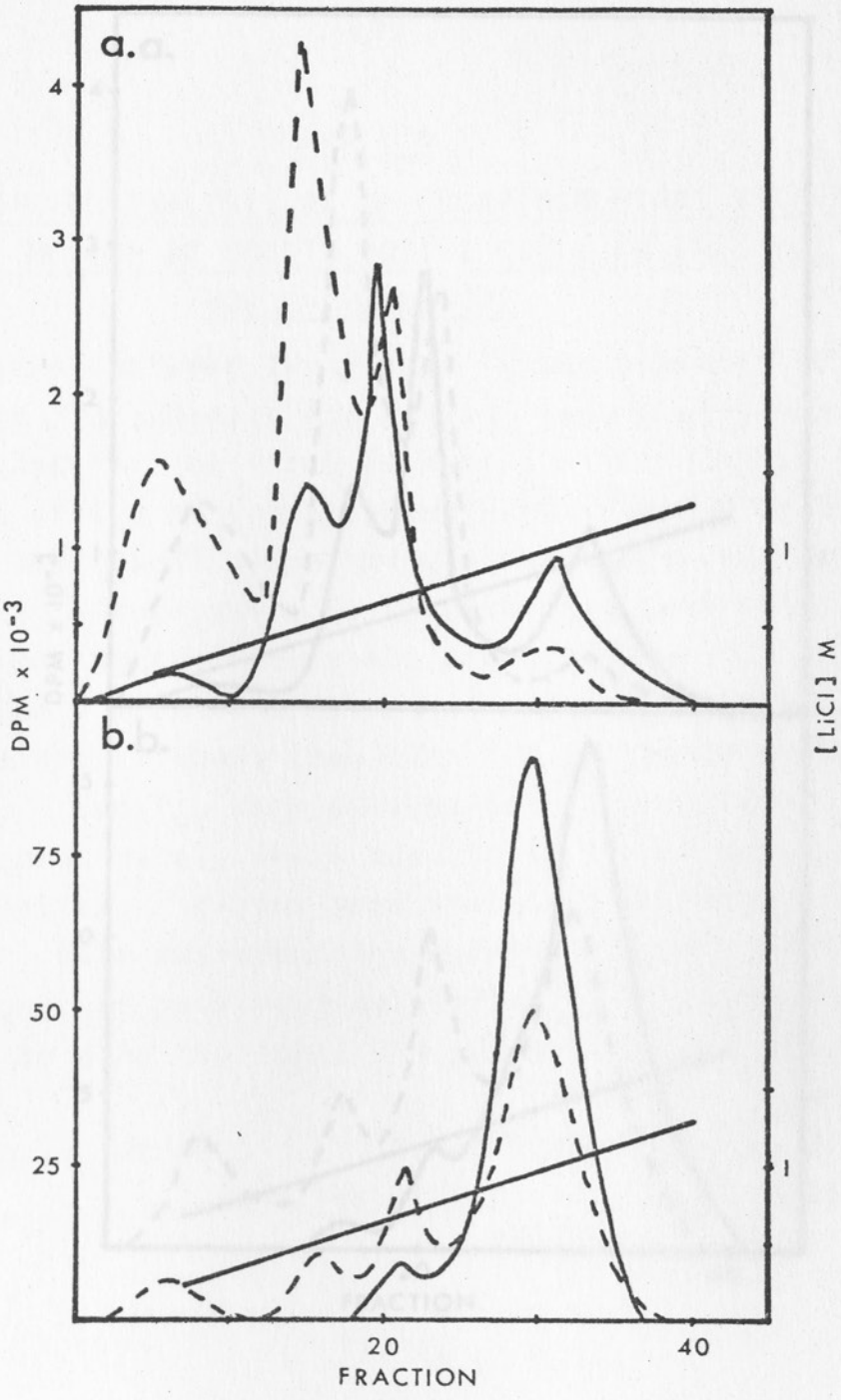



Fig. 6.7a. DEAE-cellulose chromatography of $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine labelled glycosaminoglycans from the medium of control and β-D-xyloside-treated smooth muscle cells cultured in the presence of ascorbic acid.

Cells were cultured for 8 days in the presence of ascorbic acid. For the final 24 h they were treated with DMSO alone (a) or with 0,5 mM p-nitrophenyl-β-D-xyloside in DMSO (b) together with 10 µCi/ml $|^{35}S|$ sulphate (—) and 5 µCi/ml $|^{3}H|$ glucosamine(--). At the end of the labelling period the glycosaminoglycans were isolated from the media as described in Section 6.2.4 and samples were applied to DEAE-cellulose columns.

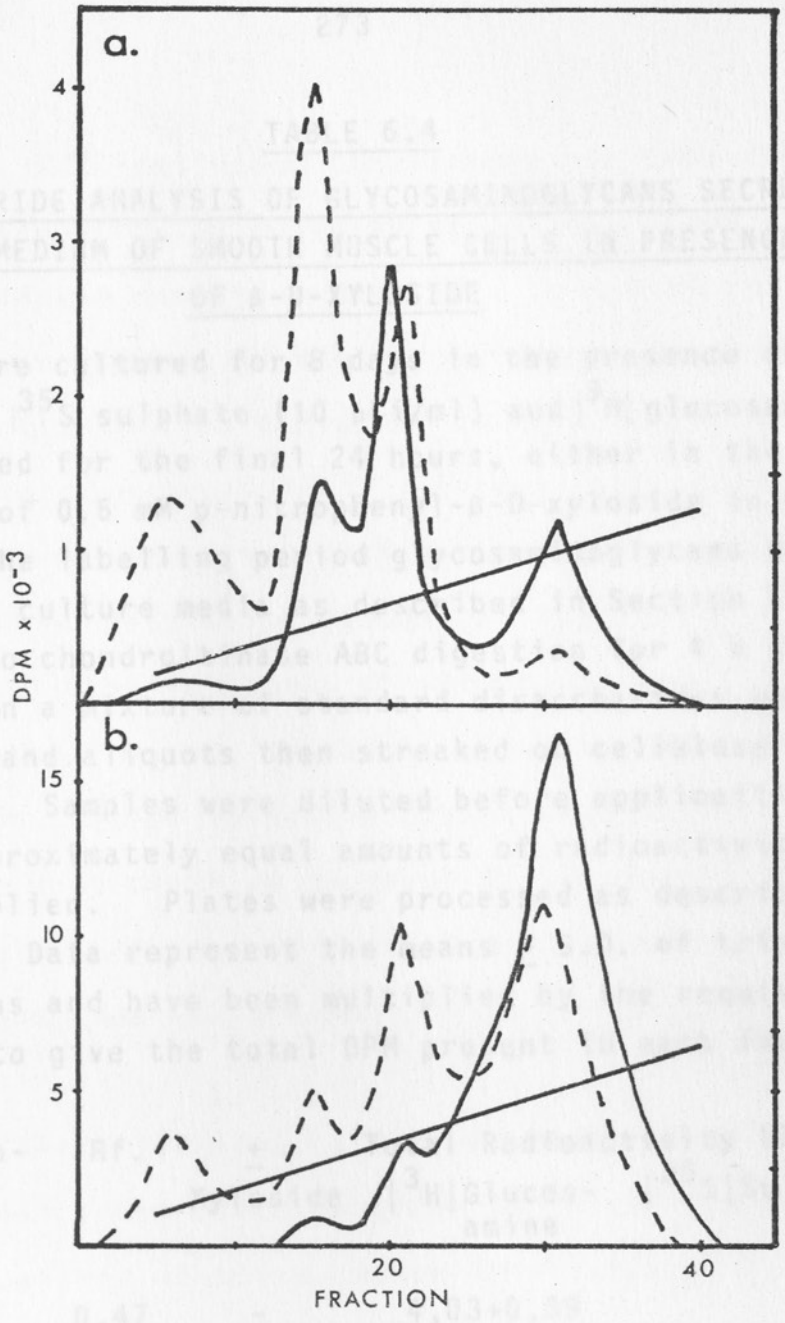



Fig. 6.7b. DEAE-cellulose chromatography of $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine labelled glycosaminoglycans from the medium of control and β-D-xyloside-treated smooth muscle cells cultured in the absence of ascorbic acid.

Cells were cultured for 8 days in the absence of ascorbic acid. For the final 24 h they were treated with DMSO alone (a) or with 0,5 mM p-nitrophenyl-β-D-xyloside in DMSO (b) together with 10 µCi/ml $|^{35}S|$ sulphate (——) and 5 µCi/ml $|^{3}H|$ glucosamine (---). At the end of the labelling period the glycosaminoglycans were isolated from the media as described in Section 6.2.4 and samples were applied to DEAE-cellulose columns.

TABLE 6.4

DISACCHARIDE ANALYSIS OF GLYCOSAMINOGLYCANS SECRETED INTO CULTURE MEDIUM OF SMOOTH MUSCLE CELLS IN PRESENCE AND ABSENCE OF β-D-XYLOSIDE

Cells were cultured for 8 days in the presence of ascorbic acid and both $|^{35}S|$ sulphate (10 μCi/ml) and $|^{3}H|$ glucosamine (5 μCi/ml) were added for the final 24 hours, either in the presence or absence of 0.5 mM p-nitrophenyl-β-D-xyloside in DMSO. At the end of the labelling period glycosaminoglycans were isolated from the culture media as described in Section 6.2.4 and subjected to chondroitinase ABC digestion for 4 h at 37°. After digestion a mixture of standard disaccharides was added to samples and aliquots then streaked on cellulose thin layer plates. Samples were diluted before application to plates to give approximately equal amounts of radioactivity in each aliquot applied. Plates were processed as described in Section 6.2.6. Data represent the means ± S.D. of triplicate determinations and have been multiplied by the requisite dilution factor to give the total DPM present in each fraction.

| Disaccharide Species | Rf. | ± Xyloside | Total Radioactivity DPM x $10^3$ $\|^{3}H\|$ Glucosamine | $\|^{35}S\|$ Sulphate | % * $^{3}H$-Glu. |
|---|---|---|---|---|---|
| ΔDi-0S | 0,47 | - | 4,03±0,39 | | 19,4 |
| | | + | 2,93±0,30 | | 2,6 |
| ΔDi-4S | 0.29 | - | 6,42±0,67 | 2,12±0,31 | 30,9 |
| | | + | 41,53±1,02 | 117,18±4,91 | 36,4 |
| ΔDi-6S | 0,18 | - | 10,32±1,04 | 4,34±0,46 | 49,7 |
| | | + | 69,07±3,45 | 220,00±8,40 | 60,8 |

* Represents the $|^{3}H|$ glucosamine present in each band as a percentage of the total.

Since the DEAE-cellulose chromatographic data suggested that β-D-xyloside-treated cultures secreted a large amount of chondroitinase-sensitive material, it was of interest to analyse the amounts of the various types of glycosaminoglycans found in the culture medium of cultures grown in the presence or absence of β-D-xyloside. It has previously been shown that the culture medium from bovine aortic smooth muscle cells contained no detectable amounts of hyaluronic acid (Table 4.2). Thus the increased levels of sulphation cannot be explained by a switch from the synthesis of a non-sulphated glycosaminoglycan to that of sulphated glycosaminoglycans. Rather β-D-xyloside must cause an increase in the levels of sulphation of the glycosaminoglycans which may normally be under-sulphated in culture (see Table 6.4).

$|^{35}S|$Sulphate-labelled culture media from control or β-D-xyloside-treated cultures grown in 75 $cm^3$ tissue culture flasks, either in the presence or absence of ascorbic acid, were collected and processed as described in Section 6.2.5. Samples were analysed by chromatography on Sephadex G-50 before and after treatment with the specific glycosaminoglycan-degrading enzymes, and the results are shown in Table 6.5. The culture media of cells grown in the presence of β-D-xyloside contained increased amounts of all glycosaminoglycan fractions when compared to control cells. Furthermore, in treated cultures the relative amount of material digested by chondroitinase ABC increased from 83,2% and 92,7% of the total to 97,2% and 96,8% of the total for cells grown either in the presence or absence of ascorbic acid,

TABLE 6.5

COMPOSITIONAL ANALYSIS OF SULPHATED GLYCOSAMINOGLYCANS SECRETED INTO THE CULTURE MEDIUM OF SMOOTH MUSCLE CELLS GROWN IN THE ABSENCE AND PRESENCE OF β-D-XYLOSIDE

Cells were cultured for 8 days in the presence or absence of ascorbic acid. |$^{35}$S| Sulphate (10 μCi/ml) was added for the final 48 h of culture, either in the presence or absence of 0,5 mM p-nitrophenyl-β-D-xyloside. At the end of the labelling period, glycosaminoglycans were isolated from the media as described in Section 6.2.4. Compositional analysis was performed as described in Section 6.2.5 using specific enzymatic digestions or nitrous acid treatment, followed by chromatography on Sephadex G-50. The data represent one of three similar experiments.

| Glycosaminoglycan Type | Ascorbate | Glycosaminoglycan Content | | | |
|---|---|---|---|---|---|
| | | Total dpm | | % of Total | |
| | | + Xyloside | - Xyloside | + Xyloside | - Xyloside |
| Chondroitin-4-or-6-sulphate + Dermatan sulphate | + | 2258143 | 269923 | 97,2 | 83,2 |
| Chondroitin-4- and -6-sulphate | + | 1473345 | 198225 | 63,4 | 61,1 |
| Dermatan sulphate | + | 785475 | 71698 | 33,8 | 22,1 |
| Heparan sulphate/heparin | + | 65070 | 54505 | 2,8 | 16,8 |
| Chondroitin-4-or-6-sulphate + Dermatan sulphate | - | 1584060 | 219498 | 96,8 | 92,7 |
| Chondroitin-4- and -6-sulphate | - | 1091495 | 165748 | 66,7 | 70,0 |
| Dermatan sulphate | - | 492565 | 53750 | 30,1 | 22,7 |
| Heparan sulphate/heparin | - | 52365 | 17285 | 3,2 | 7,3 |

respectively (Table 6.5). The increased incorporation of $|^{35}S|$ sulphate in the presence of β-D-xyloside was most marked for chondroitinase ABC-sensitive material and least for nitrous acid-sensitive material. The absolute amounts of nitrous acid-sensitive material in treated cultures increased 1,3-fold and 3-fold, compared to control cultures, for cells grown in either the presence or absence of ascorbic acid, respectively (Table 6.5). This increase was only about one-third to one-half of that of the chondroitinase ABC-sensitive material. Furthermore, the relative amounts of chondroitin-4- and -6-sulphate remained almost the same for treated and control cultures, but that of dermatan sulphate increased from being 22% of the total to 34% and 30% for cells grown in either the presence or absence of ascorbic acid, respectively. Thus treatment of aortic smooth muscle cells with β-D-xylosides caused an increase in the incorporation of $|^{35}S|$ sulphate into all glycosaminoglycans found in these cells, but the increase was most marked for the dermatan sulphate fraction and least for the heparan sulphate/heparin fraction.

Equivalent samples from control ($|^{14}C|$ glucosamine-labelled) or β-D-xyloside-treated ($|^{3}H|$ glucosamine-labelled) cultures grown in the presence of ascorbic acid were digested with the various enzymes and chromatographed on DEAE-cellulose before (Fig. 6.8a) and after digestion with chondroitinase ABC (Fig. 6.8b), chondroitinase AC (Fig. 6.8c) or treatment with nitrous acid (Fig. 6.8d). Chondroitinase ABC digested almost all of the material from the xyloside-treated cultures,

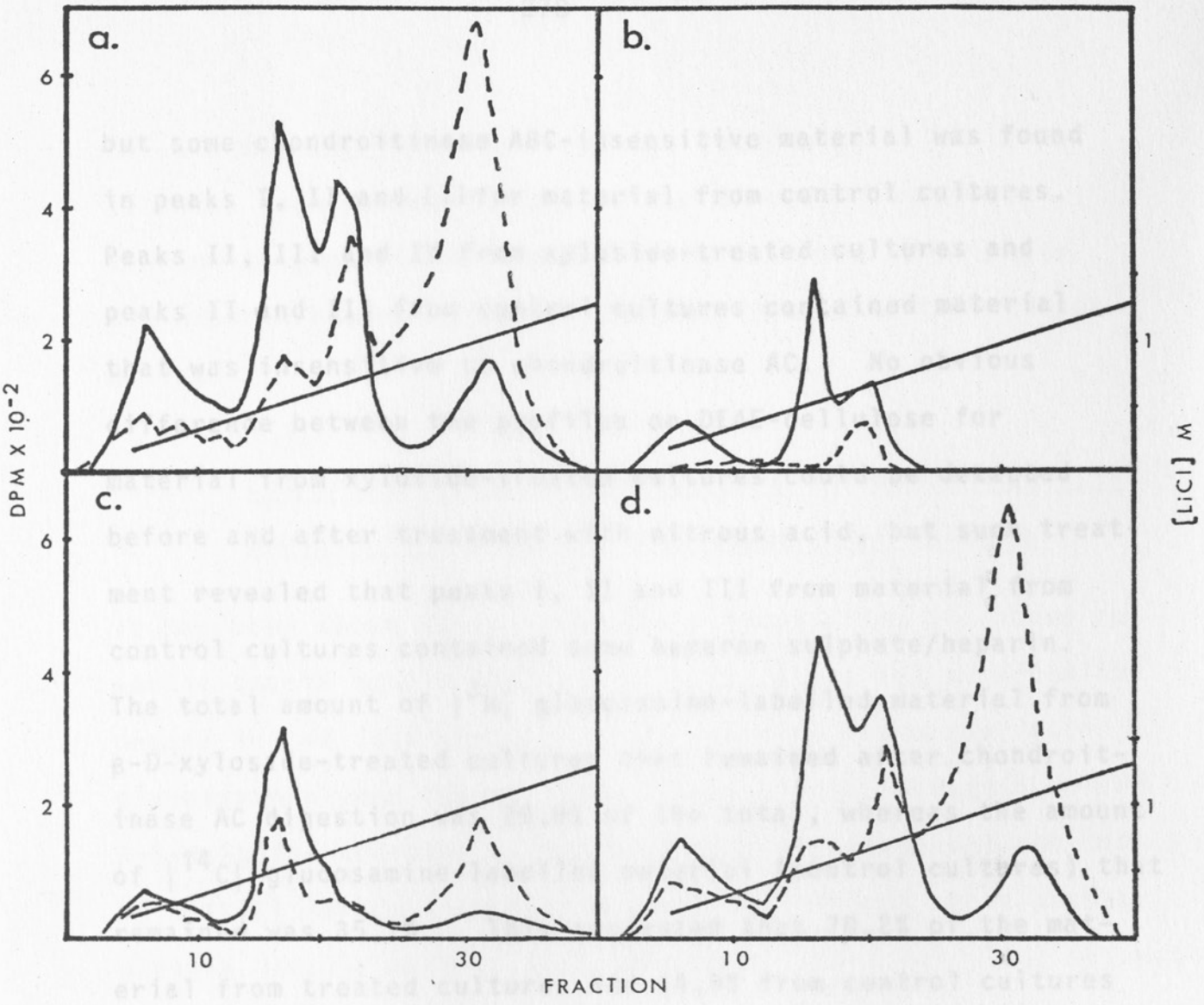



Fig. 6.8. DEAE-cellulose chromatography of glycosaminoglycans from the media of control and β-D-xyloside-treated smooth muscle cells cultured in the presence of ascorbic acid, before and after specific digestions.

Cells were cultured for 8 days in the presence of ascorbic acid. For the final 24 h they were treated with 0,5 mM p-nitrophenyl-β-D-xyloside in DMSO together with |$^{3}$H| glucosamine (---), or with DMSO alone in the presence of |$^{14}$C| glucosamine (—). Isotopes were used at a level of 5 μCi/ml culture medium. At the end of the labelling period the glycosaminoglycans were isolated from the media as described in Section 6.2.4 and samples from treated and control cultures were applied to the same DEAE cellulose column before (a) and after digestion with chondroitinase ABC (b), chondroitinase AC (c) or treatment with nitrous acid (d).

but some chondroitinase ABC-insensitive material was found in peaks I, II and III for material from control cultures. Peaks II, III and IV from xyloside-treated cultures and peaks II and III from control cultures contained material that was insensitive to chondroitinase AC. No obvious difference between the profiles on DEAE-cellulose for material from xyloside-treated cultures could be detected before and after treatment with nitrous acid, but such treatment revealed that peaks I, II and III from material from control cultures contained some heparan sulphate/heparin. The total amount of $|^{3}H|$ glucosamine-labelled material from β-D-xyloside-treated cultures that remained after chondroitinase AC digestion was 29,8% of the total, whereas the amount of $|^{14}C|$ glucosamine-labelled material (control cultures) that remained was 35,1%. This suggested that 70,2% of the material from treated cultures and 64,9% from control cultures was chondroitin-4- and -6-sulphate. This supports the data obtained by other means and shown in Table 6.5. The amount of $|^{14}C|$ glucosamine-labelled material (control cultures) that remained after chondroitinase ABC digestion was 19,4%, and that remaining after nitrous acid treatment was 81,7% of the total sample before digestion; thus in control cultures glycosaminoglycan chains were made up of 80,6% chondroitin and/or dermatan sulphate and 18,3% heparan sulphate/heparin. The data again agree well with those shown in Table 6.5.

Thus in the aortic smooth muscle cells, β-D-xyloside treatment caused an increase in the synthesis and sulphation of glycosaminoglycans which were rapidly secreted into the

culture medium. In addition, the incorporation of $|^{35}S|$ sulphate into dermatan sulphate was markedly increased, whereas incorporation into chondroitin-4- and -6-sulphate and heparin/heparan sulphate was only increased to a lesser extent.

### 6.3.2. Comparison of glycosaminoglycans synthesized on core protein in the absence and presence of xyloside

It has been shown above that, when β-D-xylosides are added to aortic smooth muscle cells in culture, there was a marked increase in the incorporation of radioactive precursors into glycosaminoglycan chains, most of which were initiated on the β-D-xyloside acceptor. However, some glycosaminoglycan chains were still formed on the core protein, although these proteoglycans usually made up less than 5% of the total $|^{35}S|$ sulphate labelled macromolecular material. The amount of radioactive label associated with protein-linked glycosaminoglycans in the presence of β-D-xyloside was only about one-third of that found for control cultures. Thus the addition of β-D-xylosides caused a decrease in the amount of proteoglycan synthesized. This observation was pursued in order to ascertain whether the glycosaminoglycans formed on the core protein in the presence of β-D-xyloside were the same as those formed on the core protein in control cultures. Samples of protein-linked glycosaminoglycans from control or treated cultures and xyloside-linked glycosaminoglycans from treated cultures were isolated from the media of control or treated cultures by chromatography on a preparative CL-6B column (Fig. 6.9). The protein-

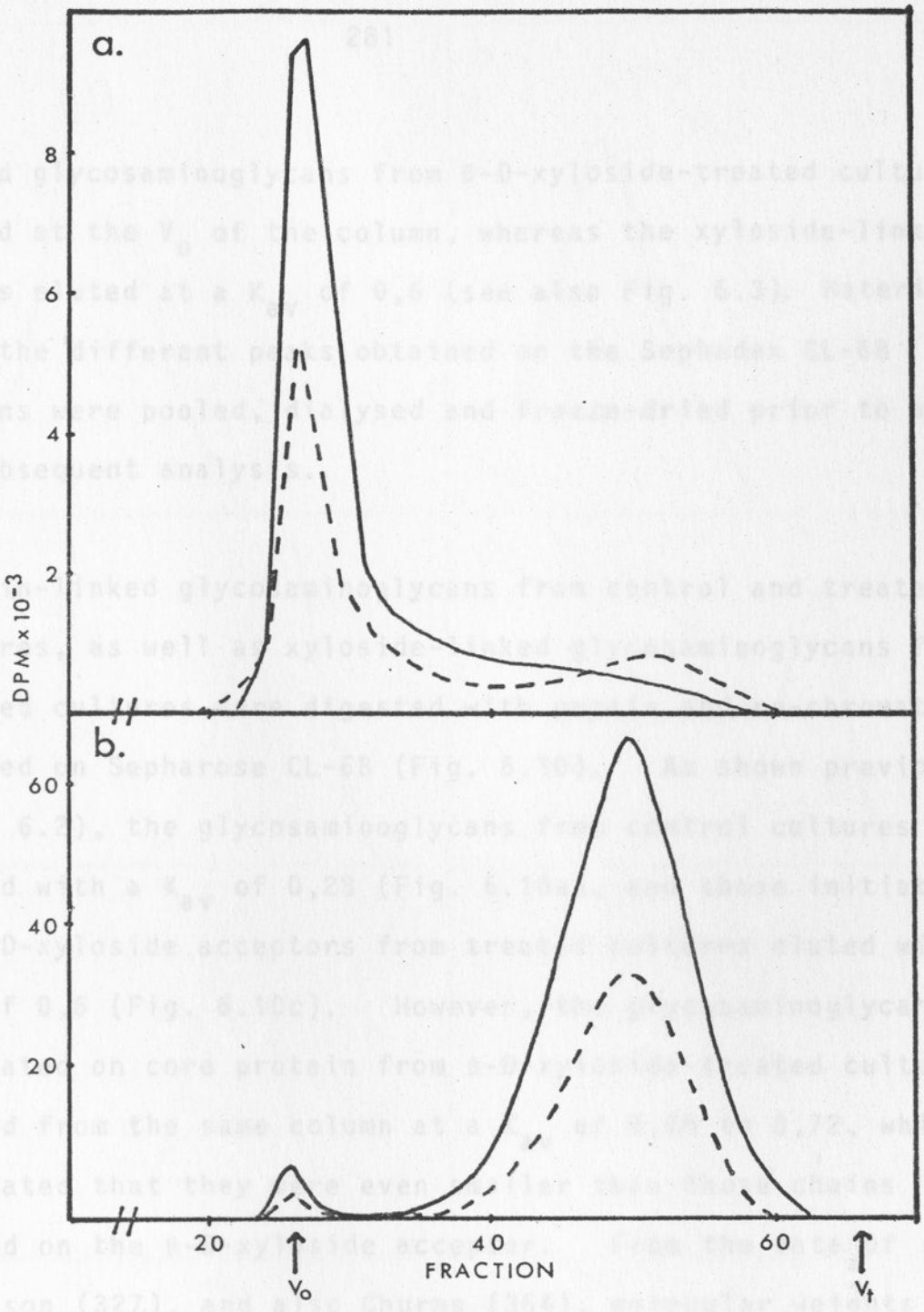



Fig. 6.9. Preparative Sepharose CL-6B chromatography of media from control or β-D-xyloside-treated smooth muscle cells cultured in the presence of ascorbic acid.

Cells were cultured for 8 days in the presence of ascorbic acid. $|^{35}S|$ Sulphate (10 μCi/ml) (-) and $|^{3}H|$ glucosamine (5 μCi/ml) (---) were added during the final 48 h in culture either in the absence (a) or presence (b) of 0,5 mM p-nitrophenyl-β-D-xyloside. Media were collected, dialysed and freeze dried and then applied to a preparative Sepharose CL-6B column as described in Section 6.2.3.

linked glycosaminoglycans from β-D-xyloside-treated cultures eluted at the $V_o$ of the column, whereas the xyloside-linked chains eluted at a $K_{av}$ of 0,6 (see also Fig. 6.3). Material from the different peaks obtained on the Sephadex CL-6B columns were pooled, dialysed and freeze-dried prior to use in subsequent analysis.

Protein-linked glycosaminoglycans from control and treated cultures, as well as xyloside-linked glycosaminoglycans from treated cultures were digested with papain and re-chromatographed on Sepharose CL-6B (Fig. 6.10). As shown previously, (Fig. 6.2), the glycosaminoglycans from control cultures eluted with a $K_{av}$ of 0,28 (Fig. 6.10a), and those initiated on β-D-xyloside acceptors from treated cultures eluted with a $K_{av}$ of 0,6 (Fig. 6.10c). However, the glycosaminoglycans initiated on core protein from β-D-xyloside-treated cultures eluted from the same column at a $K_{av}$ of 0,68 to 0,72, which indicated that they were even smaller than those chains formed on the β-D-xyloside acceptor. From the data of Wasteson (327), and also Churms (364), molecular weights for the protein-linked chains from treated cultures were estimated to be of the order of 6000 as compared to 12500 for those formed on the xyloside acceptor, and 50000 for those formed on core protein in control cultures. Thus in the presence of xyloside the glycosaminoglycans formed on the core protein were much shorter than normal, and this may be the reason why it was observed that incorporation of $|^{35}S|$ sulphate into these proteoglycans was reduced.

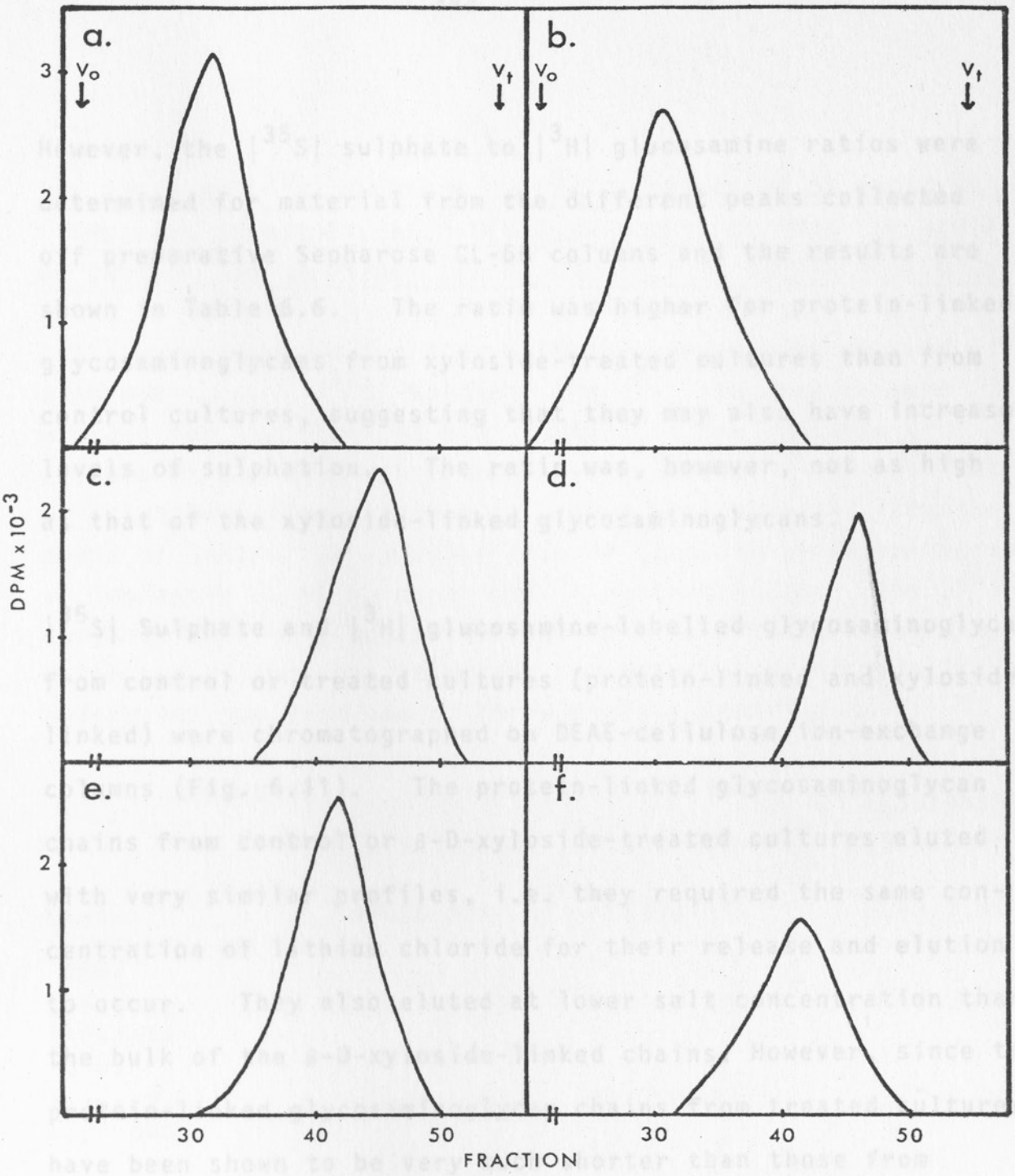



Fig. 6.10. Sepharose CL-6B chromatography of protein-linked and β-D-xyloside-linked glycosaminoglycans from control and β-D-xyloside-treated smooth muscle cultures.

Cells were cultured for 8 days in the presence (a,c,e) or absence (b,d,f) of ascorbic acid. |$^{35}$S| Sulphate (10 μCi/ml) was present during the final 24 h in culture either in the absence (a,b) or presence (c-f) of 0,5 mM p-nitrophenyl-β-D-xyloside. Media from control or treated cultures were collected and subjected to chromatography on a Sepharose CL-6B column. Material corresponding to protein-linked glycosaminoglycans from control cultures (a,b), protein-linked glycosaminoglycans from treated cultures (c,d), and xyloside-linked glycosaminoglycans from treated cultures (e,f) were pooled, digested with papain and rechromatographed on Sepharose CL-6B for size analysis.

However, the $|^{35}S|$ sulphate to $|^{3}H|$ glucosamine ratios were determined for material from the different peaks collected off preparative Sepharose CL-6B columns and the results are shown in Table 6.6. The ratio was higher for protein-linked glycosaminoglycans from xyloside-treated cultures than from control cultures, suggesting that they may also have increased levels of sulphation. The ratio was, however, not as high as that of the xyloside-linked glycosaminoglycans.

$|^{35}S|$ Sulphate and $|^{3}H|$ glucosamine-labelled glycosaminoglycans from control or treated cultures (protein-linked and xyloside-linked) were chromatographed on DEAE-cellulose ion-exchange columns (Fig. 6.11). The protein-linked glycosaminoglycan chains from control or β-D-xyloside-treated cultures eluted with very similar profiles, i.e. they required the same concentration of lithium chloride for their release and elution to occur. They also eluted at lower salt concentration than the bulk of the β-D-xyloside-linked chains. However, since the protein-linked glycosaminoglycan chains from treated cultures have been shown to be very much shorter than those from control cultures (Fig. 6.10) (6000 vs 50000), their elution from the DEAE-cellulose columns at the same salt concentration as control protein-linked chains strongly suggested that the former were more sulphated to yield the same overall total negative charge.

Thus, as the presence of β-D-xyloside caused a decrease in the length of glycosaminoglycan chains formed on core protein and an increase in their levels of sulphation, it was not possible

TABLE 6.6

THE RATIO OF $|^{35}S|$ SULPHATE TO $|^{3}H|$ GLUCOSAMINE OF PROTEIN-LINKED AND XYLOSIDE-LINKED GLYCOSAMINOGLYCANS FROM THE MEDIA OF CONTROL AND XYLOSIDE-TREATED CULTURES OF SMOOTH MUSCLE CELLS

Cells were cultured for 8 days in the presence or absence of ascorbic acid. $|^{35}S|$ Sulphate and $|^{3}H|$ glucosamine were present for the final 48 h of culture in the presence or absence of 0,5 mM p-nitrophenyl-β-D-xyloside. Protein-linked or xyloside-linked glycosaminoglycans were isolated from the media of control or xyloside-treated cultures by chromatography on Sepharose CL-6B as described in Section 6.2.3. The $|^{35}S|$ sulphate to $|^{3}H|$ glucosamine ratio was determined as already described. The data represent the mean ± S.D. of triplicate determinations from one of three similar experiments.

| | Ascorbate | Radioactivity (dpm) $\|^{35}S\|$Sulphate | $\|^{3}H\|$Glucosamine | $\|^{35}S\|$Sulphate : $\|^{3}H\|$Glucosamine |
|---|---|---|---|---|
| Control (Protein-GAG) | + | 89070±824 | 60130±375 | 1,48±0,09 |
| Xyloside (Protein-GAG) | + | 37467±2532 | 24017±2378 | 1,56±0,09 |
| Xyloside (Xyl-GAG) | + | 750798±5397 | 414795±4368 | 1,82±0,16 |
| Control (Protein-GAG) | - | 76995±5175 | 55605±3375 | 1,39±0,12 |
| Xyloside (Protein-GAG) | - | 24459±741 | 14820±1146 | 1,65±0,11 |
| Xyloside (Xyl-GAG) | - | 537600±9945 | 303729±7875 | 1,77±0,16 |

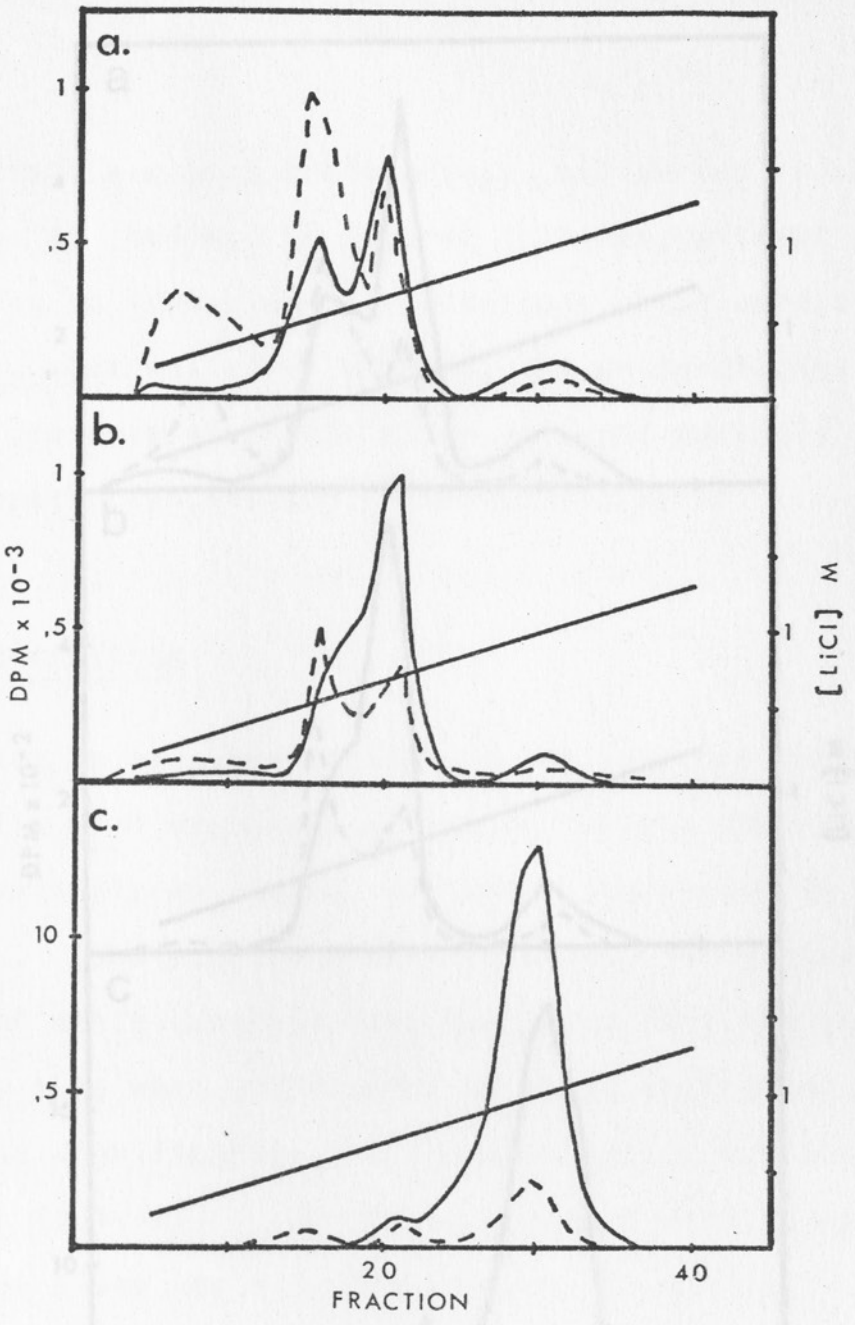



Fig. 6.11a. DEAE-cellulose chromatography of protein-linked and β-D-xyloside-linked glycosaminoglycans from control and β-D-xyloside-treated smooth muscle cells cultured in the presence of ascorbic acid.

Cells were cultured for 8 days in the presence of ascorbic acid. $|^{35}S|$ Sulphate (10 μCi/ml) (—) and $|^{3}H|$ glucosamine (5 μCi/ml) (---) were present during the final 24 h in culture in either the absence (a) or presence (b,c) of 0,5 mM p-nitrophenyl-β-D-xyloside. Media from control and treated cultures were collected and subjected to chromatography on a preparative Sepharose CL-6B column. Material corresponding to protein-linked glycosaminoglycans from control cultures (a), protein-linked glycosaminoglycans from treated cultures (b) and xyloside-linked glycosaminoglycans from treated cultures (c) were pooled, digested with papain and subjected to DEAE-cellulose chromatography as described in Section 6.2.4.

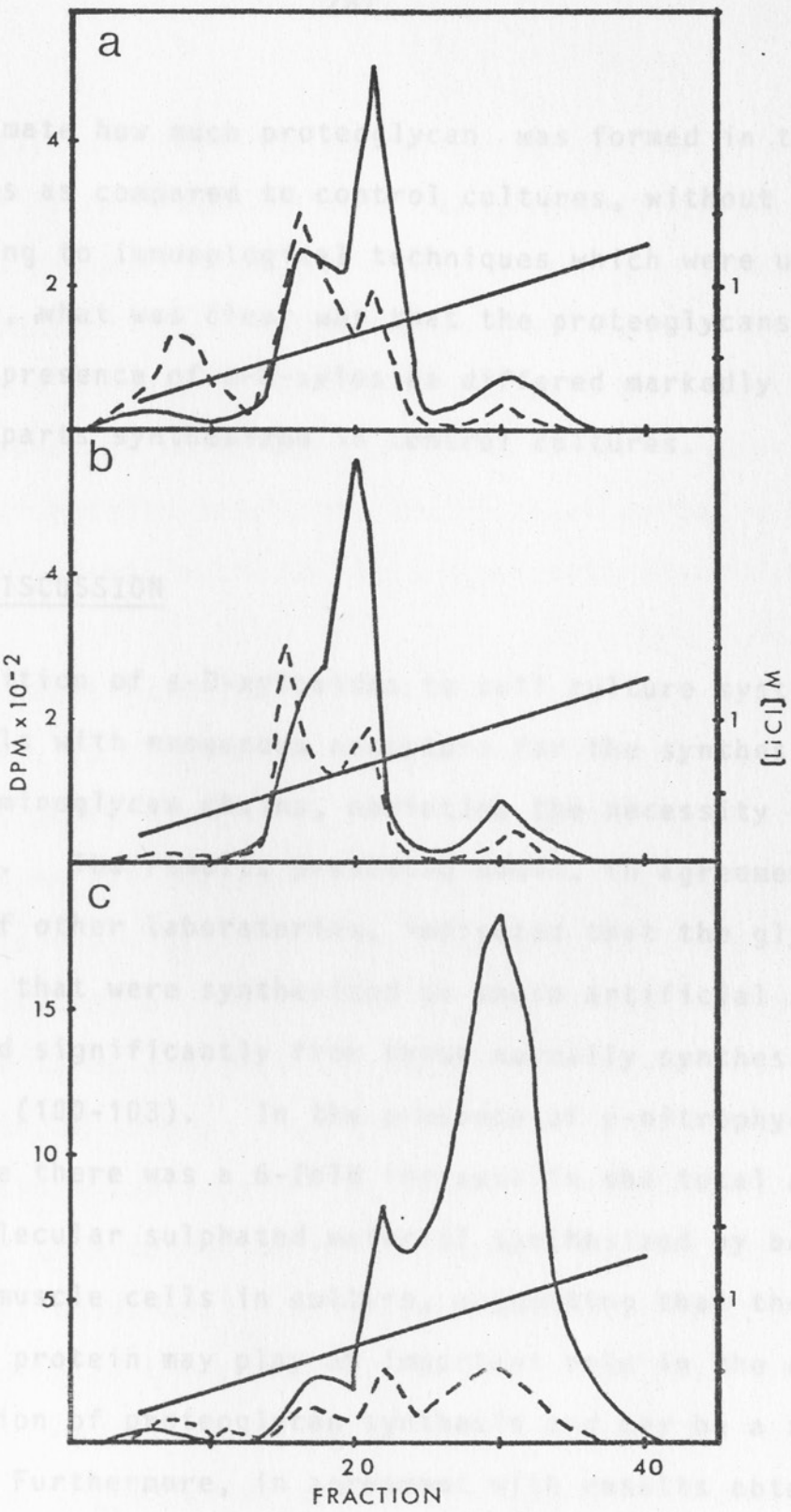



Fig. 6.11b. DEAE-cellulose chromatography of protein-linked and β-D-xyloside-linked glycosaminoglycans from control and β-D-xyloside-treated smooth muscle cells cultured in the absence of ascorbic acid.

Cells were cultured and cultured medium processed and analysed as described under Fig. 6.11a, except for the absence of ascorbic acid in this instance. (-) |$^{35}$S| Sulphate; (---) |$^{3}$H| glucosamine; (a) protein-linked glycosaminoglycans from controls, (b) protein-linked glycosaminoglycans from treated cultures, (c) xyloside-linked glycosaminoglycans from treated cultures.

to estimate how much proteoglycan was formed in treated cultures as compared to control cultures, without perhaps resorting to immunological techniques which were unavailable. However, what was clear was that the proteoglycans synthesized in the presence of β-D-xyloside differed markedly from their counterparts synthesized in control cultures.

## 6.4. DISCUSSION

The addition of β-D-xylosides to cell culture systems provides the cells with exogenous acceptors for the synthesis of new glycosaminoglycan chains, obviating the necessity for core protein. The results presented above, in agreement with those of other laboratories, indicated that the glycosaminoglycans that were synthesized on these artificial acceptors differed significantly from those normally synthesized on core protein (100-103). In the presence of p-nitrophyenyl-β-D-xyloside there was a 6-fold increase in the total amount of macromolecular sulphated material synthesized by bovine aortic smooth muscle cells in culture, suggesting that the presence of core protein may play an important role in the overall regulation of proteoglycan synthesis and may be a rate-limiting step. Furthermore, in agreement with results obtained with other cell culture systems, the glycosaminoglycans synthesized on the exogenous acceptor were shown to be more soluble and more readily secreted into the medium, and correspondingly lower amounts were found associated with the pericellular-matrix fraction (100,358-360). The core protein is almost certainly necessary for the correct "positioning" of the

proteoglycan within the extracellular matrix, and thereafter further interactions between the glycosaminoglycan chains and the cationic proteins of the matrix may stabilize this arrangement. It has been shown that the addition of β-D-xylosides to chick limb bud mesenchymal cell cultures resulted in an abnormal morphology and lower water content of the extracellular matrix, thus underlining the importance of the proteoglycans for extracellular matrix structure and function (359).

It was shown that the glycosaminoglycans synthesized on the β-D-xyloside acceptor by aortic smooth muscle cells were significantly shorter than those normally synthesized on core protein in the absence of the β-D-xyloside. This supported previous observations on the effect of this artificial acceptor on chain length of glycosaminoglycans synthesized by chondrocyte cultures and some non-connective tissue cell cultures (102,103,357,360). The shorter length may result from the increased solubility of the chains formed on the acceptor, causing them to reside for a shorter time at the site of glycosyl transfer. It has been suggested that the presence or absence of the core protein is an important factor in determining the length of the glycosaminoglycan chains, and this was supported by cell-free studies by Richmond et al. (365). They found that the chondroitin sulphate polymerizing system of microsomal preparations from embryonic chicken epiphysial cartilage behaved differently in the presence of different primers: when an endogenous peptide-glycosaminoglycan primer was present, the chains formed were longer than when the terminal pentasaccharide or hexasaccharide of chondroitin

sulphate was added as an exogenous primer to an identical system. However, the fact that the glycosaminoglycans formed on the core protein in the presence of xyloside were also shown to be shorter than those from control cultures suggested that the presence of the core protein may not be the only determining factor. The decreased length may occur as a result of an increased rate of synthesis. As a result of an increase in the number of new glycosaminoglycan chains being initiated, there would be an increased ratio between available acceptor and available precursors, chain elongating enzymes or energy supply, and as a result chain length might decrease. This view was supported by studies that showed that in β-D-xyloside-treated chicken limb bud mesenchymal cells the keratan sulphate chains formed on the core protein also decreased in length (359). These are not bound to the core protein via the normal trisaccharide linkage region and their decreased length most likely resulted from a decreased amount of available precursors, as a result of increased free chondroitin sulphate synthesis. In addition, the amount of $|^{35}S|$ sulphate incorporated into proteoglycans by aortic smooth muscle cells in the presence of β-D-xyloside decreased relative to that incorporated into proteoglycan in the absence of the acceptor. Other workers have shown that the amount of core protein in control and β-D-xyloside-treated cultures was similar (101). Thus, once again the decreased incorporation into proteoglycans in the presence of xyloside may be a result of a decreased availability of precursors etc. due to a marked increase in the rate of glycosaminoglycan synthesis. Thus glycosaminoglycan termination would appear to be a variable

process which may be regulated by the availability of initiator relative to the capacity of the polymerizing system. This is supported by observations that partial inhibition of protein synthesis caused an increase in size of glycosaminoglycan chains linked to core protein (104).

The increased incorporation of $|^{35}S|$ sulphate into macromolecular material in the presence of β-D-xyloside was shown to result from increased synthesis as well as an increased level of sulphation. However, it must be borne in mind that some perturbation of the uptake or utilization of the isotopic precursors may occur to account for the much-increased ratio of $|^{35}S|$ sulphate : $|^{3}H|$ glucosamine. This does not, however, detract from findings described here that clearly show an increase in the sulphation levels of glycosaminoglycans synthesized in the presence of β-D-xyloside by smooth muscle cells (refer Table 6.4). Other workers have reported decreased or unchanged levels of sulphation of chondroitin sulphate chains in the presence of β-D-xyloside (357,358). Only one other laboratory has reported an increase in sulphation of heparan sulphate chains formed by xyloside-treated rat hepatoma cultures when core protein synthesis was inhibited (362). The increased level of sulphation of chondroitinase ABC-sensitive chains was conclusively demonstrated in our system. The reason for this increase may reside in the ability of xyloside-linked glycosaminoglycans to associate more efficiently with the enzyme systems involved in sulphate transfer. However, in contrast to other reports (357,358), it was shown that those glycosaminoglycans initiated on core protein in the presence

of β-D-xyloside were also more sulphated than their normal counterparts in control cultures. Since these protein-linked chains from treated cultures were also very much shorter than control chains (6000 vs 50000), these proteoglycans may also be better able to associate with the sulphotransferase enzymes. Gibson et al. suggested that the major effect of β-D-xyloside in chick embryo cartilage was to cause a significant decrease in the length of the glycosaminoglycan chains of the proteoglycans and decreased sulphation followed as a result of the inferior ability of the short chain to accept sulphate (357). However, this would imply that sulphation occurs at a site separate from polymerization and not during or immediately after polymerization as is generally believed. Increased levels of sulphation may be related to the increased activity of the sulphotransferase system in the presence of β-D-xyloside.

The addition of β-D-xyloside to aortic smooth muscle cells in culture resulted in a net increase in $|^{35}S|$ sulphate incorporation into all sulphated glycosaminoglycans found in these cells, chondroitin sulphate, dermatan sulphate and heparan sulphate/heparin. However, the increase was most marked for the dermatan sulphate fraction and least for the heparan sulphate/heparin fraction, so that the relative amount of dermatan sulphate increased and that of heparan sulphate/heparin decreased slightly. This was in agreement with the findings of other workers who have generally reported an increase in the chondroitinase ABC-sensitive fraction in the presence of β-D-xyloside (100,103,107). The fact that this increase in the

bovine smooth muscle cell system was due to a specific increase in dermatan sulphate synthesis contradicts the findings of Lohmander et al., who showed a total absence of dermatan sulphate in β-D-xyloside-treated chondrocytes (360). It is possible that under normal physiological conditions different core proteins are directed to different subcellular locations rich in polymerizing enzymes for a particular glycosaminoglycan. The addition of xyloside would therefore "short-circuit" this system, and thus give rise to changes in the types of glycosaminoglycan chains synthesized. Alternatively, a particular core protein may normally be limiting and β-D-xyloside would again circumvent this limitation. To illustrate this point with our data it would mean that under normal circumstances the amount of core protein for dermatan sulphate chain acceptance would be limiting, whereas there was a ready supply of heparan sulphate/heparin-specific core protein. In the presence of β-D-xyloside the potential activity of the polymerizing enzymes for dermatan sulphate would be utilized fully, and thus increased proportions of these chains would be made in relation to heparan sulphate/heparin chains.

Thus it is clear that the core protein must play an important role in the regulation of glycosaminoglycan synthesis, and the glycosaminoglycan polymerizing system behaves differently in the presence or absence of core protein. Whether the core protein is important directly as a result of its sequence dictating what kinds of glycosaminoglycans may be attached at initiation sites, or indirectly by simply steering the molecule

to a specific site for addition of specific residues, is not known. However, because of the differences between protein-linked glycosaminoglycans found in the presence or absence of β-D-xyloside, it can be postulated that the polymerizing system also behaves differently when the rate of synthesis of glycosaminoglycans is changed.

The results of our study suggested that the core protein may be important for determining the type of glycosaminoglycan synthesized in a particular system, whereas factors like the length of glycosaminoglycan chain and degree of sulphation are largely determined by the rates of synthesis of these glycosaminoglycan chains. This was supported by observations that conditions which decreased the rate of synthesis, such as addition of protein synthesis inhibitors (104), or lowering the temperature (106), resulted in increased glycosaminoglycan length, whereas factors that increased the rate of synthesis, such as the addition of β-D-xylosides (102,103), caused decreased glycosaminoglycan chain lengths.

# CHAPTER 7

# TURNOVER OF SULPHATED GLYCOSAMINOGLYCANS IN BOVINE AORTIC SMOOTH MUSCLE CELLS

## 7.1. INTRODUCTION

The relatively few studies that have been carried out on the degradation of proteoglycans have been discussed fully in Section 1.3.1, and all the data accumulated so far have suggested a complex process. Newly synthesized glycosaminoglycans are distributed in a distinct manner into the various culture compartments and these compartments contain pools exhibiting metabolic heterogeneity (153,306). Tissue culture studies have been invaluable in the elucidation of these metabolically distinct pools since the technique allows the ready separation of these compartments to be carried out, which is not possible with intact tissue. As a result, the data obtained from studies using intact tissue have tended to be oversimplified.

As already stressed, the turnover of sulphated proteoglycans in cultured cells has not been widely studied, but already a number of important facets of this process have been documented. Generally, newly synthesized glycosaminoglycans are distributed into three pools - the extracellular, pericellular and intracellular pools. The intracellular pool is depleted either as a result of normal secretion or degradation. Glycosaminoglycans associated with the pericellular pool may either be shed into the medium or taken up by the cell for degradation. Complete degradation (hydrolysis of the carbohydrate moiety)

of glycosaminoglycans has been shown to take place intracellularly within lysosomal vesicles (173). In genetic mucopolysaccharidosis, where degradation of proteoglycans is impaired, there is only enlargement of the intracellular pool; secretion into the medium and pericellular compartments remains unaffected (94). It is thus a prerequisite for complete degradation, that extracellular molecules are internalised by the cell via receptor-mediated endocytosis (167,169). Recycling of such receptors has been proposed to occur in a manner analogous to that of low density lipoprotein receptors (170). The proteoglycan receptors on the surfaces of cultured human skin fibroblast have been shown to consist of two types: low and high affinity binding receptors (169), and both of these showed specificity for the core protein of intact proteoglycan monomers, since they have been shown not to interact with free glycosaminoglycan chains (167,169). Furthermore, the addition of sulphated glycosaminoglycan chains was shown to inhibit endocytosis (169), and this has led to speculation that sulphated proteoglycans may have to be desulphated, or at least partially, at some extracellular site prior to endocytosis for further total degradation. Such ecto-sulphatase(s), capable of partially removing sulphate residues from sulphated proteoglycans, have been shown to be associated with the pericellular compartment of rat heart smooth muscle cells (172). In addition, it has been observed that, both in human embryo fibroblasts (IMR-90) (366), and in pig medial smooth muscle cell cultures (302), the pericellular compartment contributed to the degradative process, since the amount of sulphated proteoglycans involved in the catabolic pathway far

exceeded the amount of labelled material initially present in the intracellular compartment.

Using pulse-chase radioisotope techniques, the turnover of sulphated glycosaminoglycans in bovine aortic smooth muscle cells has been studied, with a view to establishing the site and mode of degradation of these molecules.

## 7.2. METHODS

### 7.2.1. Preparation of cell cultures

Bovine foetal aortic smooth muscle cells ($A_3$) were isolated and cultured as described in Section 3.2.1, and were grown in either the presence or absence of ascorbic acid (50 µg/ml) which was added daily.

### 7.2.2. Incorporation of radioactive isotopes

Incorporation studies were usually carried out on cultures grown in 35 mm Petri dishes which were initiated at $10^5$ cells/dish. Cells were cultured for 8 days with medium changes at 3 and 6 days. Cells were pulsed for the requisite times with $|^{35}S|$ sulphate (10 µCi/ml) and/or $|^{3}H|$ glucosamine (5 µCi/ml). At the end of the pulse periods the medium was removed and the cell layers were washed three times with 2 ml of warm medium. Fresh medium containing 20 mM sodium sulphate and/or 10 mM glucosamine was then added to the cultures and the cells were returned to the incubator for the required chase periods. At each time point in pulse-chase experiments the medium

(extracellular compartment) was removed, centrifuged briefly at 1800 g for 2 min to remove any free floating cells, and retained for analysis. The cell layers were washed three times with PBS containing 30 mM KCN, and the pericellular-matrix and intracellular compartments were isolated, after treating the cell layers with a solution of Viokase and collagenase, as described in Section 3.2.7. The amount of radioactivity present in macromolecular material was assessed after descending paper chromatography as described in Section 3.2.7.

7.2.3. Molecular exclusion chromatography

Samples of the various culture compartments were analysed by gel exclusion chromatography on a Sephadex G-50 (medium) (1,5 x 100 cm) column, equilibrated with PBS containing both penicillin and streptomycin as antibacterial agents. Samples were run at 4°C and fractions analysed for their radioactivity as described in Section 3.2.4. The column was calibrated in terms of $V_o$ and $V_t$ by chromatography of purified bovine nasal septum proteoglycan monomer and radioactive $|^{35}S|$ sulphate, respectively.

Columns of Sepharose CL-6B (0,8 x 110 cm), equilibrated at room temperature with 0,5 M sodium acetate pH 6,8, were used to assess the size of the labelled proteoglycans or glycosaminoglycans in harvested culture medium, which was then re-incubated with fresh cells (see Section 7.2.5).

### 7.2.4. DEAE-cellulose chromatography

When samples were to be analysed by DEAE-cellulose chromatography they were first digested with papain (1 mg/ml) and then dialysed exhaustively against distilled-deionized water at 5°C and freeze-dried. The samples were dissolved in appropriate volumes of equilibration buffer (0,05 M sodium acetate pH 4,0) and applied to columns (1,0 x 6,0 cm) of DEAE-cellulose which were eluted initially (5 fractions) with equilibration buffer, and subsequently with a linear gradient of lithium chloride (0,2 M to 1,5 M in equilibration buffer) as described in Section 2.2.8.

### 7.2.5. Inhibition of intracellular lysosomal degradation

Cultured cells labelled as described in Section 7.2.2 were treated with lysomotrophic inhibitors as follows: fresh medium containing either chloroquine at a final concentration of 70 µM or ammonium chloride (10 mM final concentration) was added to the cells. Since the solutions of chloroquine were temperature- and light-sensitive, fresh, sterile solutions were always used. Chloroquine and ammonium chloride have been commonly used in the study of lysosomal function, largely because of their well-documented effects on lysosomal pH (367-369). Cultures were only maintained in the presence of these lysomotrophic agents for a maximum of 48 h.

### 7.2.6. Incubation of cultures with medium containing radioactive macromolecules

Medium containing macromolecules labelled with $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine was obtained following incubation of confluent cultures in 75 $cm^3$ flasks for 48 h with fresh medium which contained the isotopes and either plain DMSO (20 µl) or 0,5 mM p-nitrophenyl-β-D-xylopyranoside dissolved in that solvent. At the end of the labelling period, "hot" medium was collected by sterile techniques and then dialysed against ten volumes of complete, ice-cold medium for 48 h with four changes. Dialysed samples were made 20 mM with respect to sodium sulphate and 10 mM with respect to glucosamine, as were samples of the final medium used for dialysis. In addition, medium containing "cold" proteoglycans was prepared in exactly the same way from confluent cultures grown in the absence of the radioactive precursors. After re-sterilization by filtration, the various medium samples were incubated, as described in the text, in 35 mm Petri dishes containing $A_3$ cells grown for 6 days. Aliquots of the medium samples were chromatographed on Sepharose CL-6B as a check on the sizes of the macromolecules being added to the cultures. The radioactivity incorporated by cells incubated with the medium obtained from the dialysis procedure, namely the last batch of medium against which hot medium was dialysed, was used as controls to assess any de novo synthesis of proteoglycans during the experimental period. De novo synthesis was slightly less than the background obtained during the descending paper chromatographic procedure described above.

### 7.2.7. Analytical procedures

Determinations of cellular protein were carried out on washed cell layers after lysis by a solution of 1% (w/v) SDS using a modification of the method of Lowry *et al.* (323), as described in Section 3.2.3.

## 7.3. RESULTS

The kinetics of $|^{35}S|$ sulphate incorporation into the proteoglycans of the extracellular, pericellular-matrix and intracellular compartments during a 48 h labelling period are shown in Fig. 7.1. The intracellular $|^{35}S|$ sulphate labelled glycosaminoglycan pool showed a slow rate of incorporation with time, with low overall accumulation of radioactivity. However, incorporation into medium-associated glycosaminoglycans increased linearly with time, with either 64% or 80% of the total radioactivity being found in this compartment at the end of the 48 h incubation in the case of ascorbic acid-supplemented or deprived cultures, respectively. As shown previously, ascorbic acid-supplemented cultures exhibited increased $|^{35}S|$ sulphate incorporation into the pericellular-matrix compartment. Pulse-chase radioisotope experiments were undertaken in order to study these pathways of metabolism in the smooth muscle culture system in more detail. After a 24 h pulse with radioactive sulphate more than 80% of the total macromolecular radioactivity (pericellular-matrix and intracellular) was associated with the pericellular-matrix compartment for cells grown in the presence of ascorbic acid

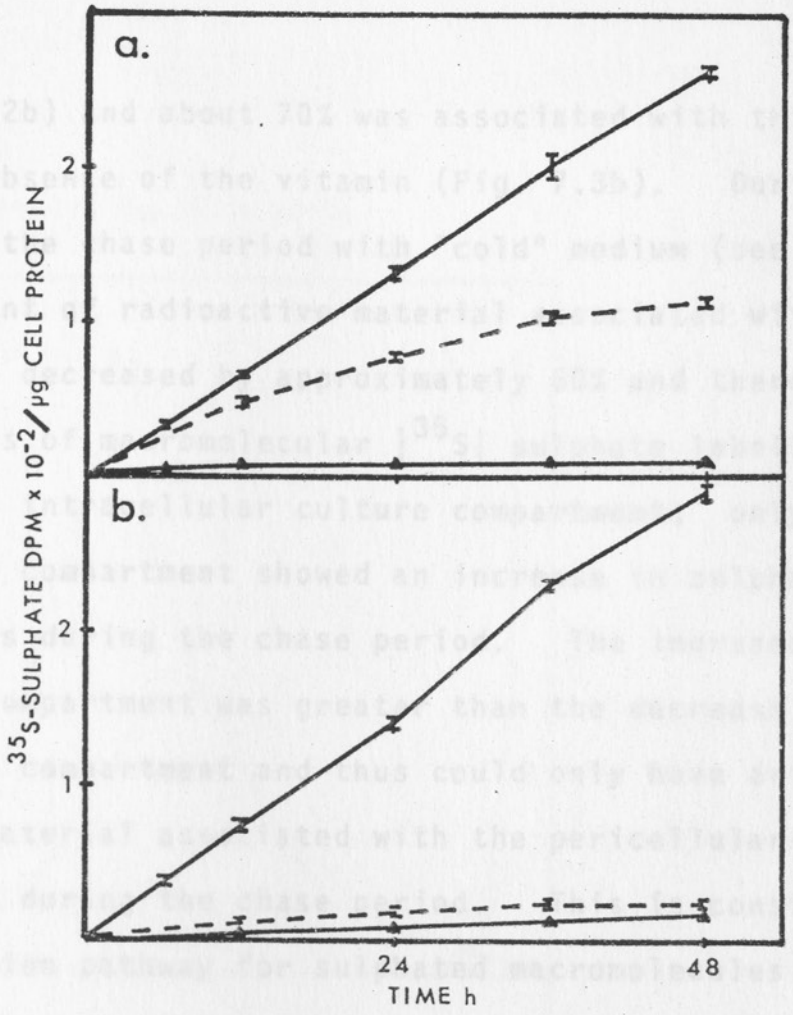



Fig. 7.1. Kinetics of incorporation of |$^{35}$S| sulphate into macromolecules present in the different culture compartments of smooth muscle cell cultures.

Cells were grown for 8 days in the presence (a) or absence (b) of ascorbic acid, with the addition of |$^{35}$S| sulphate (10 µCi/ml) during the final 48 h of culture. At each time point during incubation the medium (—), pericellular-matrix (---) and intracellular (▲—▲) compartments were collected and assayed for the total incorporated isotope as described in Section 3.2.7. The data represent the mean ± S.D. of triplicate determinations for one of two similar experiments.

(Fig. 7.2b) and about 70% was associated with this compartment in the absence of the vitamin (Fig. 7.3b). During the first 24 h of the chase period with "cold" medium (see Methods), the amount of radioactive material associated with this compartment decreased by approximately 50% and there was also some loss of macromolecular $|^{35}S|$ sulphate labelled material from the intracellular culture compartment; only the extracellular compartment showed an increase in sulphated macromolecules during the chase period. The increase in this latter compartment was greater than the decrease in the intracellular compartment and thus could only have arisen by transfer of material associated with the pericellular-matrix compartment during the chase period. This is consistent with a secretion pathway for sulphated macromolecules which involved their transfer from the intracellular to the pericellular-matrix compartment and thence to the extracellular compartment. During the remaining chase period of the experiment, both the intracellular and pericellular-matrix compartments continued to show smaller decreases in macromolecular sulphate content and the extracellular compartment also accumulated radioactivity at a decreased rate. When the specific activity data, dpm / μg cellular protein (Fig. 7.2a, 7.3a), were replotted in terms of the percentage of the total specific activity at a given time associated with each compartment (Fig. 7.2b and 7.3b), the biphasic decrease in radioactivity associated with the pericellular-matrix compartment was still apparent, especially for ascorbic acid-deprived cultures which have a less well defined extracellular matrix (Chapter 3). The initial rapid loss of

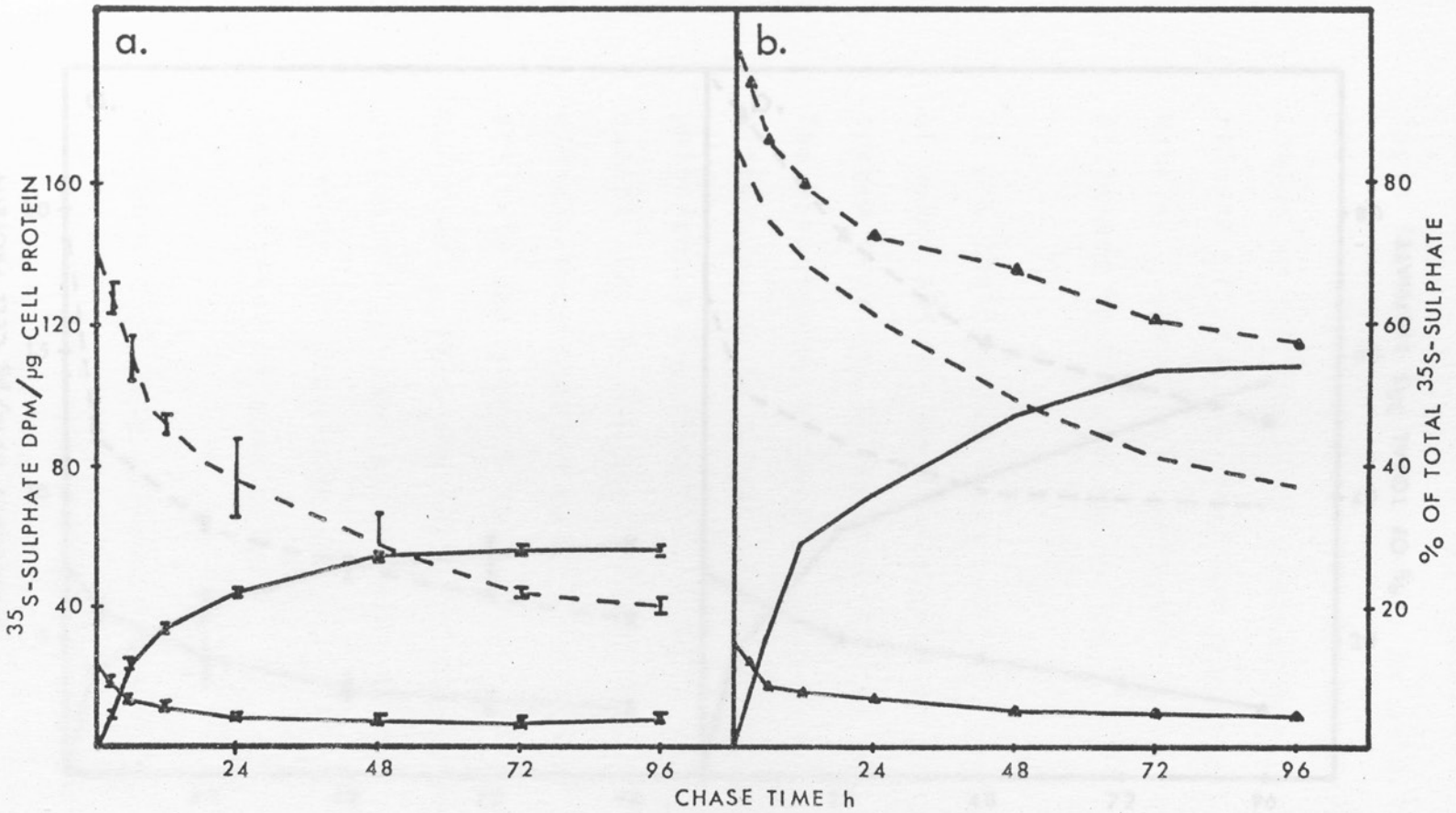



Fig. 7.2. Kinetics and percentage distribution of $|^{35}S|$ sulphate labelled macromolecules during pulse-chase experiments with cells cultured in the presence of ascorbic acid.

Cells were grown for 6 days in the presence of ascorbic acid and then pulse-labelled for 24 h with 10 µCi/ml of $|^{35}S|$ sulphate. After labelling, cells were washed with medium and fresh culture medium was added to cells for the requisite chase period (Section 7.2.2). The amounts of macromolecular radioactivity in the extracellular (—), pericellular-matrix (---) and intracellular (▲—▲) compartments were assessed as described in Section 3.2.7 both in terms of specific activity (DPM/mg total cell protein)(a) and percentage of the total radioactivity associated with a particular culture compartment at each time point (b). Furthermore, the percentage of the initial total macromolecular radioactivity present at each time point was also calculated (▲--▲). The data represent the mean ± S.D. of triplicate determinations for one of several similar experiments.

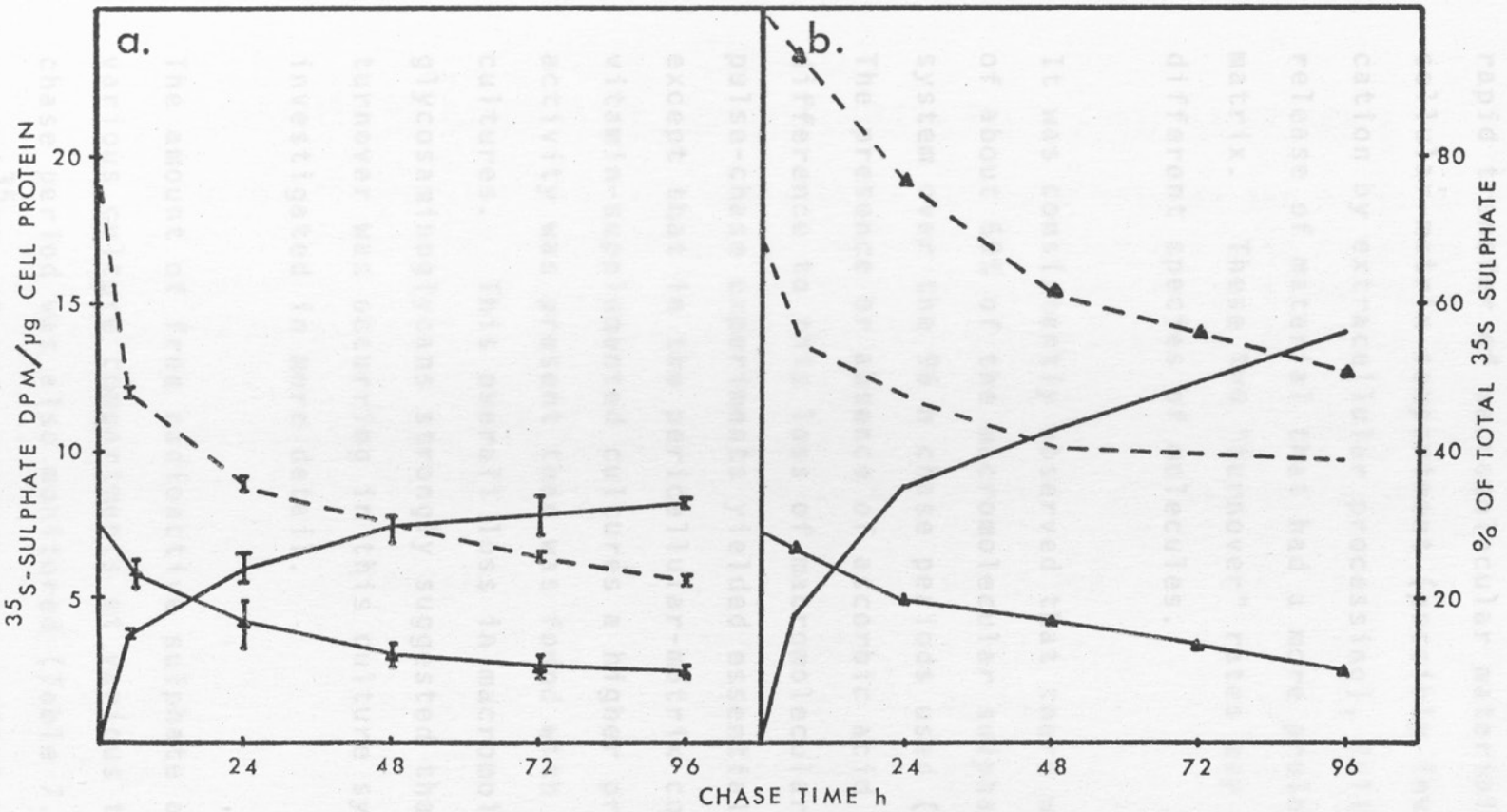



Fig. 7.3. Kinetics and percentage distribution of |$^{35}$S| sulphate labelled macromolecules during pulse-chase experiments for cells cultured in the absence of ascorbic acid.

Cells were grown for 6 days in the absence of ascorbic acid and then pulse-labelled for 24 h with 10 µCi/ml of |$^{35}$S| sulphate. After incubation, cells were washed with medium and fresh culture medium was added to the cells for the requisite chase periods (Section 7.2.2). The amounts of macromolecular radioactivity in the extracellular (—), pericellular-matrix (---) and intracellular (▲—▲) compartments was assessed as described in Section 3.2.7 in terms of both specific activity (DPM/mg total cell protein) (a) and percentage of the total radioactivity incorporated at each time point (b). Furthermore, the percentage of initial total macromolecular radioactivity present at each time point was also calculated (▲--▲). The data represent the mean ± S.D. of triplicate determinations for one of several similar experiments.

material from this compartment, followed by a subsequent slower decrease in radioactivity, suggested a process involving a rapid transfer of macromolecular material from the pericellular-matrix compartment (possibly involving some modification by extracellular processing), followed by a slower release of material that had a more prolonged sojourn in the matrix. These two "turnover" rates may be associated with different species of molecules.

It was consistently observed that there was an overall loss of about 50% of the macromolecular sulphate in the culture system over the 96 h chase periods used (Fig. 7.2 and 7.3). The presence or absence of ascorbic acid made very little difference to this loss of macromolecular radioactivity, and pulse-chase experiments yielded essentially similar profiles except that in the pericellular-matrix compartment from vitamin-supplemented cultures a higher proportion of radioactivity was present than was found with vitamin-deprived cultures. This overall loss in macromolecular sulphated glycosaminoglycans strongly suggested that a process of turnover was occurring in this culture system, and this was investigated in more detail.

The amount of free radioactive sulphate associated with the various culture compartments at various times during the 96 h chase period was also monitored (Table 7.1). The amount of free $|^{35}S|$ sulphate present intracellularly decreased very rapidly and no free $|^{35}S|$ sulphate was found associated with

TABLE 7.1

DISTRIBUTION OF RADIOACTIVE SULPHATE DURING PULSE-CHASE EXPERIMENTS

The distribution of macromolecular and free radioactive sulphate between the extracellular, pericellular-matrix and intracellular compartments was determined using the data obtained from pulse-chase experiments as described for Fig. 7.2 No free sulphate was found associated with the pericellular-matrix compartment. The data represent the mean ± S.D. for triplicate determinations.

| Time (h) | Macromolecular $\|^{35}S\|$ Sulphate (dpm) | | | | Free $\|^{35}S\|$ Sulphate (dpm) | |
|---|---|---|---|---|---|---|
| | Intracellular | Pericellular-matrix | Extracellular | Total | Intracellular | Extracellular |
| 0 | 8850±563 | 51860±1075 | 0 | 60710±488 | 4165±332 | 872±101 |
| 12 | 3693±159 | 31535±1322 | 11790±1060 | 47017±2223 | 1250±42 | 18588±640 |
| 24 | 1315±233 | 24370±1895 | 14940±28 | 40625±1633 | 530±177 | 23070±1202 |
| 48 | 1847±619 | 20215±728 | 19255±1348 | 41317±1657 | 340±71 | 26774±5298 |
| 72 | 2200±367 | 15935±587 | 19655±742 | 37790±523 | 321±37 | 33829±4086 |
| 96 | 2680±396 | 13075±253 | 17805±434 | 33560±4963 | 303±10 | 36910±1060 |

the pericellular-matrix compartment. The amount of free $|^{35}S|$ sulphate in the medium increased with time and when compared to the observed loss in macromolecular sulphate over the same time period, it was apparent that the two values were similar. Furthermore, when media samples collected at different time points during the chase period were analysed by Sephadex G-50 chromatography, it was seen that there was an increase in material associated with both the $V_o$ and the $V_t$ of the column which represented macromolecular material and free $|^{35}S|$ sulphate, respectively (Fig. 7.4). Thus there were no intermediate-sized products that accumulated in the culture medium with time.

In an attempt to assess whether the overall decrease in macromolecular sulphate and the concomitant increase in free $|^{35}S|$ sulphate which occurred in the culture medium of bovine smooth muscle cells was the result of complete degradation (lysosomal) of sulphated glycosaminoglycans or of enzymatic desulphation, parallel sets of cultures were pulsed with either $|^{3}H|$ glucosamine or $|^{35}S|$ sulphate for 24 h, followed by chase periods in the presence of either 10 mM glucosamine or 20 mM sodium sulphate. The profiles for the distribution of $|^{35}S|$ sulphate with time of chase were essentially the same as in Fig. 7.2a and 7.3a. The $|^{3}H|$ glucosamine profiles are presented in Fig. 7.5; loss in radioactive macromolecular material was also noted with these cultures, although it was not as great as that seen for cells pulsed with $|^{35}S|$ sulphate. At the end of the 96 h chase period, the amount of $|^{35}S|$ sulphate labelled macromolecular material had decreased to approximately

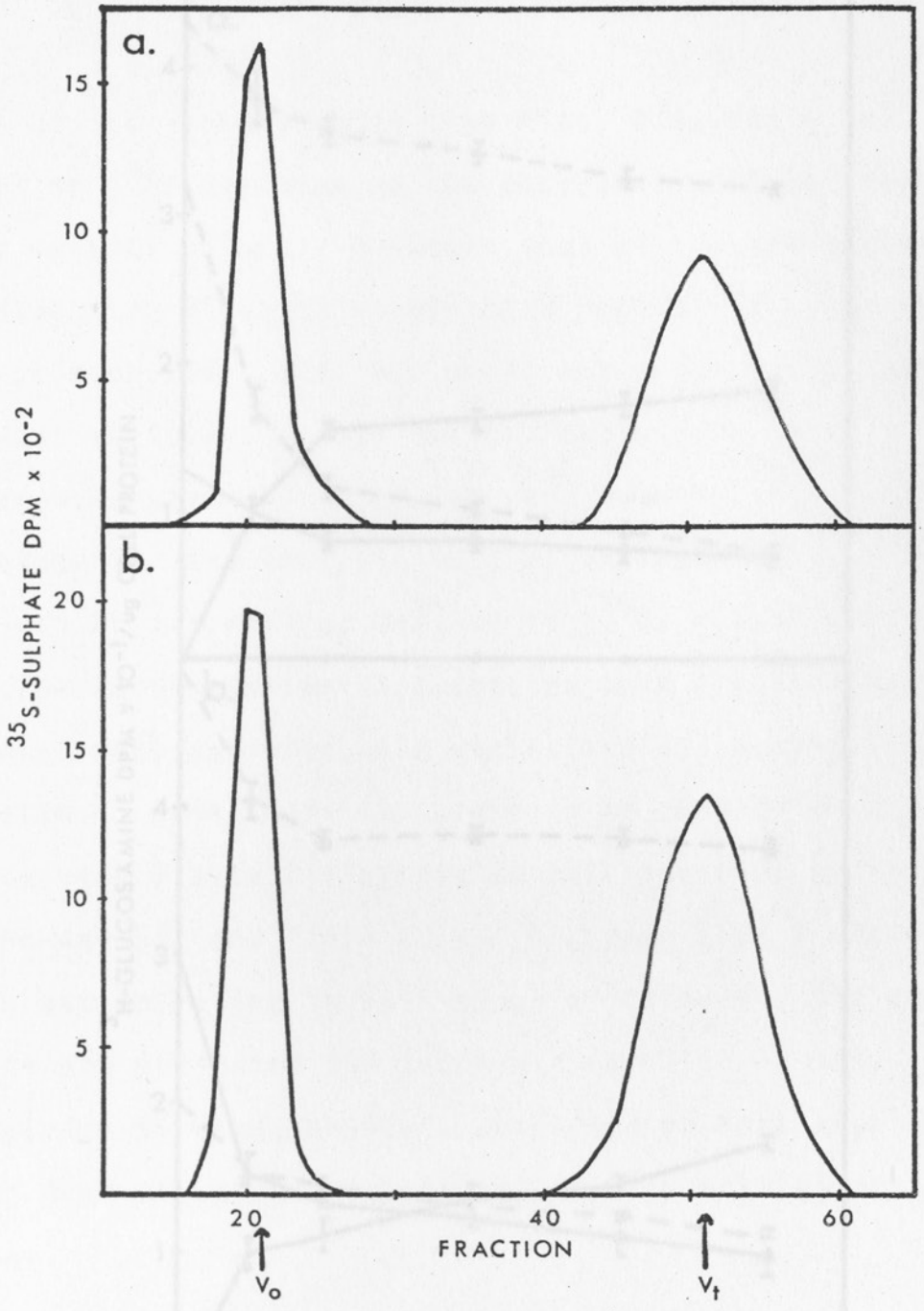



Fig. 7.4. Sephadex G-50 chromatography of culture media isolated at times during various chase times.

Cells were grown for 6 days in the presence of ascorbic acid and then pulse-labelled for 24 h with 10 µCi/ml of $|^{35}S|$ sulphate. After labelling, cells were washed with medium and fresh culture medium was added to the cells for the requisite chase periods (Section 7.2.2). Samples of culture medium at a) 24 h chase period and b) 72 h chase period were chromatographed on a Sephadex G-50 column as described in Section 7.2.3.

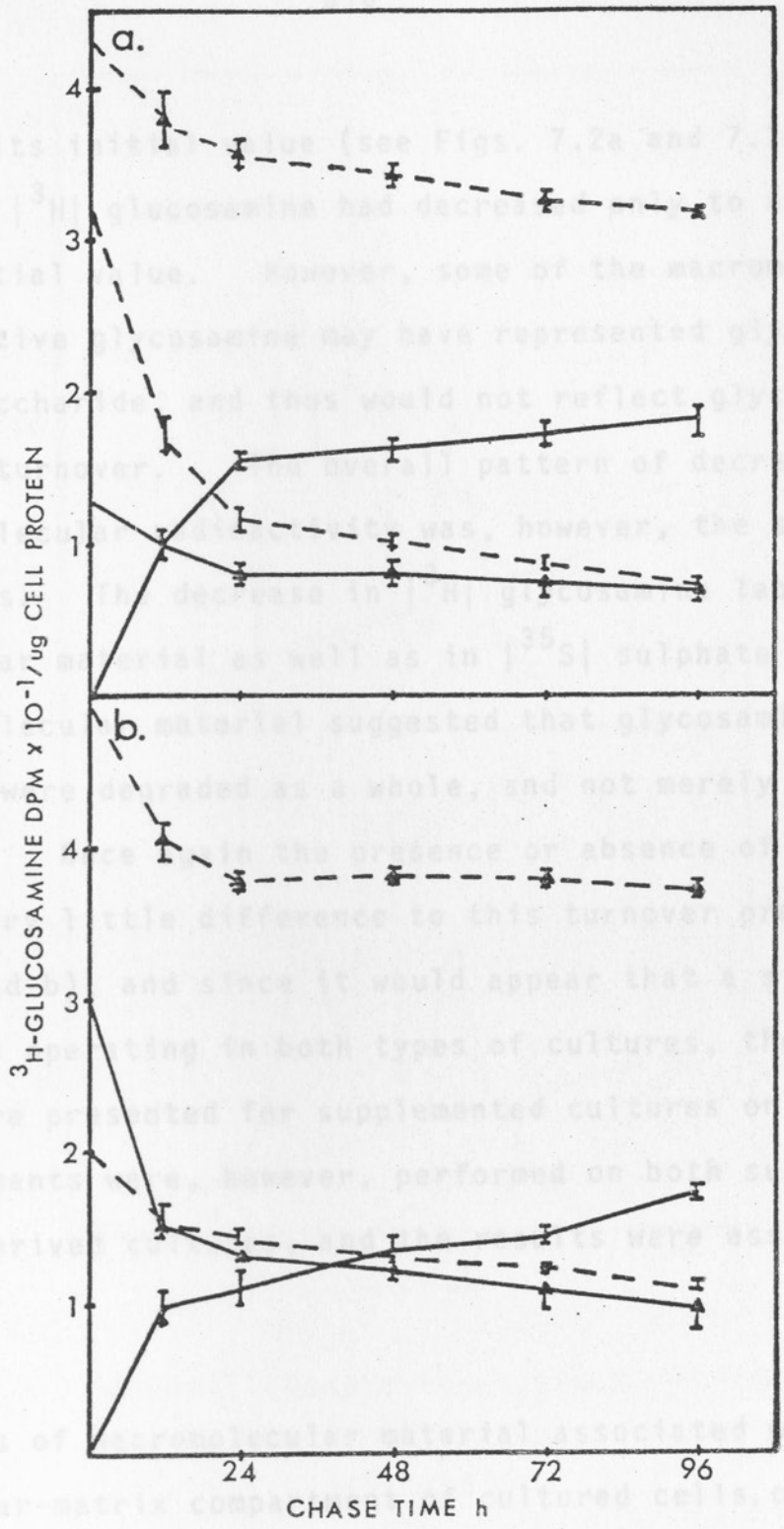



Fig. 7.5. The kinetics of pulse-chase experiments carried out on smooth muscle cell cultures pulse-labelled with |$^3$H| glucosamine.

Cells were grown for 6 days in the presence (a) or absence (b) of ascorbic acid and then pulse-labelled for 24 h with 5 μCi/ml |$^3$H| glucosamine. After labelling, cells were washed with medium and fresh medium was added to cells for the requisite chase periods (Section 7.2.2). The total amount of macromolecular radioactivity (▲--▲) and that associated with the extracellular (——), pericellular-matrix (---) or intracellular (▲——▲) compartments at each time point was assessed as described in Section 3.2.7. The data represent the mean ± S.D. of triplicate determinations for one of two similar experiments.

50% of its initial value (see Figs. 7.2a and 7.3a), whereas that of $|^{3}H|$ glucosamine had decreased only to about 70% of its initial value. However, some of the macromolecular radioactive glycosamine may have represented glycoprotein oligosaccharide, and thus would not reflect glycosaminoglycan turnover. The overall pattern of decrease in macromolecular radioactivity was, however, the same for both isotopes. The decrease in $|^{3}H|$ glycosamine labelled macromolecular material as well as in $|^{35}S|$ sulphate labelled macromolecular material suggested that glycosaminoglycan chains were degraded as a whole, and not merely being desulphated. Once again the presence or absence of ascorbic acid made very little difference to this turnover process (Fig. 7.5a and b), and since it would appear that a similar mechanism was operating in both types of cultures, the remaining data are presented for supplemented cultures only. All experiments were, however, performed on both supplemented and deprived cultures, and the results were essentially the same.

Samples of macromolecular material associated with the pericellular-matrix compartment of cultured cells, collected at 3 h after the start of the chase and at the 48 h chase period, were chromatographed on DEAE-cellulose ion-exchange columns (Fig. 7.6). Although the amount of macromolecular material in the pericellular-matrix compartment at the 48 h chase was considerably reduced, its profile on DEAE-cellulose was unchanged when compared to that of material from the start of the chase. This strongly suggested that the glycosaminoglycan chains

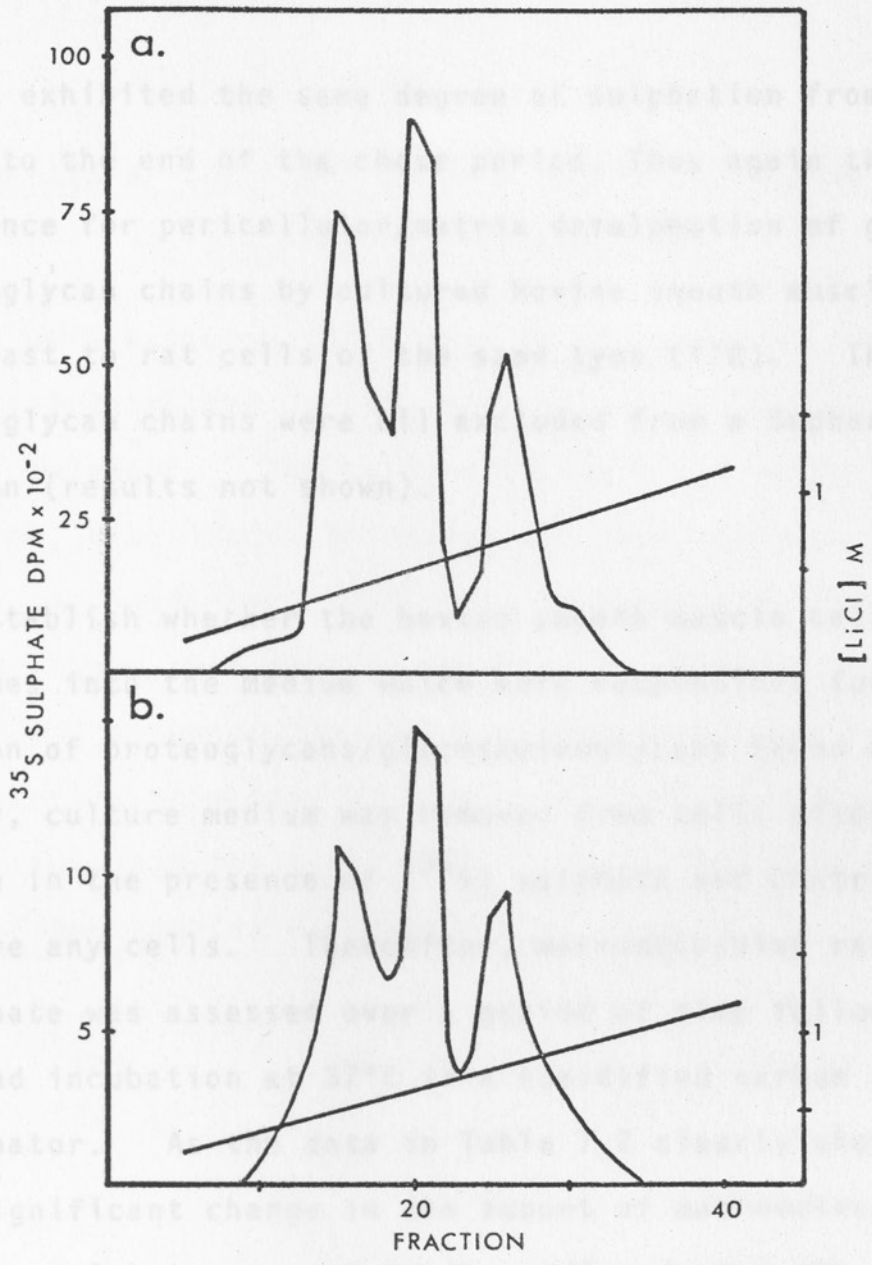



Fig. 7.6. DEAE-cellulose chromatography of radioactively-labelled pericellular-matrix material after different chase periods.

Cells were grown for 6 days in the presence of ascorbic acid and then pulse-labelled for 24 h with 10 µCi/ml of |$^{35}$S| sulphate. After labelling, cells were washed with medium and fresh medium was added to the cells for the requisite chase periods (Section 7.2.2). Pericellular-matrix material from chase periods 3 h (a) and 48 h (b) were collected and processed as described in Section 7.2.4 before being analysed on DEAE-columns. The data represent one of two similar experiments.

still exhibited the same degree of sulphation from the beginning to the end of the chase period. Thus again there is no evidence for pericellular/matrix desulphation of glycosaminoglycan chains by cultured bovine smooth muscle cells in contrast to rat cells of the same type (172). These glycosaminoglycan chains were all excluded from a Sephadex G-50 column (results not shown).

To establish whether the bovine smooth muscle cells secreted enzymes into the medium which were responsible for the degradation of proteoglycans/glycosaminoglycans found extracellularly, culture medium was removed from cells after a 24 h pulse in the presence of $|^{35}S|$ sulphate and centrifuged to remove any cells. Thereafter, macromolecular radioactive sulphate was assessed over a period of time following continued incubation at 37°C in a humidified carbon dioxide incubator. As the data in Table 7.2 clearly show, there was no significant change in the amount of macromolecular radioactive sulphate present in the medium during the incubation, nor was any free $|^{35}S|$ sulphate generated (Table 7.2). Thus the degradation of the macromolecular material that had been observed thus far required contact with the cultured cells.

Since it has been shown that proteoglycans may be internalized and degraded by lysosomal hydrolases (169,178), and, furthermore, that both chloroquine and ammonium chloride may be used to inhibit lysosomal degradation of macromolecules (367-369), these compounds were used to investigate the role of lysosomes in the degradation of proteoglycans by bovine aortic

still exhibited the same degree of sulphation from the beginning to the end of the chase period. Thus again there is no evidence for pericellular/matrix desulphation of glycosaminoglycan chains by cultured bovine smooth muscle cells in contrast to rat cells of the same type (172). These glycosaminoglycan chains were all excluded from a Sephadex G-50 column (results not shown).

To establish whether the bovine smooth muscle cells secreted enzymes into the medium which were responsible for the degradation of proteoglycans/glycosaminoglycans found extracellularly, culture medium was removed from cells after a 24 h pulse in the presence of $|^{35}S|$ sulphate and centrifuged to remove any cells. Thereafter, macromolecular radioactive sulphate was assessed over a period of time following continued incubation at 37°C in a humidified carbon dioxide incubator. As the data in Table 7.2 clearly show, there was no significant change in the amount of macromolecular radioactive sulphate present in the medium during the incubation, nor was any free $|^{35}S|$ sulphate generated (Table 7.2). Thus the degradation of the macromolecular material that had been observed thus far required contact with the cultured cells.

Since it has been shown that proteoglycans may be internalized and degraded by lysosomal hydrolases (169,178), and, furthermore, that both chloroquine and ammonium chloride may be used to inhibit lysosomal degradation of macromolecules (367-369), these compounds were used to investigate the role of lysosomes in the degradation of proteoglycans by bovine aortic

TABLE 7.2

INCUBATION OF CELL-FREE CULTURE MEDIUM CONTAINING RADIOACTIVE MACROMOLECULAR MATERIAL

Cells were cultured for 6 days in the presence of ascorbic acid and incubated during the final 24 h with 10 μCi/ml $|^{35}S|$ sulphate. The culture medium was subsequently removed, centrifuged to remove any free floating mitotic cells and maintained at 37°C for a further 4 days in a humidified carbon dioxide incubator. Aliquots were removed daily and assessed for their content of macromolecular radioactive sulphate, as described in Section 3.2.7. The data represent the mean ± S.D. for quadruplicate dishes for one of two similar experiments.

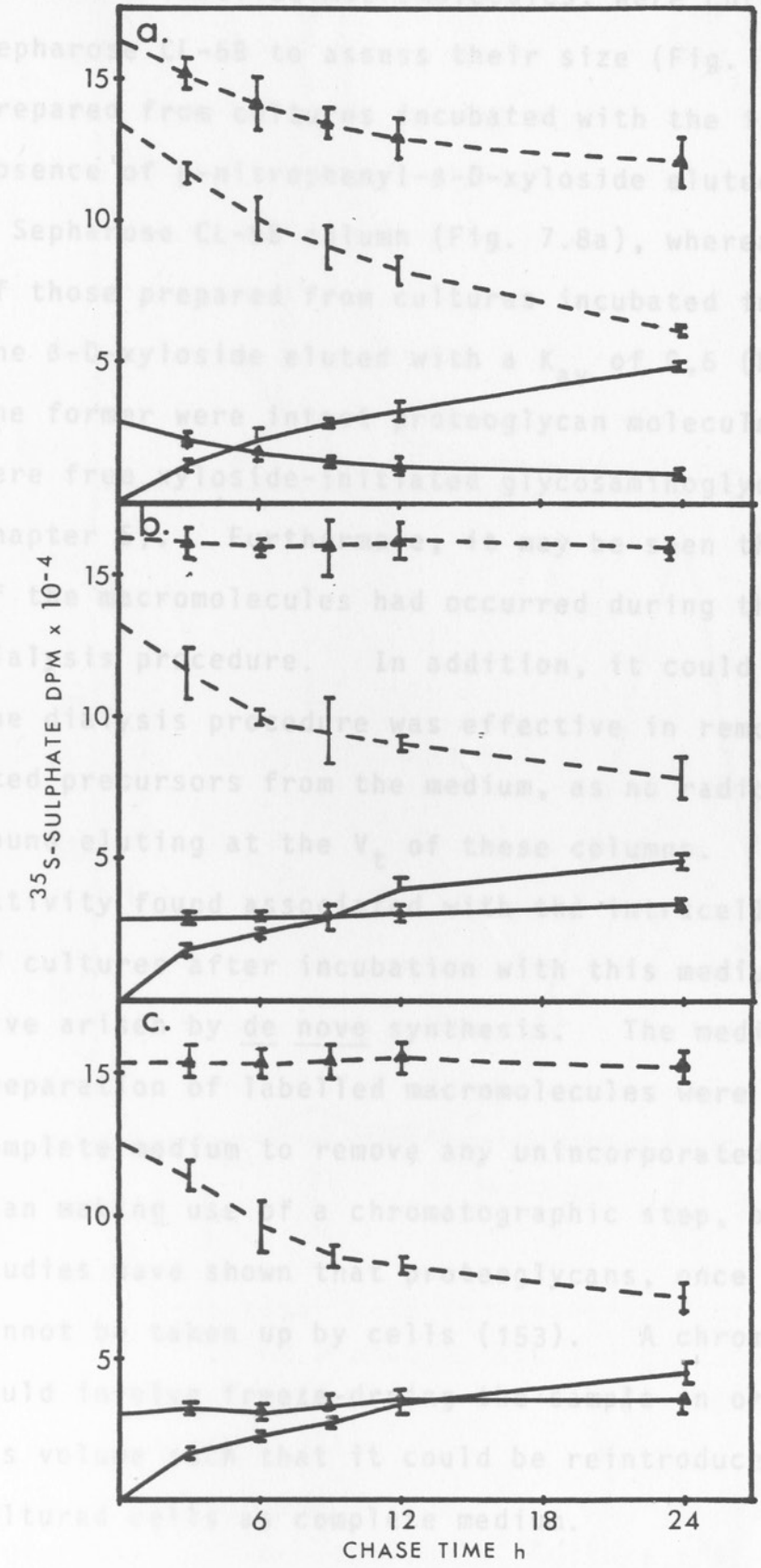
| Time (h) | Macromolecular $|^{35}S|$ Sulphate (dpm) |
|---|---|
| 0 | 279115 ± 6619 |
| 24 | 263801 ± 8423 |
| 48 | 281004 ± 5372 |
| 72 | 273017 ± 10149 |
| 96 | 268418 ± 9273 |

smooth muscle cells in culture. Consequently, cultures were pulsed for 24 h with $|^{35}S|$ sulphate and then subjected to a chase period of 24 h in the presence of the lysomotrophic agents (Fig. 7.7). The presence of either ammonium chloride (Fig. 7.7b) or chloroquine (Fig. 7.7c) resulted in almost complete inhibition of turnover of sulphated macromolecules over 24 h when compared to cultures incubated in the absence of these agents (Fig. 7.7a). The total amount of $|^{35}S|$ sulphate labelled macromolecular material present in the culture system after a chase period of 24 h in the absence of any exogenous lysomotrophic agents was routinely about 72% of the amount present at the start of the chase. Unfortunately, experiments could not be pursued for longer than 24 h, since prolonged exposure to these agents, especially chloroquine, resulted in cytotoxicity. However, the inhibition of turnover in the presence of the lysomotrophic agents for 24 h indicated that a lysosomal process was responsible for the degradation of sulphated proteoglycans/glycosaminoglycans.

In order for the degradation to occur, the proteoglycans must be internalised by the cultured cells. Kress et al. have shown that uptake of proteoglycans was achieved by receptor-mediated endocytosis (167). In order to elucidate the mechanism of internalisation carried out by the cultured bovine aortic smooth muscle cells, $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine labelled proteoglycans or glycosaminoglycans were isolated from the media of cultured smooth muscle cells as described in Section 7.2.6. These medium samples, which

Fig. 7.7. The effect of lysomotrophic inhibitors on the kinetics of macromolecular sulphate distribution among the culture compartments of bovine smooth muscle cells.

Cells were grown for 6 days in the presence of ascorbic acid, and then pulse-labelled for 24 h with 10 μCi/ml of $|^{35}S|$ sulphate. After labelling, cells were washed with medium and fresh medium was added to the cells for the required chase period in the absence (a) and presence of 10 mM ammonium chloride (b) or 70 μM chloroquine (c). The total amount of macromolecular radioactivity (▲---▲) and that present in the extracellular (——), pericellular-matrix (---) or intracellular (▲——▲) compartments at each time point was assessed as described in Section 3.2.7. The data represent the mean ± S.D. of triplicate determinations for one of three similar experiments.


a.
b.
c.
15
10
5
$^{35}$S-SULPHATE DPM x $10^{-4}$
6
12
18
24
CHASE TIME h

contained labelled macromolecules, were chromatographed on Sepharose CL-6B to assess their size (Fig. 7.8). Samples prepared from cultures incubated with the isotopes in the absence of p-nitrophenyl-β-D-xyloside eluted at the $V_o$ of a Sepharose CL-6B column (Fig. 7.8a), whereas the majority of those prepared from cultures incubated in the presence of the β-D-xyloside eluted with a $K_{av}$ of 0,6 (Fig. 7.8b), thus the former were intact proteoglycan molecules and the latter were free xyloside-initiated glycosaminoglycan chains (see Chapter 6). Furthermore, it may be seen that no degradation of the macromolecules had occurred during the preparation of dialysis procedure. In addition, it could also be seen that the dialysis procedure was effective in removing unincorporated precursors from the medium, as no radioactivity was found eluting at the $V_t$ of these columns. Thus any radioactivity found associated with the intracellular compartment of cultures after incubation with this medium, could not have arisen by de novo synthesis. The media used for the preparation of labelled macromolecules were dialysed against complete medium to remove any unincorporated isotope rather than making use of a chromatographic step, because other studies have shown that proteoglycans, once freeze-dried, cannot be taken up by cells (153). A chromatographic step would involve freeze-drying the sample in order to reduce its volume such that it could be reintroduced to fresh cultured cells as complete medium.

The uptake of radioactively-labelled proteoglycans or glycosaminoglycans by the aortic smooth muscle cells is shown in

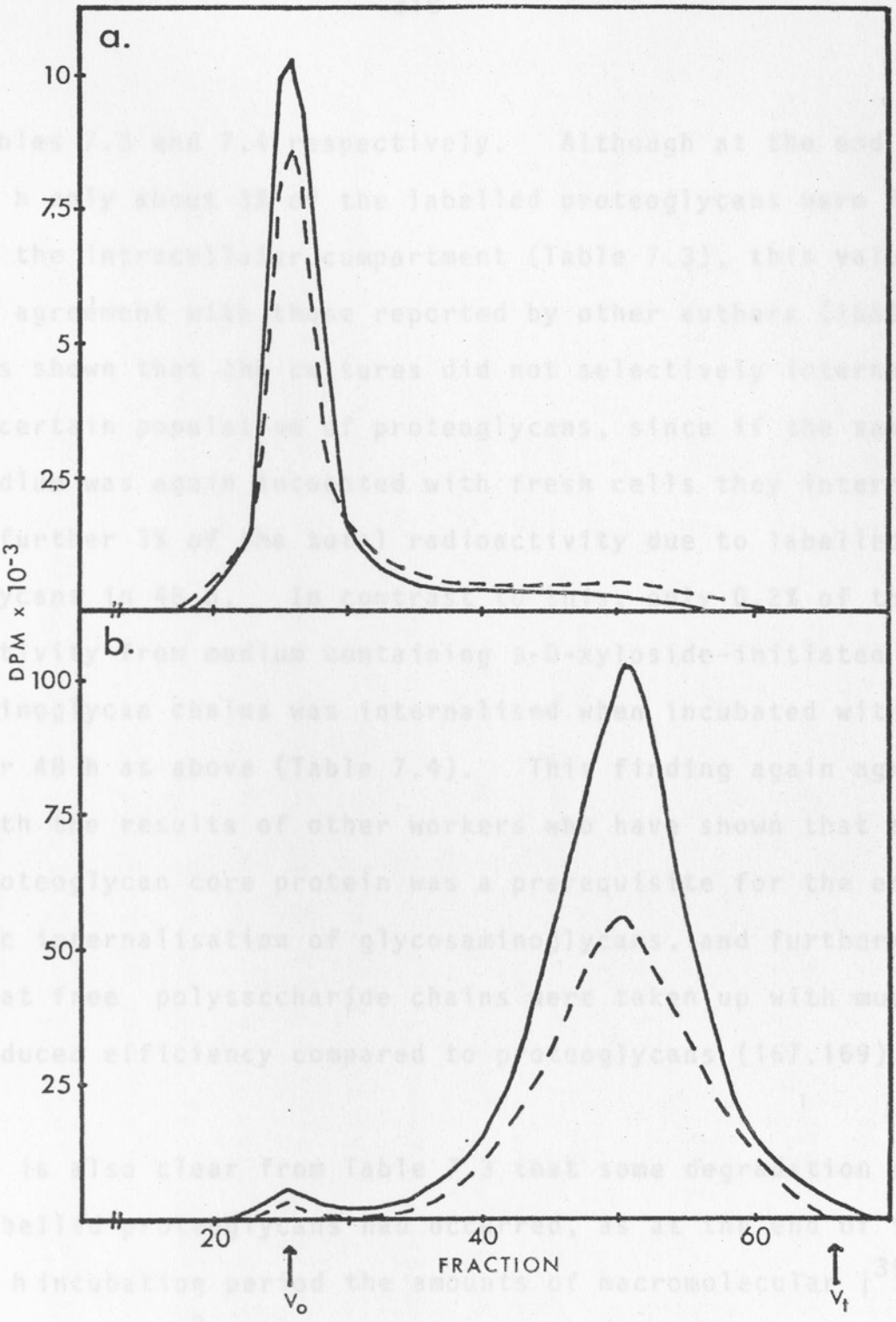



Fig. 7.8. Sepharose CL-6B chromatography of radioactively-labelled proteoglycans and glycosaminoglycans present in culture medium prior to incubation with smooth muscle cells.

Medium which contained radioactive macromolecules was prepared as described in Section 7.2.6. Culture medium samples were prepared, processed and aliquots checked by Sepharose CL-6B chromatography to assess the size of the proteoglycans (a) or free glycosaminoglycan chains (b). $|^{3}H|$ Glucosamine (--); $|^{35}S|$ sulphate (—).

Tables 7.3 and 7.4 respectively. Although at the end of 48 h only about 3% of the labelled proteoglycans were found in the intracellular compartment (Table 7.3), this value was in agreement with those reported by other authors (168). It was shown that the cultures did not selectively internalise a certain population of proteoglycans, since if the same medium was again incubated with fresh cells they internalised a further 3% of the total radioactivity due to labelled proteoglycans in 48 h. In contrast to this, only 0.2% of the radioactivity from medium containing β-D-xyloside-initiated glycosaminoglycan chains was internalised when incubated with cells for 48 h as above (Table 7.4). This finding again agreed with the results of other workers who have shown that an intact proteoglycan core protein was a prerequisite for the endocytotic internalisation of glycosaminoglycans, and furthermore that free polysaccharide chains were taken up with much-reduced efficiency compared to proteoglycans (167.169).

It is also clear from Table 7.3 that some degradation of the labelled proteoglycans had occurred, as at the end of the 48 h incubation period the amounts of macromolecular $|^{35}S|$ sulphate or $|^{3}H|$ glucosamine labelled material had been reduced to 84,6% and 79,4%, respectively, when compared to their initial values. At the same time, only the end products of this degradation process, namely free $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine were found in the medium. Furthermore, it was noted that the addition of ammonium chloride inhibited this degradation (Table 7.5), since when incubated with smooth muscle cell cultures, culture medium containing radioactively

TABLE 7.3

UPTAKE OF RADIOACTIVELY LABELLED PROTEOGLYCANS BY SMOOTH MUSCLE CELL CULTURES

Cells were grown for 7 days in the presence of ascorbic acid and then incubated for 48 h with fresh medium containing proteoglycans labelled with |$^{35}$S| sulphate and |$^{3}$H| glucosamine which was prepared as described in Section 7.2.6. At various times during the incubation period, the amount of macromolecular material associated with the various culture compartments was assessed as described in Section 3.2.7. The data represent the mean ± S.D. for quadruplicate determinations for one of three similar experiments.

| Time (h) | Macromolecular |$^{35}$S| Sulphate (dpm) | | | | | Macromolecular |$^{3}$H| Glucosamine (dpm) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Intracellular | Pericellular Matrix | Extracellular | Total | Extracellular Free |$^{35}$S| sulphate (dpm) | Intracellular | Pericellular Matrix | Extracellular | Total | Extracellular Free |$^{3}$H| glucosamine (dpm) |
| 0 | 0 | 0 | 294373± 5709 | 294373± 5709 | 0 | 0 | 0 | 283763± 13213 | 283763± 13213 | 0 |
| 24 | 5400± 238 | 6950± 665 | 273881± 26291 | 286231± 26480 | 4142± 589 | 2826± 58 | 3790± 203 | 243874± 8777 | 250491± 8631 | 25458± 1662 |
| 48 | 6492± 668 | 7400± 244 | 235150± 12906 | 249042± 12442 | 38421± 4137 | 4127± 908 | 4310± 56 | 216844± 36303 | 225281± 3703 | 55915± 3457 |

TABLE 7.4

UPTAKE OF RADIOACTIVELY LABELLED β-D-XYLOSIDE INITIATED GLYCOSAMINOGLYCANS BY SMOOTH MUSCLE CELL CULTURES

Cells were grown for 7 days in the presence of ascorbic acid and then incubated for 48 h with fresh culture medium containing free glycosaminoglycan chains initiated on β-D-xylosides and labelled with dual $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine, prepared as described in Section 7.2.6. At various times during the incubation period, the amount of macromolecular material associated with the various culture compartments was assessed as described in Section 3.2.7. The data represent the mean ± S.D. for quadruplicate determinations for one of two similar experiments.

| Time (h) | Macromolecular $|^{35}S|$ Sulphate (dpm) | | | | | Macromolecular $|^{3}H|$ Glucosamine (dpm) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Intracellular | Pericellular Matrix | Extracellular | Total | Extracellular Free $|^{35}S|$ sulphate (dpm) | Intracellular | Pericellular Matrix | Extracellular | Total | Extracellular Free $|^{3}H|$ glucosamine (dpm) |
| 0 | 0 | 0 | 2140174± 16472 | 2140174± 16472 | 0 | 0 | 0 | 1030318± 17640 | 1030318± 17640 | 0 |
| 24 | 4800± 121 | 11743± 734 | 2039816± 15072 | 2056359± 20385 | 87915± 4301 | 2218± 151 | 4983± 91 | 959767± 9203 | 966968± 34752 | 58531± 6335 |
| 48 | 6002± 559 | 16663± 849 | 1865466± 34880 | 1888131± 35662 | 222430± 17651 | 2650± 210 | 6880± 150 | 920456± 17317 | 929986± 99316 | 98003± 6472 |

TABLE 7.5

THE EFFECT OF AMMONIUM CHLORIDE ON THE UPTAKE OF RADIOACTIVELY LABELLED PROTEOGLYCANS BY SMOOTH MUSCLE CELLS

Cells were grown for 7 days in the presence of ascorbic acid, and then incubated for 48 h with fresh culture medium containing 10 mM ammonium chloride and proteoglycans labelled with $|^{35}S|$ sulphate and $|^{3}H|$ glucosamine. At various times during the incubation period, the amount of macromolecular material associated with the various culture compartments was assessed as described in Section 3.2.7. The data represent the mean ± S.D. for quadruplicate determinations for one of two similar experiments.

| Time (h) | Macromolecular $|^{35}S|$ Sulphate (dpm) | | | | | Macromolecular $|^{3}H|$ Glucosamine (dpm) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Intracellular | Pericellular Matrix | Extracellular | Total | Extracellular Free $|^{35}S|$ sulphate (dpm) | Intracellular | Pericellular Matrix | Extracellular | Total | Extracellular Free $|^{3}H|$ glucosamine (dpm) |
| 0 | 0 | 0 | 336142± 19976 | 336142± 19976 | 0 | 0 | 0 | 342607± 18738 | 342607± 18738 | 0 |
| 24 | 16593± 495 | 10793± 156 | 313064± 7719 | 340451± 8221 | 145±31 | 8113± 131 | 6417± 476 | 344087± 29757 | 358617± 29729 | 0 |
| 48 | 23913± 659 | 12630± 430 | 305708± 14991 | 342251± 14958 | 0 | 20957± 2526 | 8110± 465 | 312442± 10611 | 341509± 17935 | 162±25 |

labelled proteoglycans in the presence of 10 mM ammonium chloride did not yield increased amounts of free $|^{35}S|$ sulphate of $|^{3}H|$ glucosamine over 48 h. However, there was a not unexpected increase in the amount of radioactive material accumulated within these cells. Thus, since no free $|^{35}S|$ sulphate or $|^{3}H|$ glucosamine was found associated with the medium, no turnover had taken place. The data obtained for 48 h incubation under the different conditions has been summarised in Table 7.6. This table represents data taken from Tables 7.3, 7.4 and 7.5.

Clearly then in cultured bovine aortic smooth muscle cells extracellular proteoglycans are endocytosed and degraded intracellularly by lysosomal enzymes, the degradation products being secreted into the medium. The mechanism of this endocytosis was studied in more detail using cultures incubated in the presence of increasing amounts of labelled proteoglycan for 48 h. At the end of the incubation period the amount of radioactively-labelled macromolecular material in the different culture compartments was assayed and yielded the results shown in Fig. 7.9. Clearly at the low levels of radioactive proteoglycan offered to cells a linear relationship between available proteoglycan in the medium and the amount bound and taken up by cells existed. Using homogeneous preparations of labelled proteoglycans it was not possible to offer high enough quantities to reach saturation kinetics for binding and uptake. Thus, because the limits of the experimental restraints mentioned above, it is not feasible to ascertain the number and affinity of the proteoglycan receptors

TABLE 7.6

SUMMARY OF DATA RELATING TO THE UPTAKE AND DEGRADATION OF RADIOACTIVELY LABELLED PROTEOGLYCANS/GLYCOSAMINOGLYCANS BY BOVINE SMOOTH MUSCLE CELLS

The table was compiled from the 48 h data listed in Tables 7.3, 7.4 and 7.5. The values for the amount of "degraded" material represent the total amount of free radioactivity present in the medium after 48 h of incubation. Numbers in parentheses indicate the % of the total material (macromolecular and free radioactivity) that is represented by the accompanying figure.

| System | Total DPM $\lvert^{35}S\rvert$ $SO_4$ | | | Total DPM $\lvert^{3}H\rvert$ Glucosamine | | |
|---|---|---|---|---|---|---|
| | Bound | Internalised | Degraded | Bound | Internalised | Degraded |
| Control + proteoglycans | 7400 (2.6) ±244 | 6492 (2.3) ±668 | 38421 (13.4) ±4137 | 4310 (1.5) ± 56 | 4127 (1.5) ±908 | 55915 (20.0) ±3457 |
| Control + free-glycosaminoglycans | 16663 (0.8) ±849 | 6002 (0.3) ±559 | 222430 (10.5) ±17651 | 6880 (0.7) ±180 | 2680 (0.3) ±210 | 98003 (9.5) ± 647 |
| Control + proteoglycans + $NH_4Cl$ (10 mM) | 12630 (3.7) ±430 | 23913 (7.0) ±659 | 0 (0) | 8110 (2.3) ±465 | 20957 (6.1) ±2526 | 162±25 (0.04) |

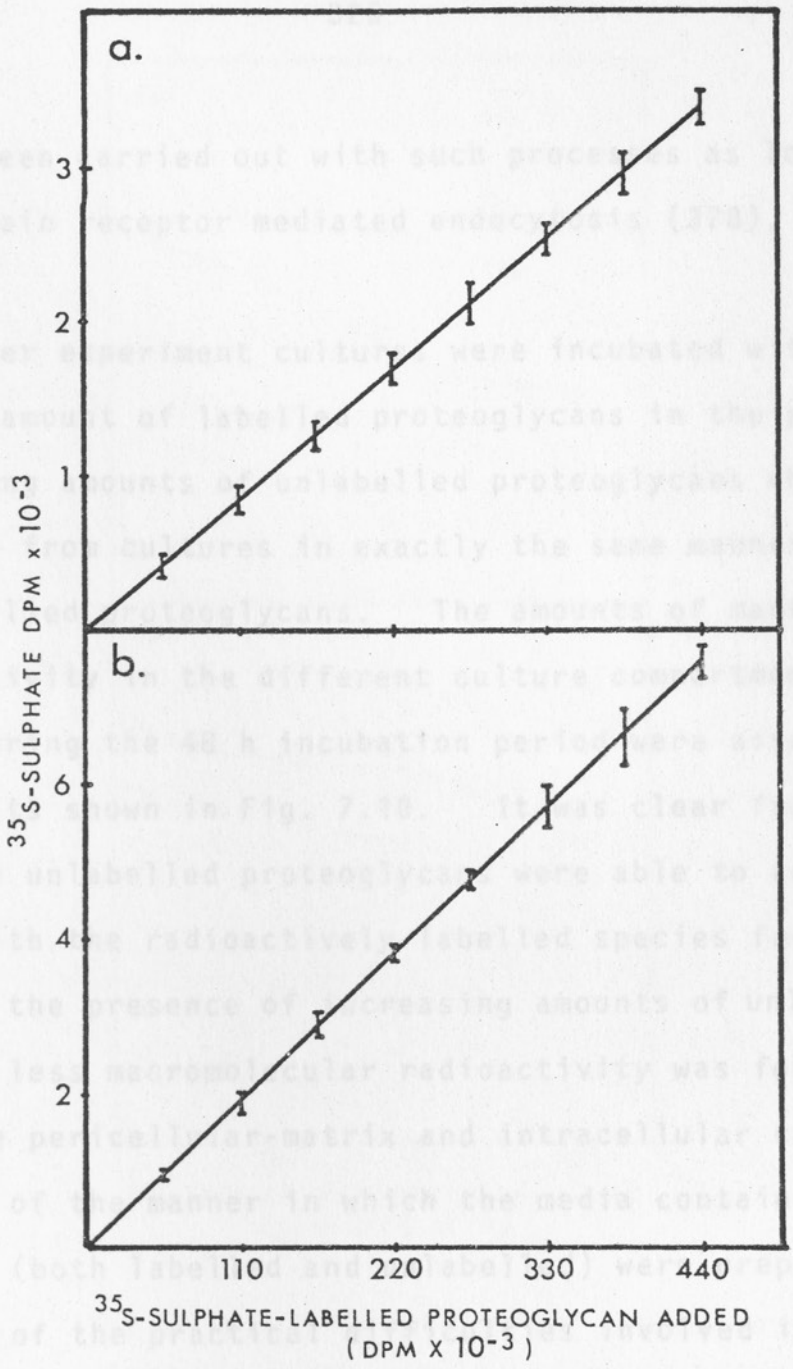



Fig. 7.9. The uptake of $|^{35}S|$ sulphate-labelled proteoglycans by bovine smooth muscle cell cultures as a function of the concentration of the labelled proteoglycans.

Cells were grown for 6 days in the presence of ascorbic acid. Medium containing increasing amounts of $|^{35}S|$ sulphate-labelled proteoglycans was added to the cells and after 48 h the amount of macromolecular radioactivity associated with the pericellular-matrix (a) or intracellular (b) culture compartments was assessed as described in Section 3.2.7. The data represent the mean $\pm$ S.D. of triplicate determinations for one experiment.

as has been carried out with such processes as low density lipoprotein receptor mediated endocytosis (370).

In another experiment cultures were incubated with a requisite amount of labelled proteoglycans in the presence of increasing amounts of unlabelled proteoglycans which were prepared from cultures in exactly the same manner as were the labelled proteoglycans. The amounts of macromolecular radioactivity in the different culture compartments at various times during the 48 h incubation period were assayed and yielded results shown in Fig. 7.10. It was clear from the data that the unlabelled proteoglycans were able to compete successfully with the radioactively labelled species for uptake, and that in the presence of increasing amounts of unlabelled proteoglycans less macromolecular radioactivity was found associated with the pericellular-matrix and intracellular compartments. Because of the manner in which the media containing proteoglycans (both labelled and unlabelled) were prepared, and because of the practical difficulties involved in concentrating them without in any way prejudicing their ability to be endocytosed, the maximum amount of unlabelled proteoglycans that could be added to the culture system to compete with the labelled species present was only a three-fold excess as compared to labelled proteoglycans. However, even with such low levels of unlabelled proteoglycans, competition for uptake was still noted. This indicated the presence of high-affinity saturable receptors for the uptake of proteoglycans.

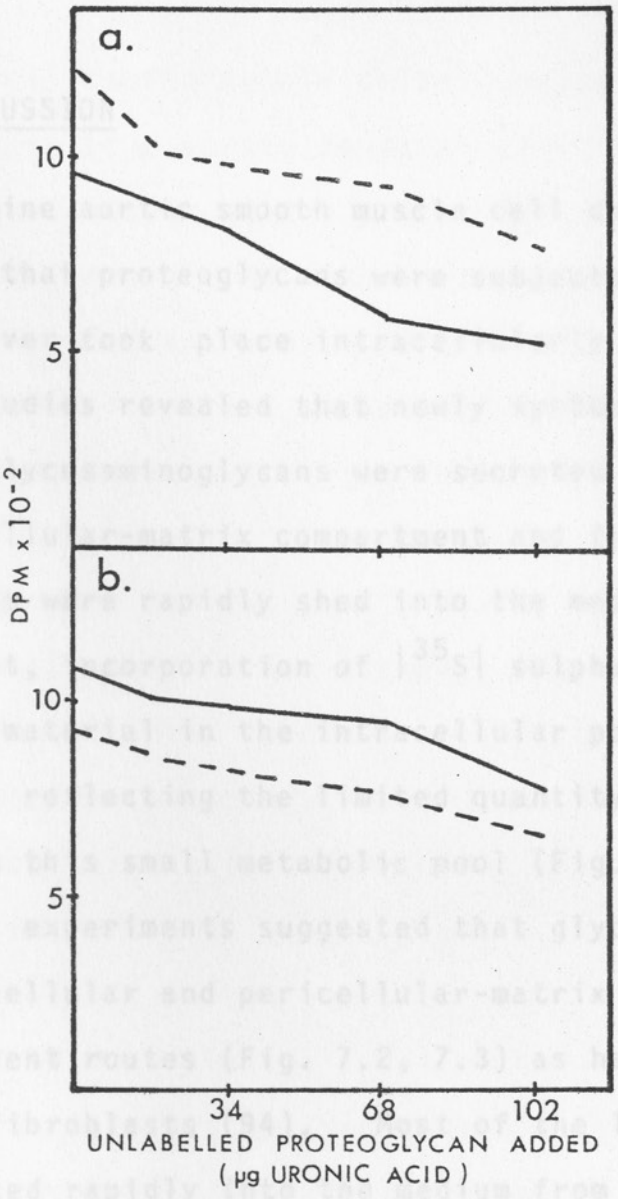



Fig. 7.10. The effect of addition of unlabelled proteoglycans on the uptake of radioactively-labelled proteoglycans by smooth muscle cells.

Cells were grown for 6 days in the presence of ascorbic acid. Medium (500 µl) containing $|^{35}S|$ sulphate (—) and $|^{3}H|$ glucosamine (---) labelled proteoglycans was added to the cells for 48 h in the presence of increasing amounts of unlabelled proteoglycans. The amount of macromolecular radioactivity associated with the pericellular-matrix (a) or intracellular (b) culture compartments was assessed as described in Section 3.2.7. The data represent the mean ± S.D. of triplicate determinations from one of two similar experiments.

## 7.4. DISCUSSION

In the bovine aortic smooth muscle cell culture system it was shown that proteoglycans were subjected to turnover and such turnover took place intracellularly in lysosomes. Kinetic studies revealed that newly synthesized $|^{35}S|$ sulphate labelled glycosaminoglycans were secreted from the cell to the pericellular-matrix compartment and from thence the majority of them were rapidly shed into the medium (Fig. 7.1 - 7.3). In contrast, incorporation of $|^{35}S|$ sulphate into macromolecular material in the intracellular pool quickly reached a plateau, reflecting the limited quantity of glycosaminoglycans in this small metabolic pool (Fig. 7.1). Pulse-chase labelling experiments suggested that glycosaminoglycans leave the intracellular and pericellular-matrix pools by at least two different routes (Fig. 7.2, 7.3) as has been shown for cultured fibroblasts (94). Most of the labelled material was secreted rapidly into the medium from the intracellular pool via the pericellular-matrix compartment, whilst a portion of the extracellular material was taken up by the cells and degraded to monomeric units. Thus several pools of glycosaminoglycans exist within the different cellular compartments. The intracellular compartment, for instance, contains some structural components associated with membranes, as well as the biosynthetic, storage and degradative pools. On the other hand, the pericellular-matrix compartment contains structural components, largely heparan sulphate, together with those glycosaminoglycans destined either for secretion or for endocytosis.

In the aortic smooth muscle cells, turnover was shown to take the form of complete degradation of the molecule. The partial desulphation of the glycosaminoglycans, extracellularly, was not demonstrated with this system. DEAE-cellulose chromatography of extracellular (pericellular-matrix) material after different chase periods revealed no significant changes in the sulphation levels of glycosaminoglycan chains (Fig. 7.6), thus it appeared that the partial desulphation reported for rat smooth muscle cells (172), was not a prerequisite step in the turnover process with bovine smooth muscle cell cultures. However, the data do not entirely preclude the action of ecto-sulphatases on proteoglycans/glycosaminoglycans outside the cell, but in this system it is not an obligatory step in the degradation process.

The degradation of proteoglycans by bovine aortic cells was shown to be inhibited by lysomotrophic inhibitors (Fig. 7.7), indicating that such degradation occurred intracellularly within the lysosomes. The proteoglycans were shown to be taken up by the cells in a process similar to that described by other workers, i.e. receptor-mediated endocytosis via high affinity receptors (167-169). In agreement with the findings of other authors, the efficiency of endocytosis was markedly increased in the presence of an intact proteoglycan core protein, since free glycosaminoglycans were only endocytosed at much-reduced levels (Tables 7.3 and 7.4) (167-169). However, those glycosaminoglycans that were endocytosed were degraded because at the end of a 48 h incubation the total macromolecular radioactivity present in the cultures had

decreased relative to that present initially (Table 7.4). After proteoglycans had been degraded by the lysosomes, the degradation products were found associated with the medium (Tables 7.1 and 7.3 to 7.5).

The presence or absence of ascorbic acid made very little difference to the overall patterns of proteoglycan degradation by bovine aortic smooth muscle cells. This indicated that if the pericellular-matrix compartment does have any significant role to play in such degradation, by inference it would be the pericellular (i.e. cell surface) component that would be responsible, since the presence (+ ascorbic acid) or absence (- ascorbic acid) of an extensive extracellular matrix made little difference to this overall process. Ascorbic acid has been shown to influence the activity of the lysosomal system in cultured human skin fibroblasts (371). It has been shown to provide some protection to the intra-lysosomal hydrolases against the inhibitory action of lysomotrophic agents such as chloroquine. However, with bovine aortic smooth muscle cells, no such protection of the lysosomal enzymes was shown to occur in the presence of ascorbic acid (Fig. 7.7). Lysomotrophic agents such as chloroquine or ammonium chloride were effective in inhibiting the activity of the lysosomal hydrolases to a similar degree in both the presence (Fig. 7.7) and absence (results not shown) of ascorbic acid.

# CHAPTER 8

## SUMMARY AND CONCLUSIONS

The work presented in this thesis was carried out with the primary objective of the elucidation of bovine aortic medial proteoglycan biochemistry. Since the advantages of cell culture techniques for the study of metabolic processes such as biosynthesis and turnover have been well-established, the initial experimentation aimed at establishing a sound knowledge of the structure, sizes and glycosaminoglycan content of proteoglycans synthesized *in vivo*, prior to their *in vitro* study by such culture techniques. The rationale being that, having established the *in vivo* pattern of what forms and types of proteoglycans are normally found associated with bovine aortic media, cell cultures established from such tissue could then be better assessed as to their "faithfulness" in the reproduction of the *in vivo* condition during culture. Therefore, in the first instance, proteoglycans were isolated from the aortic media of different aged animals immediately after slaughter and characterized as described in Chapter 2. Secondly, a clonal line of bovine aortic medial smooth muscle cells were also characterized in relation to the proteoglycans they synthesized during culture (Chapter 4), and subsequently such metabolic processes as the biosynthesis and turnover of their proteoglycans were investigated.

Although the glycosaminoglycan contents of the major arteries have been studied and changes occurring with site of origin,

maturation or in diseased states have been documented (282-284, 292-296), this is the first time that a study has been made on the aortic proteoglycans derived from different ages of animal. Since most of the studies on intact proteoglycans have been carried out using material extracted from adult animals (287-291), virtually no information is available on the changes that take place during ageing and development.

The aortic medial layer was chosen as the source of material for two main reasons: Firstly, to minimize the variability in the proteoglycans extracted due to differences in the sites of origin, and secondly, a clonal line of smooth muscle cells which had been isolated from bovine foetal aortic media was readily available for *in vitro* studies. Thus it was important that the assessment of the true phenotypic expression of the medial smooth muscle cell cultures was carried out against the background of knowledge on bovine medial proteoglycans. A further factor in the limitation of the choice of tissue for study was the fact that the medial smooth muscle cell layer is a very important constituent of the arterial wall and it has been shown to play a prominent role in the development of pathological lesions such as that which occurs in atherosclerosis (372). Thus each time material was collected for the isolation of proteoglycans, a representative sample was analysed histologically to confirm purity of the sample.

A further criterion was employed for the comparison of *in vivo* and *in vitro* produced proteoglycans, namely the $AS_1Cl_6$

and $A_3$ lines of bovine aortic smooth muscle cells were isolated from the media of bovine foetuses which were the same size as the foetuses used for the isolation of aortic proteoglycans. The cells were shown to behave as modulated smooth muscle cells in culture, exhibiting the morphological characteristics of such cells under both light and electron microscopy (Plates 3.1 and 3.2).

The isolation of proteoglycans from the aortic media of different aged cattle revealed that changes in proteoglycan size and glycosaminoglycan composition occurred during ageing. The size of the predominant proteoglycan monomer decreased with age, as did that of the glycosaminoglycan chains. Two main sizes of proteoglycan monomer were isolated from all ages, and eluted from Sepharose CL-2B with $K_{av}$'s of 0,31 and 0,56, respectively. The larger species was predominant in foetal material, whereas, in adult material, the converse was true, and the majority of these proteoglycans eluted with a $K_{av}$ of 0,56. This change in size was associated with a decrease in the length of glycosaminoglycan chains. Thus older animals had shorter chains than younger ones. However, with all age groups, the size of the glycosaminoglycan chain was very long when compared to those found associated with cartilage proteoglycans.

The sizes of the proteoglycan monomers isolated from the culture medium and cell layers of cultured smooth muscle cells parallelled those isolated from intact foetal tissue. The proteoglycans derived from the culture medium, which made

up the bulk of the material synthesized in the system, eluted from Sepharose CL-2B columns with $K_{av}$'s identical to those of the proteoglycans from foetal medial tissue. The size of the glycosaminoglycan chains was also the same as those associated with proteoglycans extracted from intact tissue.

Neither proteoglycan monomers isolated from aortic tissue, nor from cell cultures were able to form aggregates with hyaluronic acid to any great extent. This strongly suggested that such aggregation may not be of prime importance for the "trapping" of the proteoglycans in the extracellular matrix material in aortic tissue, as is the case in cartilaginous tissues. In agreement with other workers, the aortic proteoglycans were shown to contain chondroitin sulphate and dermatan sulphate in copolymeric form (287,288,290,291). Such copolymeric chains have been shown to display a considerable degree of self-association (Section 1.4.3), and thus may have a role to play in the maintenance of the structural integrity of the extracellular matrix.

The glycosaminoglycan composition of aortic proteoglycans extracted from medial tissue showed some variation with the age of animal. Chondroitin sulphate was the predominant glycosaminoglycan in all age groups; however, the proportion of chondroitin-6-sulphate appeared to increase relative to chondroitin-4-sulphate in older animals; such changes have been documented for cartilage (268,271). Dermatan sulphate was absent in tissue from young animals but was increasingly evident with older animal tissue. Small amounts of heparan

sulphate/heparin chains were found in proteoglycans extracted from all ages of animals and amounts present also appeared to increase with age. This may suggest proliferation in cells associated with medial tissue during ageing, since the two glycosaminoglycans in question are generally associated with the cell surface (242). The glycosaminoglycan composition of proteoglycans isolated from cell cultures was similar to that of the proteoglycans isolated from foetal aortas with the one big exception that the former was shown to contain dermatan sulphate. Abnormally high amounts of dermatan sulphate associated with cultured cells as compared to tissue of origin have been found by other workers (306,310), and may reflect some particular artifactual response of cultured cells.

The existence of distinct classes of proteoglycan monomer made up of discrete types of glycosaminoglycan chains attached to specific core proteins which differ in their ability to aggregate with hyaluronic acid, has been reported by other laboratories (54,299,301,303). Such a scenario was not demonstrated with the proteoglycan monomers isolated from bovine aortic medial tissue. Proteoglycan monomers derived from adult tissue contained essentially one size of proteoglycan monomer which was shown to consist of chondroitin sulphate, dermatan sulphate, heparan sulphate/heparin and some keratan sulphate-like oligosaccharides. This finding may be a reflection of the purification procedure, and, in fact, the one species mentioned may not be homogeneous; however, some new method of purification will have to be

developed to resolve this question.

The tissue culture system was used to study the pathways of glycosaminoglycan biosynthesis and degradation and the effects of various exogenous agents on these processes. Incorporation of $|^{35}S|$ sulphate was routinely used to monitor biosynthesis or metabolism of glycosaminoglycans in these studies, and thus the presence of macromolecular $|^{35}S|$ sulphated material was taken to reflect incorporation into glycosaminoglycans, although recently the existence of sulphated glycoproteins has been reported (373).

The addition of ascorbic acid to the smooth muscle cells in culture resulted in increased cell numbers. In addition the presence of ascorbic acid promoted the deposition of a copious extracellular matrix which consisted largely of collagen. In the absence of the vitamin the amount of insoluble matrix deposited was significantly decreased and was made up largely of trypsin-sensitive material with very little collagen (Chapter 3). The addition of the vitamin to culture medium neither altered the size of the proteoglycan monomer nor of the glycosaminoglycan chains synthesized by smooth muscle cells. Also, it had no effect on the ability of the monomer to aggregate with hyaluronic acid (Chapter 4). However, ascorbic acid-supplementation was shown to increase the incorporation of $|^{35}S|$ sulphate into macromolecular material, and this was shown to be both as a result of increased synthesis and increased sulphation levels of the glycosaminoglycans. These effects were shown to occur as

a direct result of the presence of the vitamin and were not mediated by its effects on the increased synthesis and deposition of collagen into the extracellular matrix. The increased incorporation of $|^{35}S|$ sulphate into macromolecular material was most prevalent for glycosaminoglycans associated with the pericellular-matrix compartment. The possibility that the increased levels of sulphation of glycosaminoglycans associated with the afore-mentioned compartments merely reflected a role of ascorbic acid as an inhibitor of ecto-sulphatase enzymes (363) was unlikely, since no evidence for such desulphation enzymes was apparent from the data obtained on the turnover of proteoglycans by bovine smooth muscle cells (Chapter 7). It must be borne in mind that, although the more extensive collagenous matrix formed in the presence of ascorbic acid was not directly responsible for the increased incorporation of $|^{35}S|$ sulphate into macromolecules associated with the pericellular-matrix compartment, this increase may occur as an indirect result of "trapping" of proteoglycan molecules by the more extensive matrix. However, such "trapping", far from being in any way artifactual, may merely reflect the sort of *in vivo* interactions between proteoglycans and matrix elements such as collagen, elastin and fibronectin which are important for the maintenance of matrix integrity in the artery wall.

The addition of β-D-xyloside to the smooth muscle cells in culture also resulted in a marked stimulation of $|^{35}S|$ sulphate incorporation into glycosaminoglycans initiated on the exogenously supplied acceptor. The glycosaminoglycans

synthesized by initiation on β-D-xylosides differed markedly from their natural counterparts synthesized on core protein in the absence of β-D-xyloside. Their chain length was considerably shorter and their molecular weights were decreased to about a quarter of that found in the absence of β-D-xyloside (Chapter 6). Glycosaminoglycan chains initiated on β-D-xylosides were rapidly secreted into the culture medium of cells and were presumably more soluble in this medium. Furthermore, it was shown that the glycosaminoglycans synthesized on the exogenously supplied acceptor had increased levels of sulphation when compared to glycosaminoglycans from control cultures. The addition of β-D-xylosides to the medium of bovine smooth muscle cell cultures also caused a marked stimulation in the synthesis of dermatan sulphate. Glycosaminoglycan synthesis initiated on core protein in the presence of β-D-xyloside also resulted in shorter chains than controls (no β-D-xyloside) and increased levels of sulphation.

Thus it appears that agents such as ascorbic acid and β-D-xylosides which caused increased synthesis of glycosaminoglycans also have a positive effect on the sulphation processes involved in glycosaminoglycan synthesis. Furthermore, the results of these studies, which utilized the afore-mentioned agents, supported the proposition that the rates of synthesis of glycosaminoglycans can affect such properties as their length and degree of sulphation (102,360). Further supportive evidence for this proposition is evident from the observation that glycosaminoglycans synthesized on core protein in the presence of β-D-xyloside differed strikingly from those

synthesized on core protein in the absence of this exogenous acceptor, and were in fact similar to those synthesized as free glycosaminoglycan chains initiated on β-D-xylosides. If the core protein was the only determinant of structure and specificity, then protein-linked glycosaminoglycans from control or β-D-xyloside-treated cultures should have identical, or very similar, properties. In relation to these findings, it was of interest to note the effects of ascorbic acid in relation to such marked changes in glycosaminoglycan structure as chain length. In spite of the stimulation of glycosaminoglycan synthesis brought about by the inclusion of ascorbic acid in the culture medium of smooth muscle cells, there was no change in the length of glycosaminoglycan chains synthesized. This marked difference between increased glycosaminoglycan synthesis by ascorbic acid and β-D-xyloside may be explained by the much-reduced stimulation induced by the former when compared to the exogenous artifactual acceptor of glycosaminoglycan chains.

However, both agents (ascorbic acid and β-D-xylosides) resulted in increased sulphation levels of glycosaminoglycans when present in the culture medium of smooth muscle cells. This was apparent when a number of different techniques were employed to assess the sulphation levels of glycosaminoglycan chains synthesized by cultured cells in the presence and absence of these exogenous compounds. Furthermore, these agents (ascorbic acid and β-D-xylosides) had specific effects resulting in the preferential increase of incorporation of

$|^{35}S|$ sulphate into dermatan sulphate (and/or heparan sulphate/heparin). These glycosaminoglycan species contain a high content of L-iduronic acid as compared to D-glucuronic acid residues which are prevalent in most sulphated glycosaminoglycan chains (Chapter 1). Of significance is the fact that the occurrence of sulphated L-iduronic acid units has been routinely reported, whereas sulphation of D-glucuronic acid has not yet been detected (351). Hyper-sulphated regions in dermatan sulphate and heparan sulphate/heparin are thus often due to the occurrence of such sulphated L-iduronic acid units, resulting in disulphated disaccharides on digestion by chondroitinase ABC. Thus the identification of such disulphated disaccharides generated by digestion by chondroitinase ABC of glycosaminoglycans synthesized by β-D-xyloside-treated cultures, and separated by thin layer chromatography, accounted for some of the increased incorporation of $|^{35}S|$ sulphate by these cultures.

Under normal tissue culture conditions the secreted glycosaminoglycans may be undersulphated, which would account for the profiles obtained on DEAE-cellulose ion-exchange chromatography for such molecules. Although both the culture medium and the pericellular-matrix compartment contained considerable amounts of chondroitin sulphate and dermatan sulphate, these glycosaminoglycans eluted from a DEAE-cellulose column at a much lower salt concentration than did commercial chondroitin sulphate (Fig. 5.7 and 6.5), indicating that their total negative charge was lower than normal. Ascorbic acid and β-D-xyloside may cause increased sulphation at the non-sulphated

sites on glycosaminoglycan chains but the effect could be most marked for the iduronic acid-containing glycosaminoglycans, since these residues could also be sulphated in the 2' position. This would go a long way to explaining the preferential increase in the synthesis of dermatan sulphate (and/or heparan sulphate/heparin) fraction, as adjudged by radioactive sulphate incorporation, whereas in fact the synthesis of all glycosaminoglycans may be stimulated to a similar degree. However, the availability of the polymerizing enzymes for the various glycosaminoglycans would also play a role in determining what types of glycosaminoglycans would exhibit increased synthesis. This is why in cultures treated with β-D-xyloside, the relative amounts of heparan sulphate/heparin synthesized appeared to decrease; presumably the polymerizing enzymes were limiting.

The presence or absence of ascorbic acid made no difference to the endocytosis and turnover of glycosaminoglycans by the smooth muscle cells. Although the glycosaminoglycans in the pericellular-matrix compartment from ascorbic acid-supplemented cultures had increased levels of sulphation, they were still endocytosed and degraded to a similar extent as those from scorbutic cultures. This was contrary to results that suggested that sulphated glycosaminoglycans may inhibit endocytosis and that extracellular desulphation may occur before such glycosaminoglycans were endocytosed (169). The existence of ecto-desulphation was not demonstrated with bovine aortic cells, and it would appear, at least in this cell type, that prior desulphation is not an obligatory step

in the process leading to endocytosis and degradation. As has been shown previously for other cell types (94), degradation of the proteoglycan in bovine aortic cells was performed intracellularly by the lysosomal vesicles, and the small molecular weight degradation products were then secreted from the cell and could be recovered in the culture medium. The addition of lysomotrophic inhibitors was shown to completely inhibit turnover and there was no loss of macromolecular material over 24 h; concomitantly, no degradation products were found in the medium.

Thus the proteoglycans from aortic medial tissue have been characterized and the changes occurring with maturation have been documented. In addition, it was shown that cultured smooth muscle cells derived from foetal bovine aortic media largely retained their differentiated functionwith respect to the synthesis of proteoglycans. Factors effecting the pathways of synthesis, secretion and degradation of the sulphated glycosaminoglycans were examined by making use of the tissue culture system.

## REFERENCES


1. Balazs, E.A. (1970) in Chemistry and Molecular Biology of the Intercellular Matrix (ed. Balazs, E.A.), Vol. 1, p. xxx, Academic Press, New York.
2. Yanagishita, M., Rodbard, D. and Hascall, V.C. (1979) J. Biol. Chem. 254, 911-920.
3. Oldberg, A., Kjellen, L. and Höök, M. (1979) J. Biol. Chem. 254, 8505-8510.
4. Axelsson, I. and Heinegård, D. (1975) Biochem. J. 145, 491-500.
5. Muthiah, P., Stuhlsatz, H.W. and Greiling, H. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 924-934.
6. Muir, H. and Hardingham, T.E. (1975) MTP Int. Rev. Sci. Biochem. Ser. One 5, 153-220.
7. Rodén, L., Baker, J.R., Cifonelli, J.A. and Mathews, M.B. (1972) Methods Enzymol. 28, 73-117.
8. Hamerman, D., Rojkind, M. and Sandson, J. (1966) Fed. Proc. 25, 1040-1045.
9. Preston, B.N., Davies, M. and Ogston, A.G. (1965) Biochem. J. 96, 449-471.
10. Scher, I. and Hamerman, D. (1972) Biochem. J. 126, 1073-1080.
11. Stockwell, R.A. (1979) in Biology of Cartilage Cells, pp. 2-4 (Cambridge University Press).
12. Faltz, L.L., Reddi, A.H., Hascall, G.K., Martin, D., Pita, J.C. and Hascall, V.C. (1979) J. Biol. Chem. 254, 1375-1380.
13. Kimura, J.H., Osdoby, P., Caplan, A.I. and Hascall, V.C. (1978) J. Biol. Chem. 253, 4721-4729.
14. Hascall, V.C. and Sajdera, S.W. (1970) J. Biol. Chem. 245, 4920-4930.

15. Heinegård, D. and Axelsson, I. (1977) J. Biol. Chem. 252, 1971-1979.

16. De Luca, S., Caplan, A.I. and Hascall, V.C. (1978) J. Biol. Chem. 253, 4713-4720.

17. Muir, H. (1977) Annals of the Rheumatic Diseases 36, 199-208.

18. Comper, W.D. and Laurent, T.C. (1978) Physiol. Rev. 58, 255-315.

19. Hardingham, T.E. (1981) Biochem. Soc. Trans. 9, 489-497.

20. Rosenberg, L., Hellman, W. and Kleinschmidt, A.K. (1975) J. Biol. Chem. 250, 1877-1883.

21. Perkins, S.J., Miller, A., Hardingham, T.E. and Muir, H. (1981) J. Mol. Biol. 150, 69-95.

22. Heinegård, D. and Hascall, V.C. (1974) J. Biol. Chem. 249, 4250-4256.

23. De Luca, S., Lohmander, L.S., Nilsson, B., Hascall, V.C. and Caplan, A.I. (1980) J. Biol. Chem. 255, 6077-6083.

24. Lohmander, L.S., De Luca, S., Nilsson, B., Hascall, V.C., Caputo, C.B., Kimura, J.H. and Heinegård, D. (1980) J. Biol. Chem. 255, 6084-6091.

25. Kornfield, R. and Kornfield, S. (1980) in Biochemistry of Glycoproteins and Proteoglycans (ed. Lennarz, W.A.), pp.1-34, Plenum Press, New York.

26. Rodén, L. and Smith, R. (1966) J. Biol. Chem. 241, 5949-5954.

27. Rosenberg, L., Wolfenstein-Todel, C., Margolis, R., Pal, S. and Strider, W. (1976) J. Biol. Chem. 251, 6439-6444.

28. Upholt, W.B., Vertel, B.M. and Dorfman, A. (1979) Proc. Natl. Acad. Sci. USA 76, 4847-4851.

29. Treadwell, B.V., Mankin, D.P., Ho, P.K. and Mankin, H.J. (1980) Biochemistry 19, 2269-2275.

30. Hascall, V.C. and Sajdera, S.W. (1969) J. Biol. Chem. 244, 2384-2396.

31. Sajdera, S.W. and Hascall, V.C. (1969) J. Biol. Chem. 244, 77-87.

32. Hardingham, T.E., Ewins, R.J.F. and Muir, H. (1976) Biochem. J. 157, 127-143.

33. Hascall, V.C. (1977) J. Supramol. Structure 7, 101-120.

34. Hardingham, T.E. and Muir, H. (1973) Biochem. J. 135, 905-908.

35. Hascall, V.C. and Heinegård, D. (1974) J. Biol. Chem. 249, 4242-4249.

36. Hardingham, T.E., Ewins, R.J.F., Dunham, D. and Muir, H. (1981) Semin. Arthritis Rhuem. 11, No. 1, Suppl. 1, 28-29.

37. Kimura, J.H., Hardingham, T.E., Hascall, V.C. and Solursh, M. (1979) J. Biol. Chem. 254, 2600-2609.

38. Solursh, M., Hardingham, T.E., Kimura, J.H. and Hascall, V.C. (1980) Dev. Biol. 75, 121-129.

39. Christner, J.E., Brown, M.L. and Dziewiatkowski, D.D. (1979) J. Biol. Chem. 254, 4624-4630.

40. Hardingham, T.E. and Muir, H. (1972) Biochim. Biophys. Acta 279, 401-405.

41. Christner, J.E., Brown, M.L. and Dziewiatkowski, D.D. (1977) Biochem. J. 167,711-716.

42. Hardingham, T.E. (1979) Biochem. J. 177, 237-247.

43. Keiser, H., Schulman, H.J. and Sandson, J.I. (1972) Biochem. J. 126, 163-169.

44. Baker, J.H. and Caterson, B. (1979) J. Biol. Chem. 254, 2387-2393.

45. Faltz, L.L., Caputo, C.B., Kimura, J.H., Schrode, J. and Hascall, V.C. (1979) J. Biol. Chem. 254, 1381-1387.

46. Heinegård, D.K. and Hascall, V.C. (1979) J. Biol. Chem. 254, 927-934.

47. Kimura, J.H., Hardingham, T.E. and Hascall, V.C. (1980) J. Biol. Chem. 255, 7134-7143.

48. Pasternack, S.G., Veis, A. and Breen, M. (1974) J. Biol. Chem. 249, 2206-2211.

49. Ogston, A.G. (1966) Fed. Proc. 25, 986-989.

50. Ogston, A.G. and Preston, B.N. (1966) J. Biol. Chem. 241, 17-19.

51. Yanagishita, M. and Hascall, V.C. (1979) J. Biol. Chem. 254, 12355-12364.

52. Axelsson, I. and Heinegård, D.K. (1978) Biochem. J. 169, 517-530.

53. Jansson, L. and Lindahl, U. (1970) Biochem. J. 117, 699-702.

54. Salisbury, B.G.J. and Wagner, W.D. (1981) J. Biol. Chem. 256, 8050-8057.

55. Palaty, V., Gustafson, B.K. and Friedman, S.M. (1970) Canad. J. Physiol. Pharmacol. 48, 54-60.

56. Wood, G.C. (1964) Int. Rev. Conn. Tiss. Res. 2, 1-31.

57. Mathews, M.B. and Decker, L. (1968) Biochem. J. 109, 517-526.

58. Palade, G.E. (1975) Science, 189, 347-358.

59. Hassell, J.R., Newsome, D.A. and Hascall, V.C. (1979) J. Biol. Chem. 254, 12346-12354.

60. Heinegård, D. (1977) J. Biol. Chem. 252, 1980-1989.

61. Kimura, J.H., Thonar, E.J-M.A. and Hascall, V.C. (1981) J. Biol. Chem. 256, 7890-7897.

62. Pratt, R.M., Yamada, K.M., Olden, K., Ohanian, S.H. and Hascall, V.C. (1979) Expt. Cell. Res. 118, 245-252.

63. Takatsuki, A. and Tamura, G. (1971) J. Antiobiot. 24, 785-794.

64. Hart, G.W. and Lennarz, W. (1978) J. Biol. Chem. 253, 5795-5801.

65. Leloir, L.R. and Cardini, C.E. (1957) J. Amer. Chem. Soc.79, 6340

66. Telser, A., Robinson, H.C. and Dorfman, A. (1965) Proc. Natl. Acad. Sci. USA 54, 912.

67. De La Haba, G. and Holtzer, H. (1965) Science 149, 1263-1265.

68. Neufeld, E.F. and Hall, C.W. (1965) Biochem. Biophys. Res. Commun. 19, 456-461.

69. Schwartz, N.B. (1975) FEBS Lett. 49, 342-345.

70. Schwartz, N.B. and Rodén, L. (1975) J. Biol. Chem. 250, 5200-5207.

71. Perlman, R.L., Telser, A. and Dorfman, A. (1964) J. Biol. Chem. 239, 3623-3629.

72. Silbert, J.E. (1964) J. Biol. Chem. 239, 1310-1315.

73. Helting, T. and Rodén, L. (1968) Biochem.Biophys.Res.Comm.31,786.

74. Neutra, M. and Leblond, C.P. (1966) J. Cell. Biol. 30, 137-150.

75. Revel, J-P. (1970) in Chemistry and Molecular Biology of the Intercellular Matrix (ed. Balazs, E.A.), pp. 1485-1502, Academic Press, New York.

76. Freilich, L.S., Lewis, R.G., Reppuci, A.C., Jr. and Silbert, J.E. (1975) Biochem. Biophys. Res. Commun. 63, 663-668.

77. Mathews, M.B. (1975) Mol. Biol. Biochem. Biophys. 19, 1-318.

78. Stoolmiller, A.C. and Dorfman, A. (1969) J. Biol. Chem. 244, 236-246.

79. Matalon, R. and Dorfman, A. (1968) Proc. Natl. Acad. Sci. USA 60, 179-185.

80. Sugahara, K., Schwartz, N.B. and Dorfman, A. (1979) J. Biol. Chem. 254, 6252-6261.

81. Malmström, A., Fransson, L.A., Höök, M. and Lindahl, U. (1975) J. Biol. Chem. 250, 3419-3425.

82. Robbins, P.W. and Lipmann, F. (1956) J. Amer. Chem. Soc. 78, 6409.

83. Adams, J.B. (1960) Biochem. J. 76, 520-523.

84. Suzuki, S. and Strominger, J.L. (1960) J. Biol. Chem. 235, 267-276.

85. Godman, G.C. and Lane, N. (1964) J. Cell. Biol. 21, 353-366.

86. Young, R.W. (1973) J. Cell. Biol. 57, 175-189.

87. Derge, J.G. and Davidson, E.A. (1972) Biochem. J. 126, 217-223.

88. De Luca, S. and Silbert, J.E. (1968) J. Biol. Chem. 243, 2725-2729.

89. Silbert, J.E. and De Luca, S. (1969) J. Biol. Chem. 244, 876-881.

90. Seno, N., Anno, K., Yaegashi, Y. and Okuyama, T. (1975) Connect. Tissue Res. 3, 87-96.

91. Faltynek, C.R. and Silbert, J.E. (1978) J. Biol. Chem. 253, 7646-7649.

92. Lindahl, U., Backström, G., Mälmstrom, A. and Fransson, L-Å. (1972) Biochem. Biophys. Res. Commun. 46, 985-991.

93. Levy, P., Picard, J. and Bruel, A. (1981) Eur. J. Biochem. 115, 397-404.

94. Frantantoni, J.C., Hall, C.W. and Heufeld, E.F. (1968) Proc. Natl. Acad. Sci. USA 60, 699.

95. Revel, J-P. and Hay, E.D. (1963) Z. Zellforsch. Mikrosk. Anat. 61, 110-114.

96. Hascall, G.K. (1980) Anat. Rec. 198, 135-146.

97. Helting, T. and Rodén, L. (1968) Biochim. Biophys. Acta 70, 301-308.

98. Okayama, M., Kimata, K. and Suzuki, S. (1973) J. Biochem. 74, 1069-1073.

99. Levitt, D. and Dorfman, A. (1973) Proc. Natl. Acad. Sci. USA 70, 2201-2205.

100. Schwartz, N.B., Galligani, L., Ho, P-L. and Dorfman, A. (1974) Proc. Natl. Acad. Sci. USA 71, 4047-4051.

101. Schwartz, N.B., Ho, P-L. and Dorfman, A. (1976) Biochem. Biophys. Res. Commun. 71, 851-856.

102. Robinson, H.G., Brett, M.J., Tralaggan, P.J., Lowther, D.A. and Okayama, M. (1975) Biochem. J. 148, 25-34.

103. Galligani, L., Hopwood, J., Schwartz, N.B. and Dorfman, A. (1975) J. Biol. Chem. 250, 5400-5406.

104. Mitchell, D. and Hardingham, T.E. (1981) Biochem. J. 196, 521-529.

105. Dietrich, C., Armelin, H.A., Nogueira, Y.L., Nader, H.B. and Michelacei, Y.M. (1982) Biochim. Biophys. Acta 717, 387-397.

106. Hardingham, T.E. and Muir, H. (1970) FEBS Lett. 9, 145-148.

107. Stevens, R.L. and Austen, K.F. (1982) J. Biol. Chem. 257, 253-259.

108. Prockop, D.J., Berg, R.A., Kivirikko, K.I. and Uitto, J. (1976) in Biochemistry of Collagen (ed. Ramachandran, G.N. and Reddi, A.H.) pp. 163-273, Plenum Publishing Corp., New York.

109. Fessler, J.H. and Fessler, L.I. (1978) Ann. Rev. Biochem. 47, 129-162.

110. Chatterjee, I.B. (1978) World Rev. Nutr. Diet. 30, 69-87.

111. Peterkofsky, B. (1972) Arch. Biochem. Biophys. 152, 318-328.

112. Schwartz, R.I. and Bissel, H.J. (1977) Proc. Natl. Acad. Sci. USA 74, 4453-4457.

113. Jeffrey, J.J. and Martin, G.R. (1966) Biochim. Biophys. Acta 121, 269-280.

114. Boxer, L.A., Allen, J.M., Watanabe, A.M., Besch, H.R. and Baehner, R.L. (1978) Blood 51, 1045-1050.

115. Verlangieri, A.J. and Mumma, R.O. (1973) Atherosclerosis 17, 37-43.

116. Ford, E.A. and Ruoff, P.M. (1965) Chem. Commun. 24, 630-631.

117. Hatanaka, H. and Egami, F. (1976) J. Biochem. (Tokyo) 80, 1215-1221.

118. Shapiro, S.S. and Poon, J.P. (1975) Biochim. Biophys. Acta 385, 221-231.

119. Berg, R.A. and Prockop, D.J. (1973) Biochem. Biophys. Res. Commun. 52, 115-120.

120. Eyre, D.R. (1980) Science 207, 1315-1322.

121. Tartakoff, A.H. and Vasalli, P. (1977) J. Exp. Med. 146, 1332-1345.

122. Tartakoff, A.M. and Vasalli, P. (1978) J. Cell. Biol. 79, 694-707.

123. Uchida, N., Smilowitz, H. and Tanzer, M.L. (1979) Proc. Natl. Acad. Sci. USA 76, 1868-1872.

124. Ledger, P.W., Uchida, N. and Tanzer, M.L. (1980) J. Cell. Biol. 87, 663-671.

125. Nishimoto, S.K., Kajiwara, T., Ledger, P.W. and Tanzer, J.L. (1982) J. Biol. Chem. 257, 11712-11716.

126. Somlyo, A.P., Garfield, R.E., Chacko, S. and Somlyo, A.V. (1975) J. Cell. Biol. 66, 425-443.

127. Kajiwara, T. and Tanzer, M.L. (1981) FEBS Lett. 134, 43-46.

128. Tajiri, K., Uchida, N. and Tanzer, M.L. (1980) J. Biol. Chem. 255, 6036-6039.

129. Orkin, R.W., Pratt, R.M. and Martin, G.R. (1976) Dev. Biol. 50, 82-94.

130. Sugahara, K. and Schwartz, N.B. (1979) Proc. Natl. Acad. Sci. USA 76, 6615-6618.

131. Kato, Y., Kimata, K., Ito, K., Karasawa, K. and Suzuki, S. (1978) J. Biol. Chem. 253, 2784-2789.

132. Lacy, P.E., Howell, S.L., Young, D.A. and Fink, J. (1968) Nature (London) 219, 1170-1179.

133. Dehm, P.E. and Prockop, D.J. (1972) Biochim. Biophys. Acta 264, 375-382.

134. Diegelmann, R.F. and Peterkofsky, B. (1972) Proc. Natl. Acad. Sci. USA 69, 892-896.

135. Ehrlich, H.P., Ross, R. and Barnstein, P. (1974) J. Cell. Biol. 62, 390-405.

136. Olsen, B.R. and Prockop, D.J. (1974) Proc. Natl. Acad. Sci. USA 71, 2033-2037.

137. Moskalewski, S., Thyberg, J., Lohmander, S. and Friberg, U. (1975) Exp. Cell Res. 95, 440-454.

138. Lohmander, S., Moskalewski, S., Madsen, K., Thyberg, J. and Friberg, U. (1976) Exp. Cell Res. 99, 333-345.

139. Lohmander, S., Madsen, K. and Hinek, A. (1979) Arch. Biochem. Biophys. 192, 148-157.

140. Berl, S., Puszkin, S. and Nicklas, W.J. (1973) Science 179, 441-446.

141. Ostlund, R.E., Leung, J.T. and Kipnis, D.M. (1977) J. Cell. Biol. 73, 78-87.

142. V. Obbhergen, E., De Meyts, P. and Roth, J. (1976) J. Biol. Chem. 251, 6844-6851.

143. Albertini, D.F. and Anderson, E. (1977) J. Cell. Biol. 73, 111-127.

144. Lenk, R., Ransom, L., Kaufman, Y. and Penman, S. (1977) Cell 10, 67-78.

145. Pollard, T.D. (1976) J. Supramolec. Struct. 5, 317(269)-334(286)

146. Boström, H. (1952) J. Biol. Chem. 196, 477-481.

147. Hardingham, T.E. and Muir, H. (1972) Biochem. J. 126, 791-803.

148. Handley, C.J. and Phelps, C.F. (1972) Biochem. J. 126, 417-432.

149. Handley, C.J. and Phelps, C.F. (1972) Biochem. J. 128, 205-213.

150. Lohmander, S., Antonopoulos, C.A. and Friberg, U. (1973) Biochim. Biophys. Acta 304, 430-448.

151. Kleine, T.O. (1974) FEBS Lett. 39, 255-258.

152. Olssen, I., Gardell, S. and Thunell, S. (1968) Biochim. Biophys. Acta 165, 309-323.

153. Kresse, H., von Figura, K., Buddecke, E. and Fromme, H.G. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 929-941.

154. Antonopoulos, C.A., Engfeldt, B., Gardell, S. and Heinegård, D. (1972) Scand. J. Clin. Lab. Invest. 29, Suppl. 123, 4.

155. Serafini-Fracassini, A. and Stimson, W.H. (1972) FEBS Lett. 26, 336-340.

156. Ali, S.Y. (1964) Biochem. J. 93, 611-618.

157. Jibril, A.O. (1967) Biochim. Biophys. Acta 136, 162-165.

158. Arboyast, B., Hopwood, J.J. and Dorfman, A. (1975) Biochem. Biophys. Res. Commun. 67, 376-382.

159. Morrison, R.I.G. (1970) in Chemistry and Molecular Biology of the Intercellular Matrix (ed. Balazs, E.A.) Vol. 3, 1683, Academic Press, London and New York.

160. Sandy, J.D., Brown, H.L.G. and Lowther, D.A. (1978) Biochim. Biophys. Acta 543, 536-544.

161. Wasteson, A., Lindahl, U. and Hallen, A. (1972) Biochem. J. 130, 729-738.

162. Sapolsky, A.I., Keiser, H., Howell, D.S. and Woessner, J.F. Jr. (1976) J. Clin. Invest. 58, 1030-1041.

163. Dingle, J.T., Barrett, A.J. and Weston, P.D. (1971) Biochem. J. 123, 1-13.

164. Morrison, R.I.G., Barrett, A.J., Dingle, J.T. and Prior, D. (1973) Biochim. Biophys. Acta 302, 411-419.

165. Sapolsky, A.I., Howell, D.S. and Woessner, J.F., Jr. (1974) J. Clin. Invest. 53, 1044-1053.

166. Sapolsky, A.I., Altman, R.D., Woessner, J.F., Jr. and Howell, D.S. (1973) J. Clin. Invest. 52, 624-633.

167. Kresse, H., Tekolf, W., von Figura, K. and Buddecke, E. (1975) Hoppe-Seyler's Z. Physiol. Chem. (1975) 356, 943-952.

168. Truppe, W. and Kresse, H. (1978) Eur. J. Biochem. 85, 351-356.

169. Prinz, R., Schwermann, J., Buddecke, E. and von Figura, K. (1978) Biochem. J. 176, 671-676.

170. Anderson, R.G.W., Goldstein, J.L. and Brown, M.S. (1977) Nature (London) 270, 695-699.

171. Prinz, R., Schwermann, J., Buddecke, E. and von Figura, K. (1977) Abstracts of Symposium on Biology of Connective Tissue, Uppsala, p. 150.

172. Diehl, T., Scott-Burden, T. and Gevers, W. (1983) Biochem. Int. 6, 29-41.

173. Hickman, S. and Neufeld, E.F. (1972) Biochem. Biophys. Res. Commun. 49, 992-999.

174. Avila, J.L. and Convit, J. (1976) Biochem. J. 160, 129-136.

175. Robinson, D. and Stirling, J.L. (1968) Biochem. J. 107, 321-327.

176. Kint, J.A., Dacremont, G., Carton, D., Oyre, E. and Hooft, C. (1973) Science 181, 352-354.

177. Avila, J.L. and Convit, J. (1975) Biochem. J, 152, 57-64.

178. Buermann, C.W., Oronsky, A.L. and Horowitz, M.I. (1979) Arch. Biochem. Biophys. 193, 277-283.

179. von Helde, E. and Buddecke, E. (1967) Hoppe-Seyler's Z. Physiol. Chem. 348, 1047-1060.

180. Mathews, M.B. (1965) Biochem. J. 96, 710-716.

181. Öbrink, B. and Wasteson, Å. (1971) Biochem. J. 121, 227-233.

182. Toole, B.P. and Lowther, D.A. (1968) Biochem. J. 109, 857-866.

183. Lowther, D.A., Toole, B.P. and Herrington, A.C. (1970) in Chemistry and Molecular Biology of the Intercellular Matrix (ed. Balazs, E.A.) pp. 1135-1153, Academic Press, London.

184. Öbrink, B. (1975) in Structure of Fibrous Biopolymers, (ed. Atkins, E.D.T. and Keller, A.), pp. 81-92, London, Butterworths.

185. Conochie, L.B., Scott, J.E. and Faulk, W.P. (1975) J. Immunol. Meth. 7, 393-398.

186. Öbrink, B. (1973) Eur. J. Biochem. 33, 387-400.

187. Radhakrishnamurthy, B., Ruiz, J.A., Jr. and Berenson, G.S. (1977) J. Biol. Chem. 252, 4831-4841.

188. Eisenstein, R., Larsson, S-E., Sorgente, N. and Kuettner, K.E. (1973) Amer. J. Pathol. 2, 443-452.

189. Öbrink, B. and Sundelöf, L-O. (1973) Eur. J. Biochem. 37, 226-232

190. Öbrink, B., Laurent, T.C. and Carlsson, B. (1975) FEBS Lett. 56, 166-169.

191. Greenwald, R.A., Schwartz, C.E. and Cantor, J.O. (1975) Biochem. J. 145, 601-605.

192. Oegema, T.R., Laidlaw, J., Hascall, V.C. and Dziewiatkowski, D.D. (1975) Arch. Biochem. Biophys. 170, 698-709.

193. Toole, B.P. (1976) J. Biol. Chem. 251, 895-897.

194. Toole, B.P. and Lowther, D.A. (1968) Arch. Biochem. Biophys. 128, 567-578.

195. Lee-Own, V. and Anderson, J.C. (1975) Biochem. J. 149, 57-63.

196. Podrazký, V., Steven, F.S., Jackson, D.S., Weiss, J.B. and Leibovich, S.J. (1971) Biochim. Biophys. Acta 229, 690-697.

197. Steven, F.S., Knott, J., Jackson, D.S. and Podrazký, V. (1969) Biochim. Biophys. Acta 188, 307-313.

198. Podrazký, V. and Adam, M. (1975) Experimentia 31, 523-524.

199. Adam, M. and Podrazký, V. (1976) Experimentia 32, 430-432.

200. Partridge, S.M., Elsden, D.F., Thomas, J., Dorfman, A., Telser, A. and Ho, P-L. (1964) Biochem. J. 93, 30c-33c.

201. Miller, E.J., Martin, G.R. and Piez, K.A. (1964) Biochem. Biophys. Res. Commun. 17, 248.

202. Podrazký, V., Štokrová, S. and Frič, I. (1975) Connect. Tissue Res. 4, 51-54.

203. Yamada, K.M., Kennedy, D.W., Kimata, K. and Pratt, R.M. (1980) J. Biol. Chem. 255, 6055-6063.

204. Jilek, F. and Hörmann, H. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 597-603.

205. Ruoslahti, E. and Engvall, E. (1980) Biochim. Biophys. Acta 631, 350-358.

206. Johansson, S. and Höök, M. (1980) Biochem. J. 187, 521-524.

207. Perkins, M.E., Ji, T.H. and Hynes, R.O. (1979) Cell 16, 941-952.

208. Hayashi, M., Schlesinger, D.H., Kennedy, D.W. and Yamada, K.M. (1980) J. Biol. Chem. 255, 10017-10020.

209. Buonassisi, V. (1973) Exp. Cell. Res. 76, 363-368.

210. Buonassisi, V. and Root, M. (1975) Biochim. Biophys. Acta 385, 1-10.

211. Andersson, L-O., Engman, L. and Henningsson, E. (1977) J. Immunol. Meth. 14, 271-281.

212. Marciniak, E. (1974) J. Lab. Clin. Med. 84, 344-356.

213. Barber, A.J., Käser-Glanzmann, R., Jakábová, M. and Lüscher, E.F. (1972) Biochim. Biophys. Acta 286, 312-329.

214. Handin, R.I. and Cohen, H.J. (1976) J. Biol. Chem. 251, 4273-4282.

215. Olivecrona, T., Bengtsson, G., Marklund, S-E., Lindahl, U. and Höök, M. (1977) Fed. Proc. 36, 60-65.

216. Robinson, D.S. (1970) Compr. Biochem. 18, 51-116.

217. Cryer, A., Davies, P. and Robinson, D.S. (1975) in Blood and Arterial Wall in Atherogenesis and Arterial Thrombosis (ed. Hautvast, J.G.A.J., Hormus, R.J.J. and van den Haar, F.) pp. 102-110, Leiden: Brill.

218. Fielding, P.E., Shore, V.G. and Fielding, C.J. (1974) Biochemistry 13, 4318-4323.

219. Ehnholm, C., Shaw, W., Greten, H. and Brown, W.V. (1975) J. Biol. Chem. 250, 6756-6761.

220. Hernell, O., Egelrud, T. and Olivecrona, T. (1975) Biochim. Biophys. Acta 381, 233-241.

221. Bengtsson, G., Olivecrona, T., Höök, M. and Lindahl, U. (1977) FEBS Lett. 79, 59-63.

222. Danishefsky, I. (1974) Adv. Exp. Med. Biol. 52, 105-118.

223. Waite, M. and Sisson, P. (1973) J. Biol. Chem. 248, 7201-7206.

224. Iverius, P-H. (1972) J. Biol. Chem. 247, 2607-2613.

225. Srinivasan, S.R., Lopez, S.A., Radhakrishnamurthy, B. and Berenson, G.S. (1970) Atheroscelrosis 12, 321-334.

226. Srinivasan, S.R., Radhakrishnamurthy, B. and Berenson, G.S. (1975) Arch. Biochem. Biophys. 170, 334-340.

227. Nakashima, Y., Di Ferrante, N., Jackson, R.L. and Pownall, H.J. (1975) J. Biol. Chem. 250, 5386-5392.

228. Vijayagopal, P., Srinivasan, S.R., Radhakrishnamurthy, B. and Berenson, G.S. (1981) J. Biol. Chem. 256, 8234-8241.

229. Brown, M.S., Faust, J.R. and Goldstein, J.L. (1975) J. Clin. Invest. 55, 783-793.

230. Goldstein, J.L., Basu, S.K., Brunschede, G.Y. and Brown, M.S. (1976) Cell 7, 85-95.

231. Iverius, P-H. (1973) Ciba Found. Symp. 185, 185-196.

232. Ross, R. and Harker, L. (1976) Science 193, 1094-1100.

233. Kumar, V., Berenson, G.S., Ruiz, H., Dalferes, E.R. and Strong, J.P. (1967) J. Atheroscler. Res. 7. 583-590.

234. Jeanes, A., Pittsley, J.E. and Senti, F.R. (1961) J. Appl. Polym. Sci. 17, 519.

235. Rees, D. (1972) Biochem. J. 126, 257-273.

236. Atkins, E.D.T. and Sheehan, J.K. (1973) Science 179, 562-564.

237. Dea, I.C.M., Moorhouse, R., Rees, D.A., Arnott, S., Guss, J.M. and Balazs, E.A. (1973) Science 179, 560-562.

238. Guss, J.M., Hukins, D.W.L., Smith, P.J.C., Winter, W.T., Arnott, S., Moorhouse, R. and Rees, D.A. (1975) J. Mol. Biol. 95, 359-384.

239. Fransson, L-Å. (1976) Biochim. Biophys. Acta 437, 106-115.

240. Coster, L. and Fransson, L-Å. (1972) Scand. J. Clin. Lab. Invest. 29, Suppl. 123, 9.

241. Malmström, A., Carlstedt, I., Aberg, L. and Fransson, L-Å. (1975) Biochem. J. 143, 369-378.

242. Kraemer, P.M. (1971) Biochemistry 10, 1437-1445.

243. Habuchi, H., Yamagata, T., Iwata, H. and Suzuki, S. (1973) J. Biol. Chem. 248, 6019-6028.

244. Oldberg, Å., Höök, M., Öbrink, B., Pertoft, H. and Rubin, K. (1977) Biochem. J. 164, 75-81.

245. Underhill, C.B. and Toole, B.P. (1979) J. Cell. Biol. 82, 475-484.

246. Cohn, R.H., Cassiman, J.J. and Bernfield, M.R. (1976) J. Cell. Biol. 71, 280-294.

247. Roblin, R., Albert, S.O., Gelb, N.A. and Black, P.H. (1979) Biochemistry 14, 347-357.

248. Ohnishi, T., Oshima, E. and Ohtsuka, M. (1975) Exp. Cell. Res. 93, 136-142.

249. Vogel, K.G. and Dolde, J. (1979) Biochim. Biophys. Acta 552, 194-200.

250. Kjellen, L., Oldberg, A., Ruben, K. and Höök, M. (1977) Biochem. Biophys. Res. Commun. 74, 126-133.

251. Kjellen, L., Oldberg, A. and Höök, M. (1980) J. Biol. Chem. 255, 10407-10413.

252. Kjellen, L., Petersson, I. and Höök, M. (1981) Proc. Natl. Acad. Sci. USA 78, 5371-5375.

253. Johnston, L.S. and Keller, J.M. (1979) J. Biol. Chem. 254, 2575-2578.

254. Toole, B.P. and Gross, J. (1971) Devel. Biol. 25, 57-77.

255. Toole, B.P. and Trelstad, R.L. (1971) Devel. Biol. 26, 28-35.

256. Toole, B.P., Jackson, G. and Gross, J. (1972) Proc. Natl. Acad. Sci. USA 69, 1384-1386.

257. Wiebkin, O.W. and Muir, H. (1973) FEBS Lett. 37, 42-46.

258. Handley, C. and Lowther, D.A. (1976) Biochem. Biophys. Acta 444, 69-74.

259. Underhill, C.B. and Toole, B.P. (1980) J. Biol. Chem. 255, 4544-4549.

260. Moscona, A.A. (1975) in Extracellular Matrix Influences on Gene Expression (ed. Slavkin, H.C. and Greulich, R.C.) pp. 833, New York, Academic Press.

261. Öbrink, B., Kuhlenschmidt, M.S. and Roseman, S. (1977) Proc. Natl. Acad. Sci. USA 74, 1077-1081.

262. Pessac, B. and Defendi, V. (1972) Science 175, 898-900.

263. Wasteson, Å., Westermark, B., Lindahl, U. and Pontén, J. (1973) Int. J. Cancer 12, 169-178.

264. Inerot, S., Heinegård, D., Audell, L. and Olsson, S-E. (1978) Biochem. J. 169, 143-156.

265. Mourão, P.A.S., Michelacci, Y.M. and Toledo, O.M.S. (1979) Cancer Res. 39, 2802-2806.

266. Honda, A., Abe, M., Murata, S. and Mori, Y. (1979) J. Biochem. 85, 519-528.

267. Sweet, M.B.E., Thonar, E.J-M.A. and Marsh, J. (1979) Arch. Biochem. Biophys. 198, 439-448.

268. Roughley, P.J. and White, R.J. (1980) J. Biol. Chem. 255, 217-224.

269. Pacifi, M., Fellini, S.A., Holtzer, H. and De Luca, S. (1981) J. Biol. Chem. 256, 1029-1037.

270. Triphaus, G.F., Schmidt, A. and Buddecke, E. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 1773-1779.

271. Mourão, P.A.S., Rozenfeld, S., Laredo, J. and Dietrich, C.P. (1976) Biochim. Biophys. Act 428, 19-26.

272. Toledo, O.M.S. and Mourão, P.A.S. (1979) Biochem. Biophys. Res. Commun. 89, 50-55.

273. Thonar, E.J-M.A., Sweet, M.B.E., Immelman, A.R. and Lyons, G. (1978) Calcif. Tiss. Res. 26, 19-21.

274. Vijayagopal, P., Radhakrishnamurthy, B., Srinivasan, S.R. and Berenson, G.S. (1980) Lab. Invest. 42, 190-196.

275. Castellot, J.J., Jr., Addonizio, M.L., Rosenberg, R. and Karnovsky, M.J. (1981) J. Cell. Biol. 90, 372-379.

276. Toole, B.P. (1973) Am. Zool. 13, 1061-1065.

277. Rollins, B.J. and Culp, L.A. (1979) Biochemistry 18, 5621-5629.

278. Hascall, V.C. and Hascall, G.K. (1981) in Cell Biology of Extracellular Matrix (ed. Hay, E.D.) pp. 39-63, Plenum Press, New York.
279. Eisenstein, R. and Kuettner, K. (1976) Atherosclerosis 27, 37-46.
280. Wight, T.N. and Ross, R. (1975) J. Cell. Biol. 67, 660-674.
281. Wight, T.N. and Hascall, V.C. (1983) J. Cell Biol. 96, 167-176.
282. Berenson, G.S., Radhakrishnamurthy, B., Dalferes, E.R., Jr. and Srinivasan, S.R. (1971) Human Pathol. 2, 57-79.
283. Buddecke, E. (1962) J. Atheroscler. Res. 2, 32-46.
284. Kaplan, D. and Meyer, K. (1960) Proc. Soc. Exp. Biol. Med. 105, 78-81.
285. Wagner, W.D., Connor, J.R. and Muldoon, E. (1982) Biochim. Biophys. Acta 717, 132-142.
286. Bach, P.R. and Bentley, J.P. (1980) Connect. Tiss. Res. 7, 185-196.
287. Kresse, H., Heidel, H. and Buddecke, E. (1971) Eur. J. Biochem. 22, 557-562.
288. Antonopoulos, C.A., Axelsson, I., Heinegård, D. and Gardell, S. (1974) Biochim. Biophys. Acta 338, 108-119.
289. Eisenstein, R., Larsson, S-E., Kuettner, K., Sorgente, N. and Hascall, V.C. (1975) Atherosclerosis 22, 1-17.
290. Ehrlich, K.C., Radhakrishnamurthy, B. and Berenson, G.S. (1975) Arch. Biochem. Biophys. 171, 361-369.
291. Oegema, T.R., Hascall, V.C. and Eisenstein, R. (1979) J. Biol. Chem. 254, 1312-1318.
292. Toledo, O.M.S. and Mourão, P.A.S. (1980) Artery 6, 341-353.
293. Stuhlsatz, H.W., Löffler, H., Mohanaradhakrishnan, V., Cosma, S. and Greiling, H. (1982) J. Clin. Chem. Clin. Biochem. 20, 713-721.

294. Kumar, V., Berenson, G.S., Ruiz, H., Dalferes, E.R., Jr. and Strong, J.P. (1967) J. Atheroscler. Res. 7, 573-581.

295. Massaro, T.A. and Glatz, C.E. (1979) Artery 5, 1-13.

296. Berenson, G.S., Srinivasan, S.R., Dolan, P.J. and Radhakrishnamurthy, B. (1971) Circulation 44, Suppl. 11, 6.

297. Kapoor, R., Phelps, C.F., Cöster, L. and Fransson, L-Å.(1981) Biochem. J. 197, 259-268.

298. McMurtrey, J., Radhakrishnamurthy, B., Dalferes, E.R., Jr., Berenson, G.S. and Gregory, J.D. (1979) J. Biol. Chem. 254, 1621-1626.

299. Breton, M., Picard, J. and Berrou, E. (1981) Biochimie 63, 515-525.

300. Buddecke, E. (1963) Hoppe-Seyler's Z. Physiol. Chem. 331, 196-218.

301. Wagner, W.D., Salisbury, B.G.J. and Connor, J.R. (1981) Fed. Proc. 40, 1841.

302. Deudon, E., Breton, M., Berrou, E. and Picard, J. (1980) Biochimie 62, 811-821.

303. Schmidt, A., Prager, M., Selmke, P. and Buddecke, E. (1982) Eur. J. Biochem. 125, 95-101.

304. Wagner, W.D., Connor, J.R. and Muldoon, E. (1982) Biochim. Biophys. Acta 717, 132-142.

305. Horn, M.C., Breton, M., Deudon, E., Berrou, E. and Picard, J. (1983) Biochim. Biophys. Acta 755, 95-105.

306. Gamse, G., Fromme, H.G. and Kresse, H. (1978) Biochim. Biophys. Acta 544, 514-528.

307. Gardell, S., Baker, J., Caterson, B., Heinegård, D. and Rodén, L. (1980) Biochem. Biophys. Res. Commun. 95, 1823-1831.

308. Baker, J. and Caterson, B. (1977) Biochem. Biophys. Res. Commun. 77, 1-10.

309. Mangkornkanok-Mark, M., Eisenstein, R. and Bahu, R.M. (1981) J. Histochem. Cytochem. 29, 547-552.

310. Wight, T.N. and Ross, R. (1975) J. Cell Biol. 67, 675-686.

311. Hermelin, B., Picard, J., Paul-Gardais, A. and Levy, P. (1974) Comp. Biochem. Physiol. 49B, 113.

312. Vijayagopal, P., Radhakrishnamurthy, B., Srinivasan, S.R., McMurtrey, J. and Berenson, G.S. (1980) Artery 6, 458-470.

313. Kresse, H. and Buddecke, E. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 151-156.

314. Deudon, E. and Picard, J. (1978) Int. J. Biochem. 9, 19-26.

315. von Figura, K., Kiowski, W. and Buddecke, E. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 1517-1525.

316. Gornall, A.G., Bardawill, C.S. and David, M.M. (1949) J. Biol. Chem. 177, 751-766.

317. Bitter, T. and Muir, H. (1962) Anal. Biochem. 4, 330-334.

318. Thonar, E.J-M.A. and Sweet, M.B.E. (1979) Biochim. Biophys. Acta 584, 353-357.

319. Thonar, E.J-M.A., Sweet, M.B.E., Immelman, A.R. and Lyons, G. (1979) Arch. Biochem. Biophys. 194, 179-189.

320. Lindahl, U., Backström, G., Jansson, L. and Hallen, A. (1973) J. Biol. Chem. 248, 7234-7241.

321. Dorfman, A. (1955) Methods in Enzymol. (ed. Colowick, S.P. and Kaplan, N.O.) 1, p. 166, Academic Press Inc., New York.

322. Saito, H., Yamagata, T. and Suzuki, S. (1968) J. Biol. Chem. 243, 1536-1542.

323. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

324. Jourdian, G.W., Dean, L. and Roseman, S. (1971) J. Biol. Chem. 246, 430-435.

325. Oegema, T.R., Jr., Hascall, V.C. and Dziewiatkowski, D.D. (1975) J. Biol. Chem. 250, 6151-6159.

326. Sweet, M.B.E., Personal Communication.

327. Wasteson, Å. (1971) J. Chromatog. 59, 87-97.

328. Ross, R.J. (1971) J. Cell Biol. 50, 172-186.

329. Jones, P.A. and Scott-Burden, T. (1979) Biochem. Biophys. Res. Commun. 86, 71-77.

330. Jones, P.A., Scott-Burden, T. and Gevers, W. (1979) Proc. Natl. Acad. Sci. USA 76, 353-357.

331. Kim, J.J. and Conrad, H.E. (1980) J. Biol. Chem. 255, 1586-1597.

332. Gamse, G., Fromme, H.G. and Kresse, H. (1978) Biochim. Biophys. Acta 544, 514-528.

333. Bierman, E.L., Stein, O. and Stein, Y. (1974) Circulation Res. 35, 136-150.

334. Chamley-Champbell, J., Campbell, G.R. and Ross, R. (1979) Physiol. Rev. 59, 1-61.

335. Schwartz, E., Bienkowski, R.S., Coltoff-Schiller, B., Goldfischer, S. and Blumenfeld, O.O. (1982) J. Cell Biol. 92, 462-470.

336. Scott-Burden, T., Davies, P.J. and Gevers, W. (1979) Biochem. Biophys. Res. Commun. 91, 739-746.

337. Carrino, D.A. and Caplan, A.I. (1982) J. Biol. Chem. 257, 14145-14154.

338. Cardinale, G.J. and Udenfriend, S. (1974) Adv. Enzymol. 41, 245-300.

339. Ramachandran, G.N. and Ramakrishnan, C. (1976) in Biochemistry of Collagen (ed. Ramachandran, G.N. and Reddi, A.H.) pp. 45-84, Plenum Publishing Corp., New York.

340. Jimenez, S., Harsch, M. and Rosenbloom, J. (1973) Biochem. Biophys. Res. Commun. 52, 106-114.

341. Murad, S., Grove, D., Windberg, K.A., Reynolds, G., Swarajah, A. and Pinnel, S.R. (1981) Proc. Natl. Acad. Sci. USA 78, 2879-2882.

342. Homig, D., Weber, F. and Wiss, O. (1974) Z. Klin, Chem. Klin. Biochem. 2, 62-65.

343. Gregory, J.D. and Lipmann, F. (1957) J. Biol. Chem. 229, 1081-1087.

344. Boxer, L.A., Albertini, D.F., Baehner, R.L. and Oliver, J.M. (1979) British. J. Haematol. 43, 207-213.

345. Adams, E. and Frank, L. (1980) Ann. Rev. Biochem. 49, 1005-1061.

346. Kivirikko, K.I. and Myllylä, L. (1980) in The Enzymology of Post-translational Modification of Proteins(ed. Friedman, R.B. and Hawkins, H.C.) pp. 53-104, Academic Press, New York.

347. Prockop, D.J., Kivirikko, K.I., Tuderman, L. and Guzman, N.A. (1979) N. Engl. J. Med. 301, 13, 77.

348. Fessler, L.I. and Fessler, J.H. (1974) J. Biol. Chem. 249, 7637-7646.

349. Kivirikko, K.I. and Prockop, D.J. (1967) J. Biol. Chem. 242, 4007-4012.

350. Höök, M., Lindahl, U., Hallen, A. and Backström, G. (1975) J. Biol. Chem. 250, 6065-6071.

351. Lindahl, U. and Höök, M. (1978) Ann. Rev. Biochem. 47, 385-417.

352. Keinman, H.K., Klebe, R.J. and Martin, G.R. (1981) J. Cell Biol. 88, 473-485.

353. Robinson, H.C., Telser, A. and Dorfman, A. (1966) Proc. Natl. Acad. Sci. USA 56, 1859-1866.

354. Telser, A., Robinson, H.C. and Dorfman, A. (1966) Arch. Biochem. Biophys. 116, 458-465.

355. Helting, T. and Rodén, L. (1969) J. Biol. Chem. 244, 2790-2798.

356. Helting, T. and Rodén, L. (1969) J. Biol. Chem. 244, 2799-2805.

357. Gibson, K.D., Segen, B.J. and Audhya, T.K. (1977) Biochem. J. 162, 217-233.

358. Gibson, K.D. and Segen, B.J. (1977) Biochem. J. 168, 65-79.

359. Lohmander, L.S., Hascall, V.C. and Caplan, A.I. (1979) J. Biol. Chem. 254, 10551-10561.

360. Lohmander, S., Madsen, K. and Hinek, A. (1979) Arch. Biochem. Biophys. 192, 148-157.

361. Schwartz, N.B. (1979) J. Biol. Chem. 254, 2271-2277.

362. Sudhakaran, P.R., Sinn, W. and von Figura, K. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 39-46.

363. Scott-Burden, T., Murray, E., Diehl, T. and Gevers, W. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 61-70.

364. Churms, S.C. in CRC Handbook of Chromatography. Carbohydrates I (ed. Zweig, G. and Sherma, J.) CRC Press, Boca, Raton, Florida.

365. Richmond, M.E., De Luca, S. and Silbert, J.E. (1973) Biochemistry 12, 3904-3910.

366. Vogel, K.G. and Kendall, V.F. (1980) J. Cellular Physiol. 103, 475-487.

367. Ohkuma, S. and Poole, B. (1978) Proc. Natl. Acad. Sci. USA 75, 3327-3331.

368. Seglen, P.O. and Reith, A. (1976) Exp. Cell. Res. 100, 276-280.

369. Carpenter, G. and Cohen, S. (1976) J. Cell Biol. 71, 159-171.

370. Hoeg, J.M., Osborne, J.C., Jr. and Brewer, H.B., Jr. (1982) J. Biol. Chem. 257, 2125-2128.

371. Coetzee, G.A., Stein, O. and Stein, Y. (1979) Atherosclerosis 31, 277-287.

372. Benditt, E.P. (1977) Scientific American 236 (2), 74-85.

373. Heifetz, A., Watson, C., Johnson, A.B. and Roberts, H.K. (1982) J. Biol. Chem. 257, 13581-13586.

## APPENDIX

Chemicals, Materials, Enzymes and Culture ware were obtained from the following suppliers:

| | |
|---|---|
| BDH Chemicals, Poole, England | : Ethylenediamine tetraacetic acid<br>Sodium dodecyl sulphate<br>Di-sodium tetraborate |
| Beckman Instruments, Fullerton, USA | : Polyallomer centrifuge tubes |
| Biorad Laboratories, Richmond, California, USA | : Bio-Gel P-2 (100-200 mesh)<br>Bio-Gel P-30 (100-200 mesh) |
| Corning Ltd., Staffordshire, England | : Tissue culture plastic ware |
| Coulter Electronics Inc., Hialeah, Florida, USA | : Isoton II |
| Difco Laboratories, Detroit, Michigan, USA | : Trypsin (1:250), tryptose phosphate broth |
| Gibco Laboratories, New York, USA | : Eagle's Minimum Essential Medium buffered with Earle's salts |
| Glaxo (Pty) Ltd., Wadeville, Transvaal South Africa | : Penicillin G, streptomycin sulphate |
| E. Merck, A.G. Darmstadt Germany | : All laboratory chemicals used (analytical grade)<br>Thin layer cellulose plates<br>L(+) ascorbic acid |
| Miles Laboratories, Goodwood, South Africa | : Bovine serum albumin<br>Hylauronic acid |

| | |
|---|---|
| Packard Instruments<br>Downers Grove, USA | : Scintillation mixture 299<br>Instagel |
| Pharmacia, Uppsala,<br>Sweden | : Sepharose CL-2B, Sepharose CL-6B<br>Sephadex G-50 (medium)<br>Sephadex G-100 (medium)<br>PD-10 columns |
| Seikagaku Kogyo Co.,<br>Ltd., Tokyo, Japan | : Chondroitinase ABC, Chondroitinase AC,<br>Hyaluronidase, keratanase, chondroitin<br>sulphate standards, Di-4S, Di-6S<br>and Di-0S standards |
| Sigma Chemical Co.,<br>St. Louis, MD, USA | : Collagenase (EC.3.4.24.3) (if pure<br>(bacterial) from Worthington, USA)<br>elastase (EC.3.4.21.11), trypsin<br>(EC.3.4.21.4), chymotrypsin (EC.3.4.21.1<br>papain (EC.3.4.22.2), amylase<br>(EC.3.2.1.1), phenylmethylsulphonyl<br>fluoride, carbazole, p-nitrophenyl-β-<br>D-xylopyranoside, chloroquine,<br>N-ethyl maleimide |
| State Vaccine Institute<br>Cape Town, South Africa | : Foetal calf serum |
| Whatman Ltd., Kent,<br>England | : 3MM chromatography paper<br>DE-52 Diethylaminoethyl cellulose<br>GF/C glass microfibre filters |

Radioactive chemicals:

| | |
|---|---|
| New England Nuclear,<br>Boston, USA | : L-\|5-$^{3}$H\|-Proline 20-40 Ci/nmole |
| The Radiochemical Centre,<br>Amersham, Bucks, England | : Sulphur-35 25-40 Ci/mg<br>D-\|6-$^{3}$H\| glucosamine hydrochloride<br>20-40 Ci/mmole<br>D-\|U-$^{14}$C\|glucosamine hydrochloride<br>>200 mCi/mmole |

: L-|4,5-$^{3}$H| Leucine 130-190 Ci/mmole

L-|U-$^{14}$C| Phenylalanine > 450 mCi/mmole

L-|U-$^{14}$C| amino acid mixture, code CFB 104