BIOSYNTHESIS AND DEGRADATION OF PROTEOGLYCANS IN CULTURED SMOOTH MUSCLE CELLS

Thesis presented by

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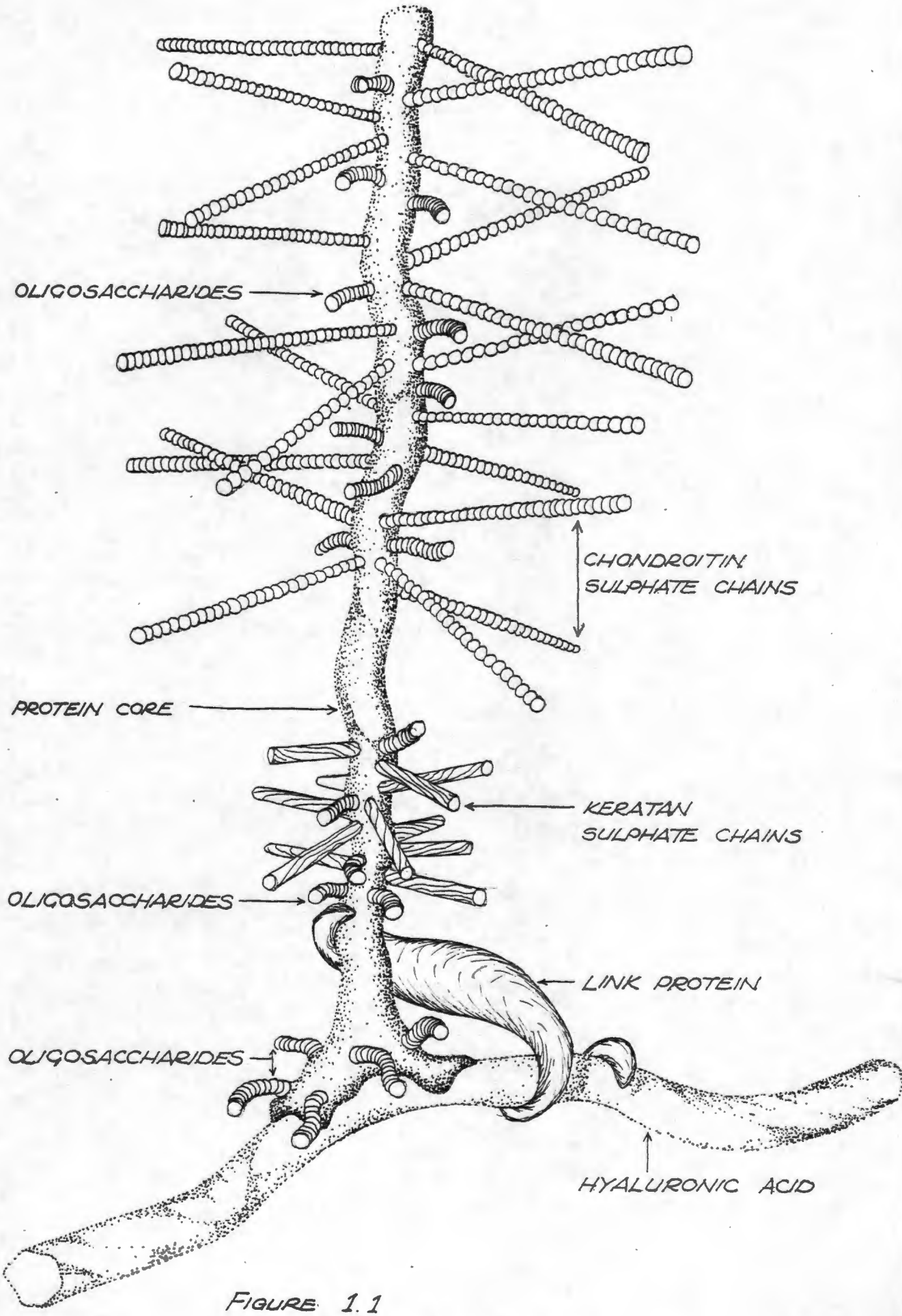

FIGURE 1.1
ARTISTIC REPRESENTATION OF PROTEOGLYCAN AGGREGATION

To my friend, John.
I am grateful to you for
doing all the graphics
for this thesis and for
giving me emotional support.

ABSTRACT

Smooth muscle cells isolated from neonatal rat hearts synthesize and secrete radioactively labelled proteoglycans into two distinct extracellular compartments, the pericellular (cell surface/matrix layer) and the culture medium (extracellular).

Cultures grown in the presence of ascorbic acid synthesize proteoglycans that are more highly sulphated than those produced in the absence of ascorbate. The glycosaminoglycan chains associated with the proteoglycans synthesized by rat smooth muscle cells were heparan sulphate, chondroitin sulphate and dermatan sulphate. There was no evidence for the synthesis of hyaluronic acid by these cells. Most of the heparan sulphate was found to be associated with the pericellular and intracellular compartments, whereas the extracellular compartment contained the bulk of the chondroitin sulphate. In the presence of ascorbate there was an increase in dermatan sulphate content of the pericellular compartment at the expense of heparan sulphate, whilst in the absence of ascorbate the heparan sulphate content of this compartment was significantly increased.

Hyaluronic acid and the antibiotic Tunicamycin had no effect on the biosynthesis of sulphated macromolecules produced by the rat smooth muscle cells. However, p-nitrophenyl-β-D-xyloside increased by 10-fold the amount of radioactive sulphate incorporation into macromolecules in the extracellular compartment. This increase was due to increased sulphation of glycosaminoglycan chains synthesized in the presence of the exogenous acceptor, as evidenced by the sulphate/uronate ratio of these sulphated macromolecules. Furthermore, heparan sulphate secretion into the extracellular compartment was decreased whilst dermatan sulphate increased in the presence of xyloside.

Pulse-chase experiments with radioactive sulphate were used to study the pathways and kinetics of secretion in the rat smooth muscle cell system. The data from these studies are consistent with a very rapid intracellular sulphation mechanism followed by rapid secretion to the pericellular compartment of macromolecular sulphated proteoglycans.

Subsequently some of these molecules then travel to the extracellular compartment. The time that different proteoglycan species remain associated with the pericellular compartment is influenced by the different matrix connective tissue proteins found in this compartment as a result of ascorbate supplementation or deprivation.

During the course of these investigations, it was observed that the pericellular compartment contributed to catabolism of sulphated macromolecules. The sulphated proteoglycans associated with this compartment are acted upon by a sulphatase or sulphatases to give rise to free radioactive inorganic sulphate and macromolecules which have been desulphated. That this process occurs in the pericellular compartment only was proven by the use of intracellular lysomotrophic inhibitors and by the continuous exposure of sulphate labelled macromolecules to the extracellular extract. Neither resulted in the release of radiolabelled inorganic sulphate from sulphated macromolecules.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ADP = adenosine diphosphate

APS = adenosine-5'-phosphosulphate

ATP = adenosine triphosphate

Ci = Curie ($3,7 \times 10^{10}$ disintegrations per second)

cm = centimeter

cm^3 = cubic centimeter

CMP = cytosine monophosphate

DPM = disintegrations per minute

EDTA = ethylenediamine tetraacetic acid

g = acceleration due to gravity

GTP = guanidine triphosphate

GuHCl = guanidinium hydrochloride

hrs = hours

$K_{av} = \frac{V_e - V_o}{V_t - V_o}$

KCN = potassium cyanide

K_d = dissociation constant

K_m = Michaelis-Menten constant

ℓ = litre

M = molar

mg = milligram

min = minutes

ml = millilitre

mm = millimeter

mM = millimolar

MW = molecular weight

NaF = sodium fluoride

nm = nanometer

PAPS = phosphoadenosine-5-phosphosulphate

PBS = phosphate buffered saline

pH = negative logarithm of the hydrogen ion concentration

r.p.m. = revolutions per min

SD = Standard Deviation $\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}$

μ (prefix) = micro ($10^{-6}x$)

μg = microgram

μl = microlitre

v/v = volume per volume

w/v = weight per volume

% = percent

[ ] = concentration

CONTENTS

CHAPTER I

INTRODUCTION

1.1. MOLECULAR STRUCTURE OF PROTEOGLYCANS

The name proteoglycan was derived to define a large group of heterogeneous molecules made up of many polysaccharide chains attached to a core protein (Muir, H., 1958). These large molecular weight molecules are ubiquitous in the body and have been studied using both chemical and physicochemical methods (Rosenberg, L. et al, 1976).

Six of the seven known types of linear polysaccharide chains, or glycosaminoglycans as they are called, may be covalently linked to the protein core. Thus proteoglycans may contain more than one type of glycosaminoglycan chain associated with the same protein. It is not known for certain whether hyaluronate is covalently linked to protein. The majority of work on proteoglycans has been carried out on material isolated from cartilage with the result that most of our knowledge regarding these molecules relates to proteoglycans of cartilaginous origin.

The typical cartilage proteoglycan is made up of a protein backbone of not more than 3 000 amino acids (MW 3×10^5), about 100 chondroitin sulphate chains (unit MW 2×10^4) and 50 keratan sulphate chains (unit MW up to 1×10^4). There are in addition N- and O-linked oligosaccharide chains associated with the structure (unit MW up to 2×10^3). The chondroitin sulphate and the keratan sulphate glycosaminoglycans are concentrated in specific regions of the molecule to give a chondroitin-rich region and a keratan-rich portion. Therefore the overall composition of the molecule is thus approximately 80 - 84% chondroitin sulphate, 7 - 12% keratan sulphate, 1 - 3% oligosaccharides and 7% protein (Muir, H., 1977)(Fig. 1.1).

Rosenberg (1976) has been able to visualise two types of proteoglycan macromolecules in cartilage by electron microscopy. The one is a shorter form which consists of a central filament of 170 - 190 nm with 20 - 22 side chains, each 52 - 54 nm long, whereas the longer form

consists of a central filament of 320 - 340 nm with 34 side chains of similar length as those seen in the small molecule.

Proteoglycans having different compositional make-up have also been found. A keratan sulphate proteoglycan has been isolated from bovine corneal stroma (Axelsson, I. and Heinegard, D., 1975), and rat liver cells produce a heparan sulphate proteoglycan (Oldberg, A., et al., 1977). A similar type of heparan sulphate proteoglycan has also been isolated from bovine aorta as well as a dermatan sulphate-chondroitin sulphate proteoglycan (Ehrlich, K.C. et al, 1975; Jansson, L. and Lindahl, U., 1970; Kapoor, R. et al, 1981).

The current model for cartilage proteoglycan monomer core protein envisages a hyaluronic acid binding region of constant size and composition, rich in cysteine, methionine and asparagine, and a large domain of variable length for the attachment of glycosaminoglycan chains. This polysaccharide attachment region has a repetitive amino acid sequence containing serine and glycine in equimolar amounts (Heinegard, D. and Hascall, V.C., 1974). However, newly synthesized core protein, obtained using cell-free translation of total RNA from chick cartilage, has been found to be of uniform size prior to addition of glycosaminoglycan chains which is inconsistent with a core protein structure having a glycosaminoglycan attachment domain of variable length (Upholt, N.B. et al, 1979). Limited proteolysis of chondroitin sulphate proteoglycan from different species has led to the proposal that the protein backbone exists as a series of repeating sequences. It is believed that the protein core contains a polysaccharide attachment region (xyl-gal-gal) at every sixth amino acid but not all these attachment sites are utilised for the formation of completed glycosaminoglycan chains (Wusternan, F.S. and Davidson, E.A., 1975). However, Mathews, M.B. (1971) proposed that there are short and long repetitive amino acid sequences, the short ones consisting of 2-8 amino acids, while the long ones consist of about 35 amino acids.

So far seven different glycosaminoglycan chains have been identified. These chains are made up of repeating disaccharide units and are variable in length, chemical composition and degree of sulphation (Tables I and II). Electron microscopic examinations of the sizes of

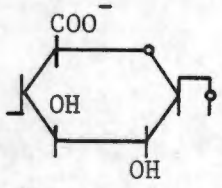
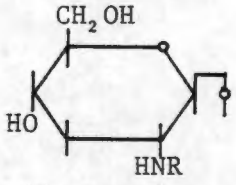
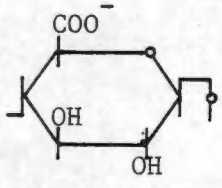
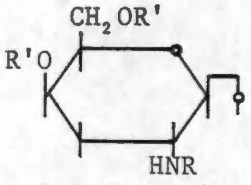
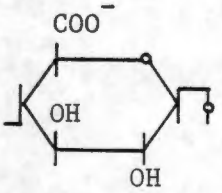
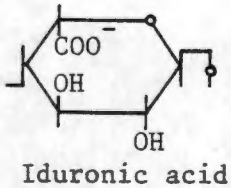
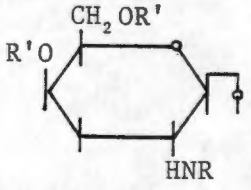
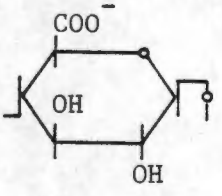
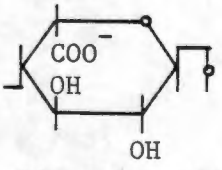
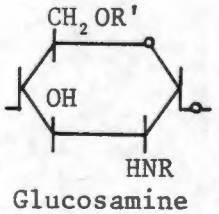
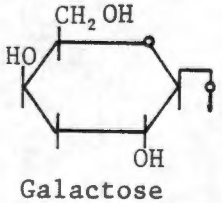
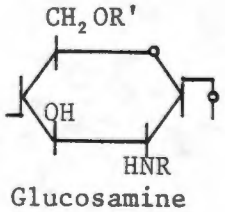
TABLE I

TYPES AND PROPERTIES OF GLYCOSAMINOGLYCANS

POLYSACCHARIDE	MOL. WT. ($x10^{-3}$)	DISACCHARIDE REPEATING UNIT		TYPE OF SULPHATION	SULPHATE PER DISACCHARIDE
		HEXURONIC ACID	HEXOSAMINE		
Hyaluronate	4-8000	D-Glucuronic acid	D-Glucosamine	-	-
Chondroitin 4- and 6-Sulphate	5-50	D-Glucuronic acid	D-Galactosamine	O-Sulphate	0,1 - 1-3
Dermatan Sulphate	15-40	L-Iduronic acid or D-Glucuronic acid	D-Galactosamine	O-Sulphate	1,0 - 3,0
Keratan Sulphate	4-19	D-Galactose	D-Glucosamine	O-Sulphate	0,9 - 1,8
Heparan Sulphate		D-Glucuronic acid or L-Iduronic acid	D-Glucosamine	O-Sulphate + N-Sulphate	0,4 - 2,0
Heparin	6-25	D-Glucuronic acid or L-Iduronic acid	D-Glucosamine	O-Sulphate + N-Sulphate	1,6 - 3,0

TABLE II

STRUCTURE OF GLYCOSAMINOGLYCANS

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

proteoglycans showed them having a broad Gaussian distribution (Kimura, J.H. et al, 1978). It is currently thought therefore that this polydispersity is due to the number and length of the glycosaminoglycan chains attached to the single protein core (Fellini, S.A. et al, 1981; Heinegard, D., 1977).

The mechanism that generates this chain variation is not yet clear. It may be due to local nutritional or environmental effects on biosynthesis and degradation of proteoglycans. It has been shown that there is variation in synthesis of glycosaminoglycan chains due to many factors. In the case of glycosaminoglycan synthesis studied in tissue culture, such factors as the age of the culture (Pacifici, M. et al, 1981; Fellini, S.A. et al, 1981) or the tissue (Sweet, M.B.E. et al, 1979) from which they were derived have a pronounced effect on the rates of synthesis (Kato, Y. et al, 1978; Mitchell, D. and Hardingham, T., 1981), as well as the hormonal status of the culture (Stevens, R.L. and Hascall, V.C., 1981).

1.2. BIOSYNTHESIS OF PROTEOGLYCANS

The biosynthesis of proteoglycans involves both the synthesis of the protein core and the sulphated glycosaminoglycan chains. The interrelationship of the two types of macromolecules immediately suggested some degree of coordinated control during their synthesis. By comparing the rates of formation of the individual components and by the use of specific protein synthesis inhibitors, it has been established that the protein biosynthesis precedes the polysaccharide addition (Telser, A., Robinson, H.C. and Dorfman, A., 1965; Barland, P. et al, 1968; Olssen, I., 1969).

The protein core is synthesized on the rough endoplasmic reticulum by the conventionally accepted pathway (Upholt, W.B. et al, 1979), whereas the Golgi apparatus is the principal site of biosynthesis of glycosaminoglycan chains (Herwitz, A.L. and Dorfman, A., 1968). A large intracellular precursor pool of core protein having a molecular weight of 370 000 has been found to exist in studies using cultured chondrocytes (Kimura, J.H. et al, 1981). Translation of putative core

protein mRNA isolated from chick cartilage in a cell-free system gives rise to a core protein having a molecular weight of 340 000, whereas the protein synthesized by cultured bovine chondrocytes has a lower molecular weight (310 000) (Treadwell, B.V. et al, 1980). It is assumed that the cell-free translational product is carbohydrate-free but may still have its small hydrophobic "signal" peptide present, since this would only be removed during transfer through the endoplasmic reticulum (Blobel, G. and Dobbestein, B., 1975). The size of the polypeptide from intact cartilage proteoglycan monomer has been estimated to be 200 000 - 230 000 using physicochemical measurements (Hascall, V.C. and Sajdera, S.W., 1970). The nature and size of the "signal" extension peptide has as yet not been established.

Once completed, the nascent polypeptide core is sequestered into the cisternal space of the endoplasmic reticulum from whence it moves to the Golgi apparatus prior to secretion.

It is not known whether the high mannose type oligosaccharide present in some proteoglycans is added whilst the protein core is present in the cisternae of the endoplasmic reticulum (Kornfeld, R. and Kornfeld, S., 1980) or in the cis compartment of the Golgi apparatus (Rothman, J.E., 1980). It is well-established that the process of N-glycosylation proceeds via the formation of dolechol-linked saccharides that serve as direct donors of the saccharide chains to the asparagine residues in peptides. In the Golgi apparatus the mannose-rich oligosaccharides are partially processed to their final complex form before the final processing of the proteoglycan molecule takes place (Hanover, J.A. et al, 1980). Although the Golgi apparatus seems to be the site for the biosynthesis of glycosaminoglycan chains (Herwitz, A.L. and Dorfman, A., 1968; Kimata, K., 1971; Neutra, M. and Leblong, C.P., 1966; Silbert, J.E. and Freilich, L.S., 1980), the site of addition of the O-linked oligosaccharide synthesis is still uncertain as is the stage at which this addition takes place. The Golgi apparatus would appear to be the main site of glycosylation during proteoglycan synthesis (Fleisher, B., et al, 1969) in spite of the finding that nearly all types of glycosyltransferase activity may also be associated with the endoplasmic reticulum (Strouss, J.E., 1979; Hanover, J.A. et al, 1980; Kim, Y.S. et al, 1971; Ko, K.W. and Raghapathy, E., 1972). These

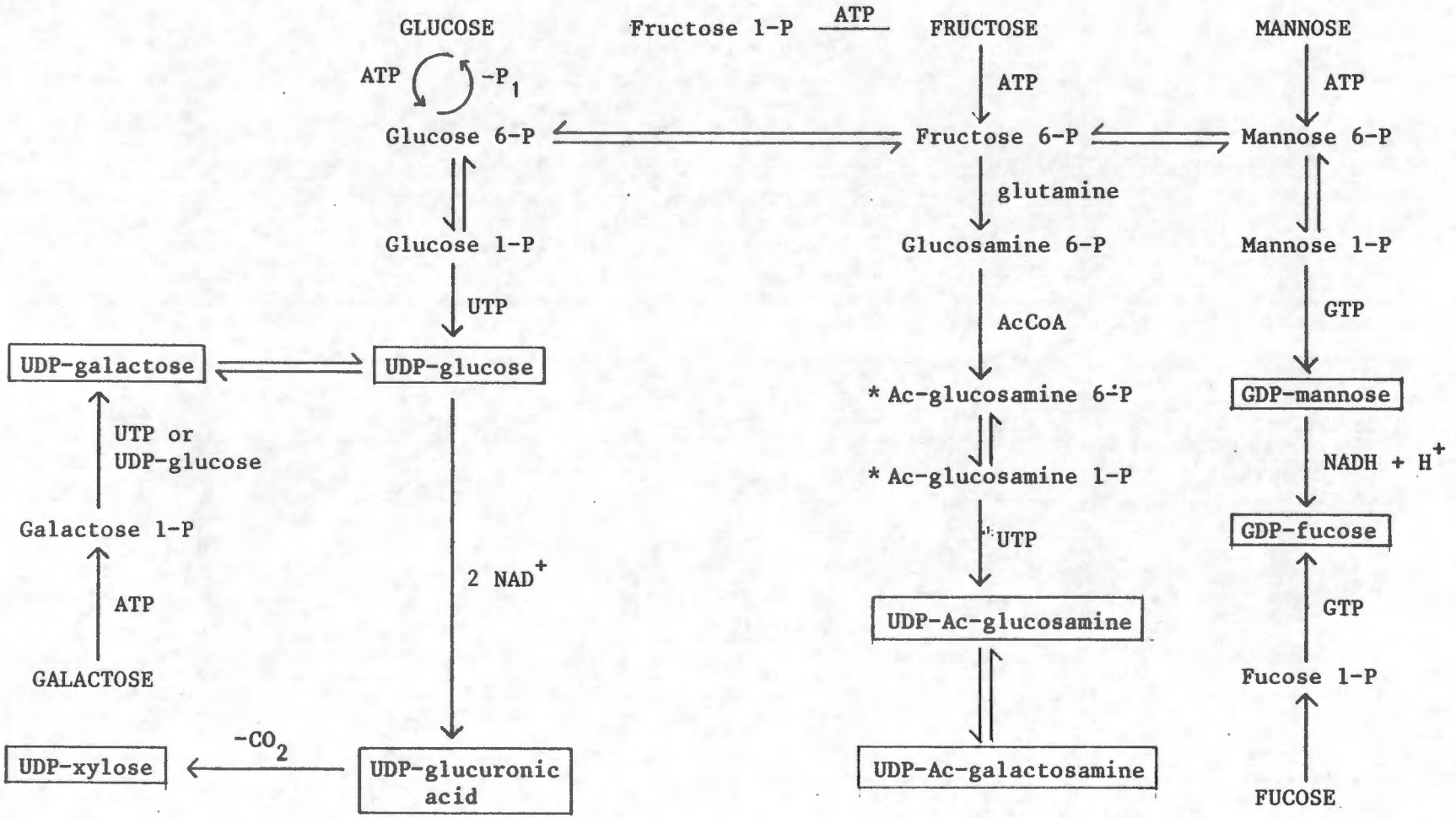
findings may be due to artifactual contaminations that have arisen during isolation. Since in N-glycosylation there is a requirement for a lipid-linked intermediate prior to preassembly and transfer to the polypeptide chain, it is not yet known whether an analogous situation exists in the case of O-glycosylation. Although the presence of a lipid-linked saccharide that could be involved in assembly of O-linked oligosaccharides has been reported, there is no evidence for its participation in assembly of these chains. In studies using the antibiotic tunicamycin, which inhibits glycoprotein synthesis by 90-95% (Pratt, M.R. et al, 1979), no inhibition of O-glycosylation (Hanover, J.M. et al, 1980) was observed.

The cell carbohydrate moeities are derived initially from glucose metabolism (Table III) and the nucleotide monosaccharide is required for the biosynthesis of the carbohydrate chains (Topper, Y.G. and Lipton, M.M., 1953). In glycosaminoglycan synthesis all the sugars and their derivatives are activated using the normal uridine diphosphate pathway except fucose that utilizes GTP and sialic acid CMP activation (Perlman, R.L. et al, 1964). The details of steps involved in chain initiation, propagation and termination during glycosaminoglycan biosynthesis are still unknown.

Initiation, the attachment of xylose to a peptidyl serine or threonine hydroxyl group, is a multi-step mechanism involving (a) xylosylation, (b) the intracellular site of xylose transfer, (c) the interaction of xylosyltransferase with the other glycosyltransferases, (d) the regulation of the chain synthesis by xylosyltransferase (Robinson, C.H. et al, 1975). Once again, details of these steps are still not well understood in spite of a number of in vitro cell free studies. The protein core has been shown to be the acceptor of choice for the activated xylose (K_m 0,064), when assayed using xylose transferase. Tryptic peptides or serine containing polypeptides, derived from acid hydrolysis, were less active while the synthesized tripeptide Ser-Gly-Gly showed the least activity (Baker, J.R. et al, 1972). Xylose transferase is a glycoprotein (Schwartz, N.B. and Dorfman, A, 1975) and its activity is dependent on the presence of divalent cations with Mn^{++} being the cation of choice over Mg^{++} (Robinson, H.C. et al, 1966). Xylosylation is followed by the addition of two galactosyl residues and then

TABLE III

PATHWAY OF MONOSACCHARIDE FORMATION



* Ac = Acetyl

sequentially by the alternate addition of hexuronic acid and N-acetylated hexosamines (Fig. 1.2)(Helling, T. and Roden, L., 1969a and b, Brandt, H.E. and Distler, J.J., 1969).

It appears that the initiation of polysaccharide biosynthesis is the same for chondroitin sulphate, heparin (Stern, E.L. et al, 1971; Grebner, E.E. et al, 1966), and heparan sulphate. There is evidence that in the case of dermatan sulphate there is another type of O-glycosidic bond formed between N-acetyl galactosamine and the peptidyl acceptor (Akiyama, E.S., 1981) which is similar to the acceptor for keratan sulphate glycosaminoglycan chains (Heinegard, T., 1981).

Glycosyltransferases and their acceptors are predominantly associated with membranes (Brandt, A.E. et al, 1975). Attempts to solubilize and purify these enzymes have so far been unsuccessful.

Chain propagation has not been studied extensively. It is, however, known that the two specific sugars are added alternatively (Telser, A. et al, 1966), are unsulphated (Silbert, J.E., 1964; Perlman, R.L. et al, 1964) and that the transferases work in a concerted manner (Hopwood, J.J. et al, 1974; Olsson, I. and Gardell, S., 1971).

As already mentioned above, there is some evidence for a lipid soluble disaccharide intermediate, i.e. glucuronosyl-N-acetylglucosaminyl pyrophosphodolichol being involved in the biosynthesis of hyaluronate and/or heparin-like polysaccharides (Hopwood, J.J. and Dorfman, A., 1977; Turco, S.G. and Heath, E.C., 1977). It is, however, more likely that this lipid intermediate is involved in the N-linked glycosylation, as has been established for glycoprotein synthesis. In the biosynthesis of the different glycosaminoglycan chains the polymerisation products are as shown in Tables I and II.

The action of C_5 epimerase appears to take place on the nascent polysaccharide chain during its synthesis rather than at the stage of chain completion. The epimerase reaction brings about the conversion of glucuronic acid to L-iduronic acid by C-5 epimerization (Malmstrom, A. et al, 1975) and occurs during the formation of dermatan sulphate, heparin and heparan sulphate.

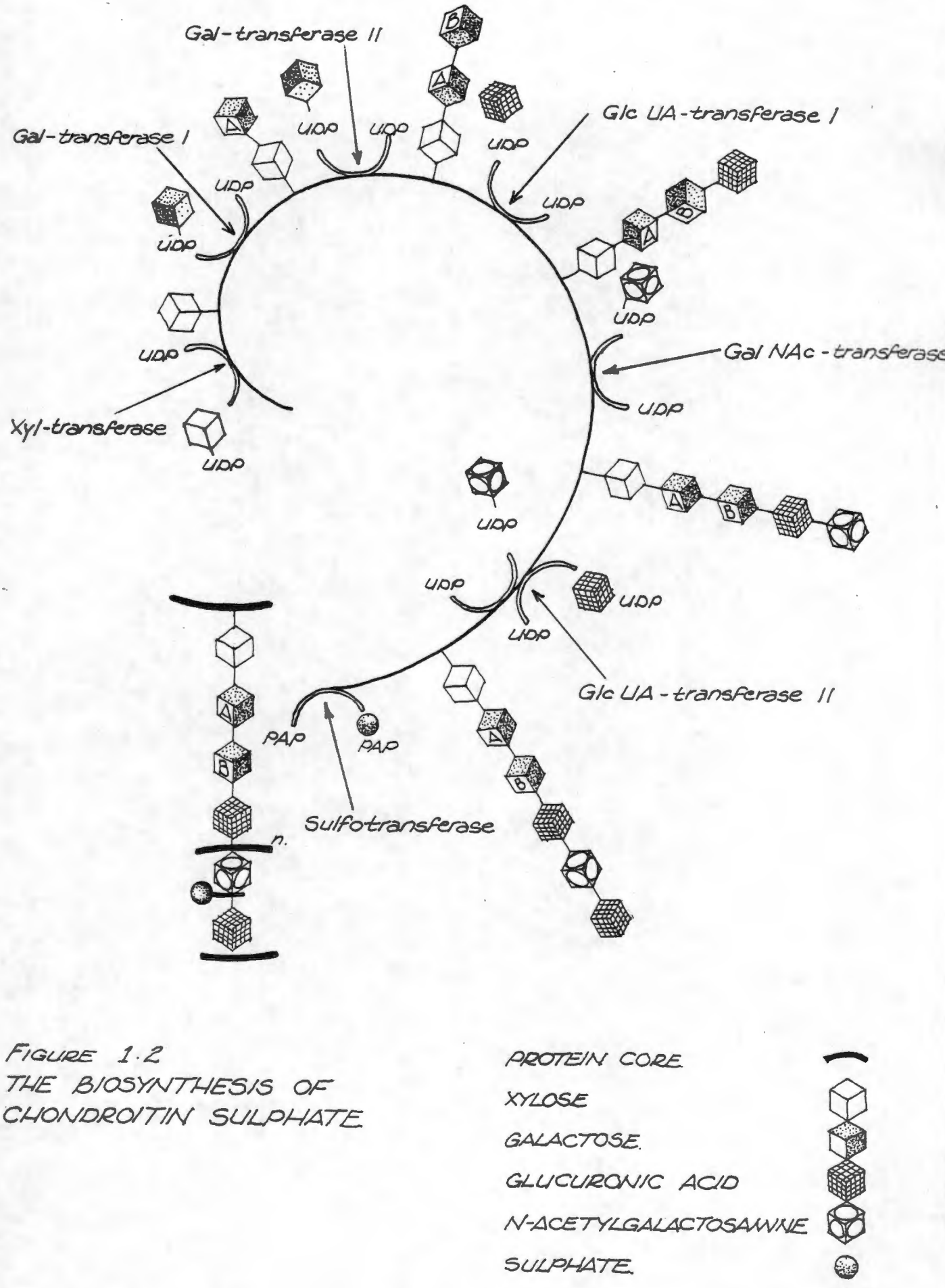


FIGURE 1.2
THE BIOSYNTHESIS OF CHONDROITIN SULPHATE

PROTEIN CORE
XYLOSE
GALACTOSE
GLUCURONIC ACID
N-ACETYLGALACTOSAMINE
SULPHATE

The mechanism of sulphation involves specific O- and N-sulphation enzymes and seems to be associated with the smooth endoplasmic reticulum. Indirect evidence for this was obtained by electron microscopy and autoradiography (Peterson, M. and Leblong, C.P., 1964; Godman, G.C. and Lane, N., 1964; Neutra, M. and Leblong, C.P., 1966). The formation of activated sulphate takes place in two steps

(1) $ATP + SO_4^{2-} \rightleftharpoons APS + 2PO_4^{3-}$

(2) $APS + ATP \longrightarrow PAPS + ADP$

(D'Abromo, F. and Lipman, F., 1951; Korn, E.D., 1959; Adams, J.B. and Meaney, M.F., 1961). Sulphation occurs rapidly after the polymerization of the monosaccharide unit in the growing glycosaminoglycan chain. It is suspected that three different enzymes are involved in the specific sulphation of sulphoamino groups and the C_4 and C_6O-sulphate groups (De Luca, S. et al, 1973; Richmond, M.E. et al, 1973; Suzuki, S. and Strominger, J.L., 1960). Some workers have demonstrated that certain enzymes transfer sulphate to non-sulphated monosaccharide units present in a predominantly sulphated chain, while others have suggested that the 6-transferase activity works in concerted effort with a 4-sulphatase (Nakanishi, Y. et al, 1981). This is based on the finding that chains ending in N-acetyl galactosamine-6-sulphate are able to accept another glucuronic acid molecule, whereas those ending in N-acetyl galactosamine-4-sulphate are not. This observation may be, on the other hand, a mechanism to control elongation (Silbert, J.E., 1978; Telser, A. et al, 1966). In a pig aortic microsomal fraction it has also been shown that there is rapid N-sulphation followed by O-sulphation, and also that O-sulphation occurs on previously N-sulphated compounds (Levy P. et al, 1981). There is little knowledge on the mode of chain termination in glycosaminoglycan synthesis but the addition of sialic acid residues might 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 (Gottschalk, A., 1969).

1.3. SECRETION AND TURNOVER OF PROTEOGLYCANS

A number of in vitro and in vivo experiments have been performed in attempts to establish the details of the pathways of proteoglycan

metabolism. By using radioactively labelled precursors it has been established that there is more than one intracellular pool of proteoglycans that also have different metabolic activities (Olssen, I. et al, 1968). This may represent different populations of proteoglycans as discussed under Section 1.4, but this has not as yet been established.

There are at least four different metabolic compartments. There is the intracellular compartment, which includes the location of synthesis, storage and degradation. The half-life of proteoglycans in this compartment has been found to be relatively short (Lohmander, S. et al, 1973). This may be due to secretion of the newly synthesized proteoglycans, degradation of the internalized molecules, or both (Kleine, T.O. et al, 1974). The remaining three metabolic compartments are all extracellular and are made up of the aggregating, non-aggregating and the cell-surface proteoglycans as discussed in Section 1.4. The non-aggregating proteoglycans are smaller than their aggregating counterparts (Heinegard, D. and Hascall, V.C., 1979), and have a shorter half-life. In guinea pig costal cartilage the smaller proteoglycans had a half-life of 3 days, whilst the bigger species had a half-life of 65 days (Lohmander, S., 1977).

The turnover rates of various glycosaminoglycan chains seems to be different. In vivo studies using rat kidney, hyaluronic acid seems to turn over faster than other glycosaminoglycans (Barry, D.N. and Browness, J.M., 1975). In vitro experiments using neonatal epiphyseal cartilage slices showed that chondroitin sulphate had a turnover rate of 70 hours compared to hyaluronic acid which turns over every 120 hrs (Handley, C.J. and Phelps, C.F., 1972(a)). This, in comparison with bovine corneas incubated with radioactive label in vitro, showed that chondroitin sulphate turned over every 10 days, whilst keratan sulphate every 30 days (Handley, C.J. and Phelps, C.F., 1972(b)). Glycosaminoglycans of the same type seem to turn over at the same rate in different tissues.

In in vitro studies of incorporation of $|^{35}S|$-sulphate into pig laryngeal cartilage proteoglycans, radioactivity appeared in chondroitin sulphate after 2 min, with a delay of 35 min in the appearance of extractable proteoglycans, but no precursor-product relationship was found between

the different pools. It has been suggested that molecules of all sizes are synthesized at the same time (Hardingheim, T.E. and Muir, H., 1972a). This does not exclude that there are different biosynthetic pools. In slices of calf cartilage, four pools of chondroitin sulphate were extracted which were metabolically and chemically different (Kleine, T.O. et al, 1973).

There seem to be age-dependent changes in turnover rates. With increasing age, turnover seems to be slower (Maroudas, A. and Evans, H., 1974; Vogel, K.G. et al., 1981).

1.3.1. Degradation of proteoglycans.

Degradation, as discussed in the following section, is considered only under normal and not pathological conditions, and as such may be termed turnover of proteoglycans. It has been shown that proteoglycan aggregates are not depolymerized before leaving the matrix area, and this is confirmed by the total absence of a hyaluronidase in cartilage matrix (Arboyast, B. et al, 1975). In organ cultures it was found that proteoglycans may be released from matrices by proteolysis of the core protein at or near the hyaluronic acid binding region, giving a product similar in size to native proteoglycan. However, this partially degraded molecule was unable to re-aggregate with hyaluronic acid in vitro (Sandy, D.J. et al, 1978). The protein core binding to the extracellular matrix proteins, which has been discussed under Section 1.4, is the most probable explanation for the retention of partially degraded proteoglycan molecules in the extracellular matrix. The effect of enzymatic degradation of the carbohydrate chains does not appear to influence the integrity of the matrix until there has been considerable destruction of carbohydrate.

In bone explant cultures the medium contains a latent neutral proteinase which has been isolated. This protease, which can degrade cartilage proteoglycans, has been proposed as the enzyme that participates in extracellular matrix degradation. The soluble fragments resulting from degradation by the enzyme may be endocytosed and digested within the lysosomal system (Vaes, G. et al, 1978); Seller, A. et al, 1978).

The uptake, internalization and subsequent lysosomal degradation has been studied using fibroblast cultures (Kresse, H. and Buddecke, E. et al, 1975).

Kresse and Buddecke have shown that the transfer of proteoglycans from the culture medium to the interior of the cells takes place via receptor mediated endocytosis. There are two types of binding sites on the cell surface for proteoglycan uptake. There are high affinity binding sites which show saturation kinetics whilst the low affinity binding sites appear to be non-saturable (Prinz, R. et al, 1978). The finding of a high turnover rate for proteoglycan receptors suggests a recycling mechanism similar to that proposed for low density lipoproteins (Anderson, R.G.W. et al, 1977).

It has been demonstrated that the high affinity receptors recognize the protein moeity of the proteoglycan specifically since uptake experiments using protein-free glycosaminoglycan chains have shown little binding by these molecules (Truppe, W. and Kresse, H., 1978). It has not been clearly established whether the endocytosed proteoglycans are still sulphated. Typically, only some 10% of added sulphated proteoglycans are internalised by the culture cells used by the various investigators. There are suggestions that sulphated proteoglycans inhibit endocytosis (Prinz, R. et al, 1977) and that desulphation is required before uptake. There also seems to be a minimal length of five disaccharide units required for interaction with the receptor (Prinz, R. et al, 1978).

In in vitro experiments using cultured leucocytes it has been found that there is a reversible electrostatic interaction which is pH dependent between several glycosaminoglycans and some lysosomal proteins. This leads to inhibition of lysosomal enzymes, and has been shown to occur particularly with heparin (which contains both N- and O-sulphates) but also to some extent with chondroitin 4- and 6-sulphate. Dermatan sulphate and hyaluronic acid showed very little inhibition in this study (Avila, J.L., 1978; Avila, J.L. and Convit, J., 1975). This binding of glycosaminoglycans to lysosomal proteins has been suggested as a regulatory mechainsm for lysosomal enzyme activity and is probably

unrelated to the role of lysosomes in the degradation of internalised glycosaminoglycans *in vivo*. It has also been found that there is a relatively high concentration of chondroitin sulphate present in lysosomes which may be involved in the regulatory function as proposed by Avila, J.L. and Convit, J. (1975).

Using cultured fibroblasts, it has been found that there is some desulphation of proteoglycan molecules before total degradation by lysosomal enzymes. Inhibition of aryl sulphatases leads to inhibition of chondrosulphohydrolases. The lysosomal enzymes have been shown to work in a concerted manner whereby desulphation is followed by sequential action by lysosomal β-glucuronidases, 4- and 6-sulphatases and N-acetyl galactosaminidases to bring about total degradation of the internalised proteoglycan (Buermann, C.W. *et al*, 1979; von Held, E. and Buddecke, E., 1967).

1.4. EXTRACELLULAR ASSOCIATION OF PROTEOGLYCANS

The predominant components of extracellular matrix material are collagen and proteoglycans with glycoproteins of the fibronectin type being found associated with these two macromolecules. Matrix material in elastic tissue also contains elastin as another proteinaceous component and this is also associated with specific glycoproteins.

The extracellular matrix is of great importance in the formation and maintenance of correct tissue morphology and in the regulation of morphogenic events. Furthermore, it provides an environment for the continued functioning of the cells associated with it. The deposition of insoluble proteoglycans into the extracellular matrix may be a result of interactions between themselves and the proteinaceous components in the matrix - collagen, elastin and glycoproteins, apart from the formation of aggregates with hyaluronic acid.

1.4.1. Binding to collagen

The many studies on the interaction of glycosaminoglycans/proteoglycans

with collagen have resulted in an abundance of circumstantial evidence for such an event. The interactions are of an electrostatic nature at physiological pH and ionic strength (Öbrink, B. and Wasteson, A., 1971). Light scattering studies suggest a two-stage binding process, a primary interaction which gives rise to a spatial rearrangement followed by secondary binding or aggregation.

The primary specific binding seems to be between collagen and the protein (Kobayashi, T.K. and Pedrini, V., 1973; Toole, B.P., 1976; Greenwald, R.A. et al, 1975). In isolated renal basement membrane it was found that the proteoglycan molecule was linked to collagen by disulphide bonds (Parthasarathy, N. and Spiro, R.G., 1981). No details of this interaction are as yet known. The secondary binding involves the interaction of the negatively charged sulphate and carboxyl groups on the glycosaminoglycan chains with the positively charged guaninido group of arginine and the ε-amino group of lysine on the collagen molecule (Podrazky, V. et al, 1971; Steven, F.S. et al, 1969). Dermatan sulphate has been shown to have the strongest interaction with collagen followed by heparan sulphate, heparin and chondroitin sulphate (Öbrink, B., 1973). Enzymatic studies have shown that the chondroitin sulphate - dermatan sulphate proteoglycan is closely associated with collagen from bovine aorta, and released by collagenase digestion (Radhakrishnamurthy, B. et al, 1977). Hyaluronic acid (Greenwald, R.A. et al, 1975) and keratan sulphate show negligible binding to collagen but the former shows some association by mutual steric interaction (Öbrink, B., 1973). Light scattering studies clearly showed stronger interactions with glycosaminoglycans containing L-iduronic rather than D-glucuronic acid since in the case of 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 interaction is proportional to chain length and charge density (Öbrink, B., 1973).

Proteoglycans have been shown to bind to all types of collagen (Toole, B.P., 1976; Toole, B.P. and Lowther, D.A., 1968). Interaction of cartilage proteoglycans with isolated α-chains from type I collagen have shown them to have a preference for α_2 and β_{12} components, but by increasing the amounts of proteoglycan, α_1 and β_{11} components also bound

(Lee-Owen, V. and Anderson, J.C., 1975; Oegema, T.R. et al, 1975). Also in vitro competitive-interaction experiments showed that in the presence of equal amounts of collagen type I and II, the proteoglycan molecule bound type I collagen preferentially (Lee-Owen, V. and Anderson, J.C., 1976).

The exact binding site(s) on the collagen molecule has not been established but basic amino acids are expected to play a major role. The number of binding sites on the collagen monomer varied between two and five (Öbrink, B. et al, 1975; Obrink, B. and Sundelöef, L.O., 1973).

Using data obtained from light scattering studies, it has been calculated that one cartilage proteoglycan can bind to two collagen molecules. There is a direct relationship between the size of proteoglycans and their binding capacity (Öbrink, B. and Sundelöef, L.O., 1973).

Heparin, heparan sulphate and cartilage proteoglycans have been shown to retard the assembly of collagen fibres and cause alterations in the organization of the fibril in vitro. Chondroitin-sulphate and hyaluronate were less effective. The molecules that delayed fibril formation were also effective in producing an increase in degree of aggregation of fibrillar collagen. This could be an important factor in the organization and functioning of connective tissues at all stages of growth and development (Mathews, M. and Decker, L., 1968).

1.4.2. Binding to elastin.

The interaction of proteoglycans with elastin has not been studied in detail as yet. There is some evidence that an electrostatic interaction takes place between bovine nasal cartilage proteoglycans and α elastin or tropoelastin (Podrazký, V. and Adam, M., 1975; Adam, M. and Podrazký, V., 1976). Elastase digestion of bovine aorta solubilized most of the heparan sulphate proteoglycans and some of the dermatan sulphate-chondroitin sulphate species as well (Radhakrishnamurthy, B. et al, 1977). This has been taken to show that these glycosaminoglycans have preferential association with elastin in the arterial extracellular matrix.

1.4.3. Binding to fibronectin.

Studies using either rutherium red, a specific proteoglycan stain, or chemical cross-linking, have established that proteoglycans and fibronectin are associated in the extracellular compartment (Perkins, M.E. et al, 1979, Graham, J.M. et al, 1975).

It has been shown that hyaluronic acid, heparin and, to a lesser extent, heparan sulphate, bind to fibronectin. There was, however, no binding of chondroitin sulphate to the glycoprotein.

The binding is specific and not merely due to electrostatic interaction of the macromolecules. Scatchard analyses revealed non-linear, high affinity binding on fibronectin at saturable non-competitive sites (for HA K_D x 10^{-7} M, heparin 10^{-7} - 10^{-9}). There is no evidence yet whether interaction of hyaluronate and fibronectin is similar to the cartilage proteoglycan binding to hyaluronate. The fibronectin-hyaluronate interaction is not inhibited by the presence of collagen, which is also known to bind to fibronectin. This suggests that there are at least two distinct binding sites on the fibronectin, one for collagen and one for hyaluronic acid.

The binding of heparin, heparan sulphate or hyaluronate to fibronectin-collagen complexes results in the formation of a more stable complex than that of fibronectin and collagen alone (Ruoslahti, E. and Engvall, E., 1980; Yamada, K.M. et al, 1980). Glycosaminoglycans also caused agglutination of gelatin-coated plastic beads when fibronectin was present but had no effect when it was absent. These observations have given rise to the proposal that all three components are necessary for the formation of an insoluble extracellular matrix (Jilek, F. and Hörmann, H., 1979; Ruoslahti, E. and Engvall, E., 1980).

1.4.4. Binding to hyaluronate.

The aggregation of cartilage proteoglycans with hyaluronic acid has been studied in great detail. The majority of the work utilized material extracted from cartilage or from chondrocyte cultures. In 1969 Hascall and Sajdera showed that when bovine nasal cartilage was analyzed in the ultracentrifuge in an associative solvent (0,4 M GuHCl)

the aggregated proteoglycans had a high sedimentation coefficient. In dissociative solvent (4 M GuHCl) the monomer had a higher sedimentation coefficient than free hyaluronic acid and could be separated from the latter. Under the electron micrograph, hyaluronic acid has been shown to appear as a single unbranched chain (Fessler, J.H. and Fessler, I.H., 1966).

The typical cartilage proteoglycan monomer, consisting of a protein core with distinct keratan sulphate and chondroitin sulphate domains, has a globular, elongated region containing four or five intramolecular disulphide bridges on one end of the molecule. This domain is also low in carbohydrate, interacts with hyaluronic acid (Perkins, S.J. et al, 1981) and has a molecular weight of 90 000 (Heinegard, D. and Hascall, V.C., 1974). It has been found constant in size in all monomers analyzed so far.

Chemical modification studies using acetylation, N-bromo succinylation and reactions with 2-methyl maleic anhydride have implicated a number of amino acid residues as being important for binding to hyaluronic acid. Modifications with 2-nitrophenylsulphenyl chloride and butanedione indicated the same amino acids, namely arginine, tryptophan and lysine. 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. Reduction of the disulphide bridges with dithiothreitol prevented binding but this was restored after reoxidation (Hardingham, T.E. et al, 1976). When the ε-amino group of lysine was dansylated there was complete loss of binding which showed that this group contributed to the stability of the interaction but was not necessarily at the active site since acetylation did not prevent interaction (Heinegard, D. and Hascall, V.C., 1979). The core protein interaction with hyaluronic acid is specific and has a high affinity for hyaluronic acid chains containing a minimum of five disaccharide units (K_D x 10^{-8} - 5 x 10^{-8} M) (Christner, J.E. et al, 1978; Nieduszunski, I.A. et al, 1980; Hardingham, T.E. and Muir, H., 1973; Hascall, V.C. and Heinegard, D. 1974). Other polysaccharides such as dextran sulphate, chondroitin sulphate or sodium alginate cannot mimic hyaluronic acid binding

(Hardingham, T.E. and Muir, H., 1972b). When 60% of the carboxyl groups of glucuronic acid were modified with diazomethane to form methyl esters there was no interaction. Reduction of glucuronic acid to glucose or modification by glycine substitution also resulted in loss of binding activity. The interaction appears to require at least four consecutive unmodified carboxyl groups on the hyaluronic acid (Christner, J.E. et al, 1977). The decasaccharide must also contain at least one N-acetylglucosamine residue since chondroitin which only differs from hyaluronate at the hydroxyl position at C_4, does not interact even at high concentrations (Christner, J.E. et al, 1979; Hascall, V.C. and Heinegard, D., 1974).

The binding of the monomer to hyaluronic acid is stabilized by link protein (Heinegard, D. and Hascall, V.C., 1974). Two types of link proteins seem to exist in cartilage. They are structurally similar since they share the same amino acid composition and have the same electrophoretic behaviour. However, although they differ in their carbohydrate content (Baker, J.R. and Caterson, B., 1979), no functional difference between them has been established. The molar ratio of the link protein to protoglycan monomer seems to be 1:1 (Kimura, J.H. et al, 1980).

The link protein is not essential for hyaluronic acid-proteoglycan binding since interaction may occur in its absence (Schwann, D.A. et al, 1976; Christner, J.E. et al, 1978). However, link-free aggregates are less stable under physiological conditions and are easily disaggregated (Hardingham, D., 1979). In in vitro experiments it has been shown that link proteins can bind to the monomer in the absence of hyaluronate (Caterson, B. and Baker, J.R., 1978). However, it is the interaction between all three components that results in a stable ternary complex being formed which is also relatively insusceptible to digestion by proteases (Heinegard, D. and Hascall, V.C., 1974). 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 monomer. This suggests that the formation of stable tertiary complexes is a sequential process involving link-monomer complex formation first, and subsequent binding of this to

hyaluronic acid (Kimura, J.H. et al, 1980; Treadwell, B.V. et al, 1980). This stepwise interaction, however, does not account for the observation that hyaluronate can combine with a proteoglycan monomer to form a complex that is only stabilized on addition of link protein. The composition of an aggregate is thus 96% proteoglycan monomer, 10% hyaluronic acid and 3% link protein. The exact relationship between the three molecules has to be discovered as yet. It has been calculated that as many as 100 proteoglycan monomers can bind to one hyaluronic acid molecule MW 1 x 10^6 at one time (Rosenberg, L. et al, 1975). Fifteen hyaluronic disaccharide units appear to be involved in formation of a stable link protein-monomer aggregate. The monomer binds about 5 disaccharides whilst the link protein occupies a further adjacent 10 disaccharides (Faltz, L.L. et al, 1979). The distance between interacting monomers on the hyaluronic acid seem to be about 40 disaccharide units (Heinegard, D. and Hascall, V.C., 1974). Electron micrographs of proteoglycan aggregates show the major axis of the proteoglycan to be perpendicular to the hyaluronic acid backbone (Kimura, J.H. et al, 1978). Since the hyaluronic acid binding region is parallel with the long axis of the binding region of the hyaluronate, it must mean that the proteoglycan long axis is also perpendicular to the long axis of the binding region which gives it a T or L-shaped structure (Perkins, S.J. et al, 1981) (Fig. 1.1).

1.4.5. Cell-surface proteoglycans/glycosaminoglycans

Proteoglycans, apart from being one of the fundamental components of the extracellular matrix, are also one of the components that make up the "glycocalyx" of mammalian plasma membranes. Most of the data on their role in the plasmalemma have involved cultured cells although some work has been done using isolated hepatocyte plasma membrane (Kjellen, L. et al, 1981).

The predominant proteoglycan/glycosaminoglycan found associated with cell membranes is heparan sulphate (Kraemer, P.M., 1971), although both hyaluronic acid and chondroitin sulphate proteoglycan have also been identified (Underhill, C.B. and Toole, B.P., 1979; Cohn, R.H. et al, 1976).

It is not clear yet whether the glycosaminoglycans/proteoglycans are inculcated into the membrane or whether they are localized on the surface of the membrane. Attempts to release the total membrane associated proteoglycans from the cell surfaces by repeated washing with PBS, alterations in pH and by treatment with various concentrations of EDTA have been unsuccessful. This suggests that the molecules are held in association with the surface by more than mere ionic or electrostatic interactions (Vogel, K. and Dolde, J., 1978). Mild trypsin treatment released most of the glycosaminoglycans associated with the cell surface from cultured cells under conditions causing no cell damage or death. This could indicate that these proteoglycans are covalently bound to the membrane through an inculcated polypeptide.

It has been found that binding of heparin and heparan sulphate to liver cells is reversible and saturable. Heparin could also displace heparan sulphate binding but the reverse was not true. This binding was shown to be specific in that the attempts to displace bound heparin or heparan sulphate using hyaluronate, chondroitin sulphate, dermatan sulphate and under-sulphated heparan sulphate were unsuccessful (Kjellen, L. et al, 1977). This suggest that a certain high level of sulphation may also play a role in the interaction of heparin and heparan sulphate with membranous components.

It has been shown that the synthesis of surface associated heparan sulphate is not effected by the presence of exogenous xyloside in the culture medium, whilst synthesis of medium-associated heparan sulphate was enhanced (Johnston, L.S. and Keller, M.J., 1979). This may indicate that the cell-associated proteoglycans have their own separate biosynthetic pathway (Kraemer, P.M., 1971). Evidence for this comes from tandem label experiments which have indicated that the cell surface heparan sulphate proteoglycans were derived from a small intracellular membrane-associated compartment.

As constituents of membranes, these proteoglycans could serve as receptors and in this respect it has been found that the lipoprotein lipase receptor on endothelial cells appears to be heparan sulphate (Cheng, C-F

et al, 1981). Small molecular weight hyaluronic acid chains have been shown to be able to mask cell surface receptors (Underhill, C.B. and Toole, B.P., 1979) and it is possible that this is a function shared with other cell-associated glycosaminoglycans.

Cell-cell aggregation has also been found to be dependent on the presence of intact endogenous hyaluronate associated with the cell surface (Underhill, C.B. and Dorfman, A., 1978). Chondroitin and oligosaccharides of hyaluronate consisting of six or more sugars were active in blocking both binding to receptors and cell-cell aggregation mediated by the cell surface-associated hyaluronate, whilst sulphated glycosaminoglycans had no effect in this regard.

CHAPTER 2

KINETICS OF CELL GROWTH AND PROTEOGLYCAN PRODUCTION

2.1. INTRODUCTION

The biosynthesis and secretion of proteoglycans has been studied using cultured chondrocytes (Hascall, V.C. et al, 1976; Kimata, K. et al, 1974) and fibroblasts from various sources (Sjöberg, I. and Franssen, L.A., 1977; Sjöberg, I. et al, 1979; Vogel, K.G. and Kendall, F.V., 1980, inter alia) but information concerning the production of proteoglycans by cultured smooth muscle cells is as yet limited (Deudon, E. et al, 1980; Gamse, G. et al, 1978). Likewise, although the types and structures of proteoglycans associated with cartilaginous tissue are well documented (Hardingham, T., 1980), much less is known about the organization and species of proteoglycans found in the arterial wall (Burke, J.M. and Ross, R., 1979). Extracellular molecules that make up the extracellular matrix are in contact with the cell surface and form the structural network in which the cells are associated. These extracellular matrix molecules influence cell shape and cellular adhesion. The cross-linked proteins, collagen and elastin, form the fibrous network of the extracellular matrix, whilst glycoproteins such as fibronectin, chondronectin and laminin in association with proteoglycans contribute the non-fibrous space-filling components (Kleinmann, H.K. et al., 1981). Proteoglycans are mainly responsible for the control of matrix permeability. They also regulate the electrolyte and water content of the matrix (Maroudas, A., 1976; Comper, W.D. and Laurent, T.C., 1978), whereas the glycoproteins are more important for biological adhesion (Kleinmann, H.K. et al., 1981).

Apart from their role in the extracellular matrix, proteoglycans such as heparan sulphate have also been identified as integral membrane components (Kyellen, L. et al, 1981). A number of glycosaminoglycans such as heparan sulphate, chondroitin sulphate and hyaluronic acid have been shown to be associated with the cell surface (Kraemer, P.M., 1971; Vogel, K.G. and Kelley, R.O., 1977; Vogel, K.G. and Dolde, J., 1977). These cell surface proteoglycans may play an important role in cell adhesion (Culp, L.A. et al, 1978) and cell-cell interaction (Underhill, C.

and Dorfman, A., 1978). For instance, a number of studies have indicated that during transformation to the oncogenic state there are marked changes in heparan sulphate content of cell membranes (Underhill, C.B. and Keller, J.M., 1975, 1977; Johnston, L.S. et al, 1979; inter alia).

The aim of this study was to investigate the ability of rat smooth muscle cells to produce proteoglycans and also to characterise the amounts and type made under culture conditions.

2.2. METHODS AND MATERIALS

2.2.1. Preparation of cell culture

Smooth muscle cells were isolated as follows: hearts were dissected out from neonatal rats (1-3 days old), cut into small pieces with a pair of scissors and washed twice in sterile calcium and magnesium-free phosphate buffer saline, pH 7.3. Thereafter the tissue was trypsinized at room temperature (20°C) in freshly prepared 0,1% sterile trypsin. The first harvest of cells obtained after 10 min of digestion was discarded but the next four successive 30 min harvests were collected, the cells pelleted by centrifugation and resuspended in Eagle's minimum essential medium, buffered with Earle's salts. The medium also contained 10% heat-inactivated (30 min at 56°C) foetal calf serum, 60 µg/ml penicillin G and 100 µg/ml streptomycin sulphate. The resuspended cells were pooled after collecting the last digest and seeded into 75 cm^3 tissue culture flasks at 4-5 x 10^6 cells per flask. The cultures were maintained at 37°C for 90 min prior to removal of supernatant medium. The attached cells were washed once with fresh medium to remove cardiomyocytes (Blondel, B. et al, 1971) and after further medium addition cultured at 37°C in a humidified CO_2 incubator (95% air/5% CO_2). Cultures were passaged shortly before confluence at a ratio of 1:4 and the secondary cultures were trypsinized (0,05% trypsin containing 0,002% EDTA) and frozen in liquid nitrogen at approximately 1 x 10^6 cells per 1 ml aliquots in medium containing 10% dimethylsulphoxide. For re-use the frozen cells were thawed and the cell suspension was transferred to 75 cm^3 flasks containing 10 ml of fresh

medium. The medium was changed the next day to ensure the removal of the dimethylsulphoxide. For experimental procedures involving growth of cells in petri dishes, cells were seeded at 10^5 cells per 35 mm culture dish in 2 ml of medium. Medium changes were carried out routinely every three days. All cells used for this project were from the 22nd isolate carried out and designated R22. They were used between the 17th and 25th passage unless otherwise stated, since the proteinaceous composition of the matrix they produced during these passages was consistent and constant in quantity; refer to Table 2.1 showing comparison of matrix proteins. The morphology of cells was continuously monitored by light microscopy and the typical appearance of the culture is shown under the Results section (Plate 2.1).

2.2.2. Determination of cell number

Cell numbers were determined in a coulter counter after dispersion of cells with a mixture of 0,25% (w/v) viokase and 0,05% (w/v) collagenase. Trypsin was inadequate for this purpose late in culture since cells produced an extensive cross-linked trypsin-insensitive matrix which enmeshed cells and resulted in erroneous cell numbers.

2.2.3. Quantitation of matrix protein production

The amount of matrix proteins produced per day during the culture period was determined as follows. Culture medium was removed and cells washed three times with distilled deionized water. Cellular components were dissolved by the addition of 1 ml of 1% (w/v) sodium dodecyl sulphate (SDS) for 30 min and thedishes, with adherent matrix proteins, washed extensively with distilled deionized water, followed by 70% (w/v) ethanol and allowed to dry. The protein on the dishes was dissolved overnight with 1 ml of 2 M sodium hydroxide at 37°C in a humid environment. A 100 µl aliquot of the dissolved protein was taken to determine the amount of protein present by the method of Lowry et al, using bovine serum albumin as standard.

2.2.4. Determination of matrix protein composition

Radioactively labelled matrix components were quantitised according to the procedure of Jones, P.A. et al, 1979. Cell cultures were grown for

Plate 2.1. LIGHT MICROSCOPY OF SMOOTH MUSCLE CELLS IN CULTURE.

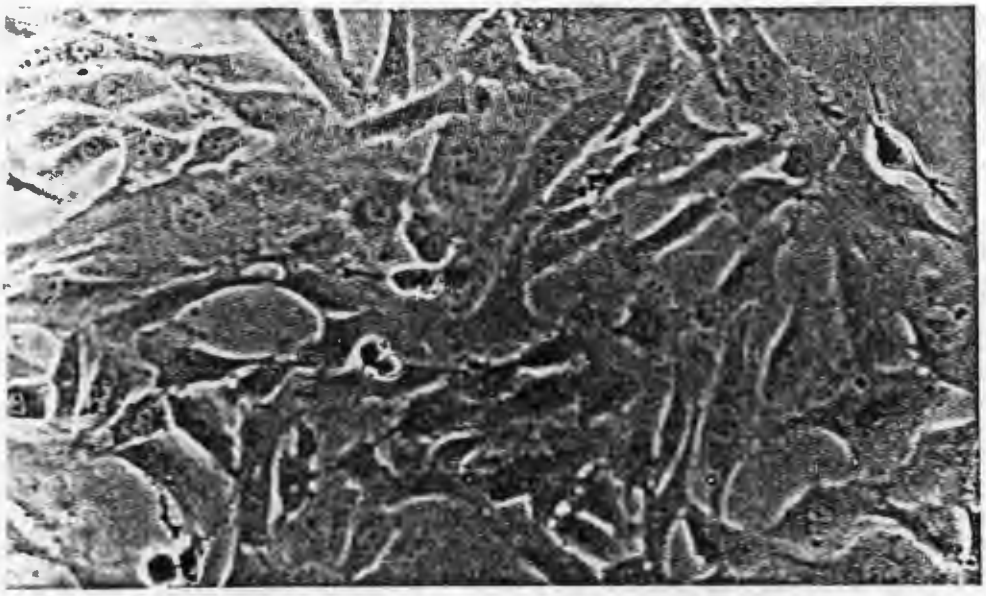
Phase contrast micrographs of (R22) cells in culture:

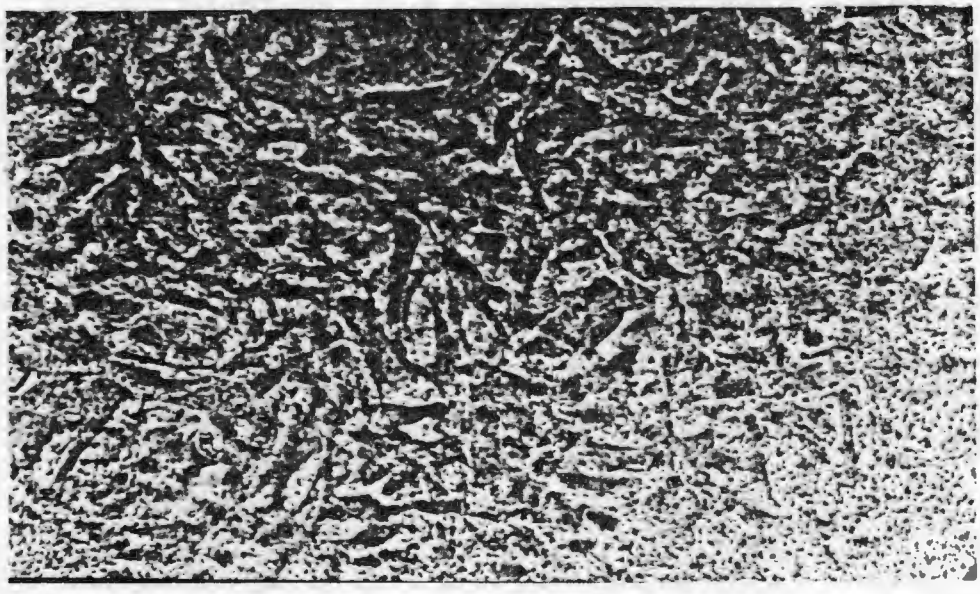
(A) Cells 24 hrs after plating.

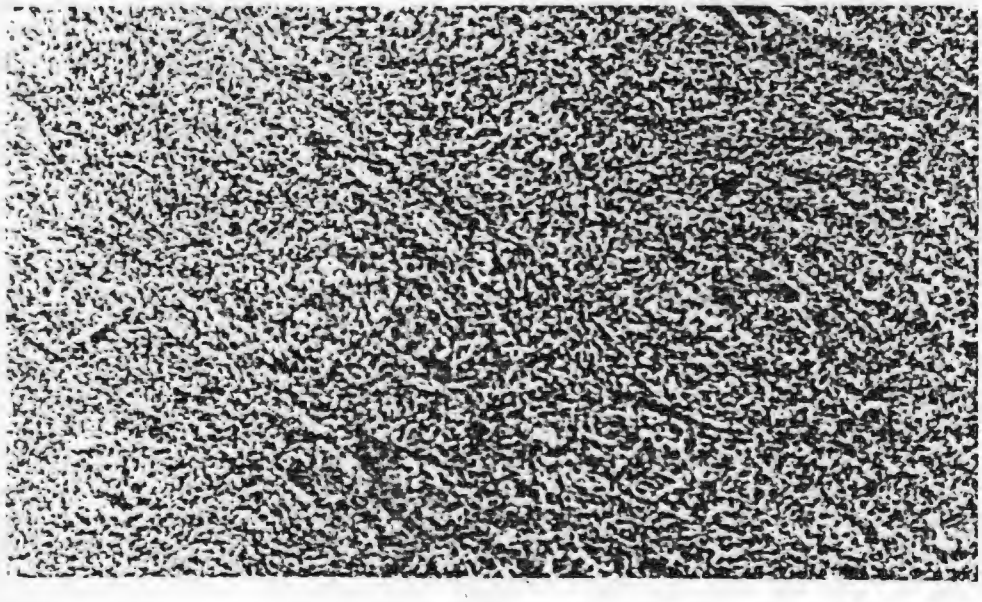
(B) Eight days after plating.

(C) After eighteen days in culture, dark spots clearly showing an extensive extracellular matrix.

Magnification = X250.







seven days in the presence or absence of 50 µg/ml culture medium of ascorbic acid which was added daily. On the seventh day, 10 µl of L-|3,4(n)-H|-Proline was added with fresh medium, and the cells incubated for a further 24 hrs. Following incubation, medium was removed and the radiolabelled matrices prepared as described under Section 2.2.3, except that 1 ml of 0,5 M NH_4OH was used to lyse cells instead of 1% SDS. Dried radioactively labelled matrices were subjected to sequential proteolytic digestion to determine their proteinaceous composition. Enzymes were all used at concentrations of 20 µg/ml in 0,1 M Tris HCl, pH 7.6 containing 10 mM $CaCl_2$. Proteolytic digestion of matrices was performed for 3 hrs, first with trypsin, then elastase and finally collagenase. After each 3 hr digestion period, aliquots were removed for counting. dishes were washed thrice with 2 ml distilled deionized water and the next enzyme addition made. Counting of samples was carried out using scintillation mixture 299 (see appendix) in a Beckman Liquid Scintillation Counter Model LS 9000.

2.2.5. Assay of macromolecular $|^{35}S|$-Na_2SO_4 incorporation into the intracellular, pericellular and extracellular culture compartments.

Cells were cultured as described in Section 2.2.1 for the requisite number of days prior to addition of 10 µCi of $|^{35}S|$-Na_2SO_4/ml culture medium for 24 hrs. At the end of the labelling period the volume of the culture medium was measured and retained as the extracellular compartment and the cell layers washed three times with 2 ml of cold phosphate buffered saline (PBS) containing 30 mM of both NaF and KCN to prevent secretion of macromolecules from the intracellular compartment (Kim, J.J. and Conrad, A.E., 1980). Cell layers were treated with 1 ml of warm 0,1% trypsin in PBS containing 0,004% EDTA for 5 min at 37°C and the resultant cell suspension transferred to a centrifuge tube after gentle aspiration with a Pasteur pipette. Culture dishes were rinsed with an additional 0,5 ml of cold PBS and combined with the cell suspension. Samples were centrifuged for 2 min in a bench centrifuge at 3000 r.p.m. Supernatants were removed and retained as the "pericellular compartment". Cell pellets were lysed by sonification in 1 ml of distilled-deionized water and this fraction was designated as the intracellular compartment. Pericellular fractions were either heated to 100°C for 10 min or 10 µl of foetal calf serum was added to inactivate the trypsin present. The

separation of macromolecular sulphated molecules from unincorporated free radioactive sulphate was routinely carried out as follows. Aliquots (100 µl) of samples from the extracellular, pericellular or intracellular fractions were spotted at the origins of sheets (2 x 60 cm) of Whatman No. 3 MM chromatography paper. Chromatographs were eluted overnight by descending chromatography using 1-butanol, glacial acetic acid, 1 N NH_4OH (2:3:1,5) as solvent (Kim, J.J. and Conrad, A.E., 1980). Under these conditions labelled precursors were eluted from the origin. The macromolecular labelled material did not migrate off the origin in the solvent system used and was quantitised by cutting out paper strips at that position. Chromatograph strips were counted in scintillation cocktail as described under Section 2.2.4. Corrections for background counts due to non-specific binding of labelled precursor to macromolecules at the chromatograph origin were routinely carried out on each chromatogram by running 100 µl aliquots of fresh medium containing the requisite amount of radioactivity as used in any given experiment.

2.2.6. Preparation of proteoglycan monomer from 10-day and 19-day old cultures

Proteoglycan monomers were prepared according to the procedure of Eisenstein, R. et al, 1975. Culture cells were labelled with $|^{35}S|$-Na_2SO_4 either from day 6 to day 10, or from day 10 to day 19. At the end of the labelling period medium was removed and the volume measured. Cell layers were washed twice with 4 ml of PBS, the washes were pooled with the medium fraction. Cell layers were treated with 10 ml of 4 M GuHCl containing 0,05 M Na acetate; 0,1 M 6-amino-n-hexanoic acid; 0.01 M EDTA; 250 mM phenylmethylsulphonyl fluoride and 500 µM benzamidine, all at pH 5.8. Extractions were performed at 4°C for 24 hrs to dissociate the proteoglycans from the other extracellular matrix components. Medium samples combined with PBS washes were made 4 M with respect to guanidinium hydrochloride by addition of solid and a mixture of proteolytic inhibitors added from stock solutions as described above. After additions, the pH of samples was adjusted to 5,8 by addition of glacial acetic acid. After extraction with 4 M GuHCl solid caesium chloride was added to samples to give a final density of 1,5 g/ml and samples were placed in Beckman SW65 polyallomer

tubes (13 ml). The tubes were centrifuged in a SW 65 titanium rotor for 48 hrs at room temperature at an average of 100 000 g. After centrifugation the gradients were fractionated (2.7 ml) using a tube slicer and the uronic acid, sulphate and protein content determined for each fraction (Fig. 2.1) along with the mean density of the fraction. For normal preparative purposes only the bottom 3 ml which contained proteoglycan monomer was collected and dialysed initially against running tap water. Dialysis tubing (molecular weight cut off of 12 000) was processed before use by boiling in a solution of 0,005% EDTA containing 0,2% (w/v) $NaHCO_3$. After dialysis for 36 hrs as described above, samples were dialysed for a further 12 hours against 0,5 M Na acetate pH 6,8. Dialysed samples were freeze dried after the addition of bovine nasal septum proteoglycan monomer (1 mg/ml), prepared as described by Hascall, V.C. and Sajdera, W., 1969), as carrier. The analytical procedure was carried out by Sepharose 4B column chromatography to assess the size of the labelled monomer. Samples of 5 mg were dissolved in buffer (0,5 M Na acetate pH 6,8) and applied to a 6 x 1000 mm column equilibrated with 0,5 M Na acetate buffer pH 6,8. Samples were eluted with the same buffer, fractions collected and counted to determine the elution profile. Columns were calibrated with respect to V_o and V_t using bovine nasal septum proteoglycan monomer and radiolabelled sulphate respectively.

2.2.7. Estimation of cell protein.

To determine the amount of cell protein, aliquots of 50 µl of the intracellular fractions were taken and analysed as described by Lowry, O.H. et al, 1951, using bovine serum albumin as standard.

2.3. RESULTS AND DISCUSSION

2.3.1. Cell growth and matrix protein composition

Rat smooth muscle cells (R22) showed an initial fast rate of proliferation for the first six days after plating at 10^5 cells per dish (Fig. 2.2). After six days, the cells reached confluency and their rate of division slowed down; however, they do continue to divide and start piling up on top of each other. Viable cultures may be maintained for up to four

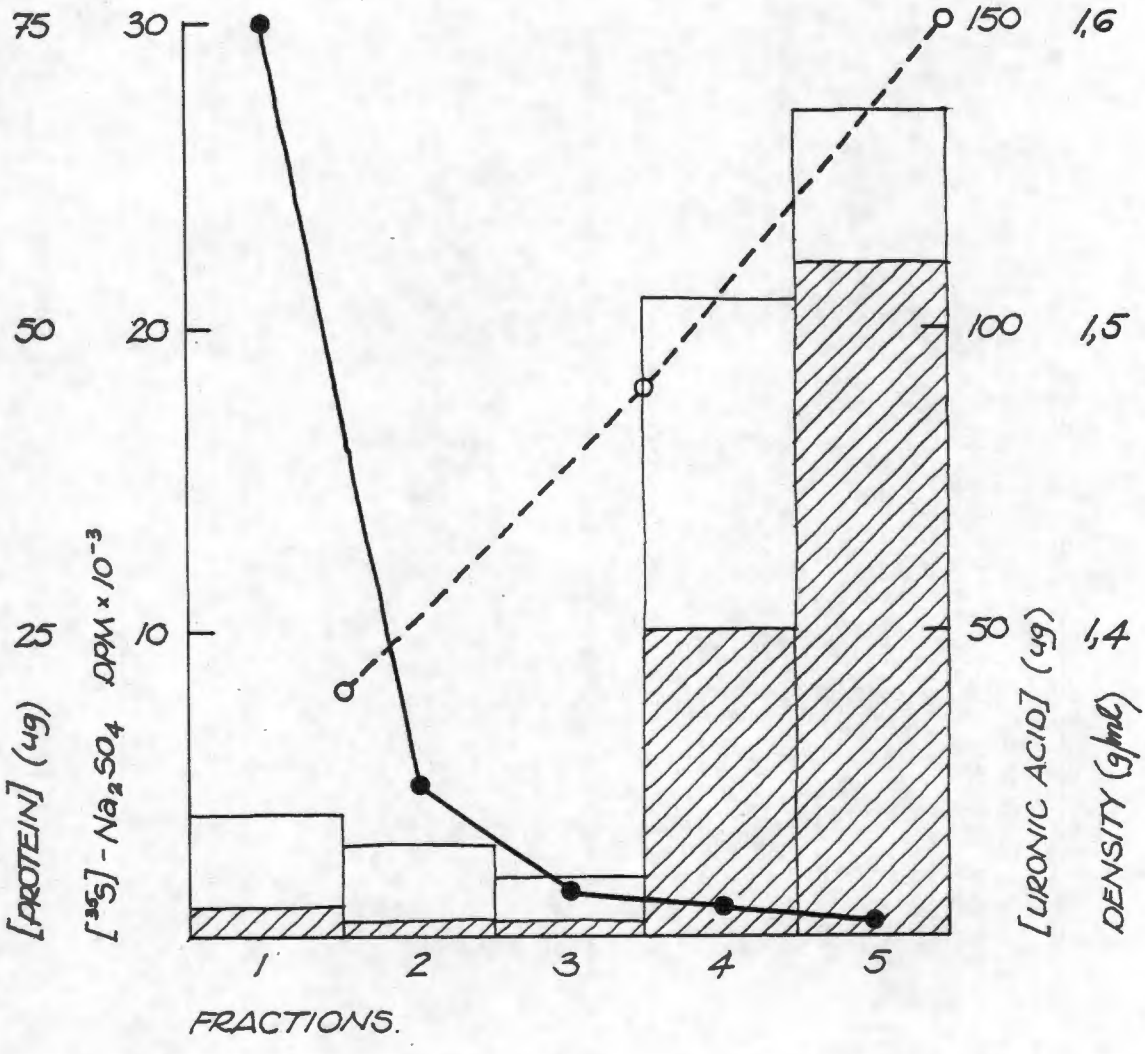


Figure 2.1. ANALYSIS OF DISSOCIATIVE DENSITY GRADIENT CENTRIFUGATION.

Cells (R22) were cultured for 8 days with the addition of 50 µCi $|^{35}S|$-Na_2SO_4 to the culture medium for the final 48 hrs (Section 2.2.6). Culture medium and cell layers were treated with 4 M guanidinum chloride as described in Section 2.2.6 and after mixing with CsCl to a final density of 1,5 g/ml, the solutions were centrifuged for 48 hrs. The centrifuge tubes were divided into 5 fractions (2,7 ml each). Symbols above represent protein concentration —●— and density --O--. The open bars represent the amount of radioactive sulphate and closed bars represent the uronic acid content of each fraction, with fraction five being the bottom one-fifth of the centrifuge tube.

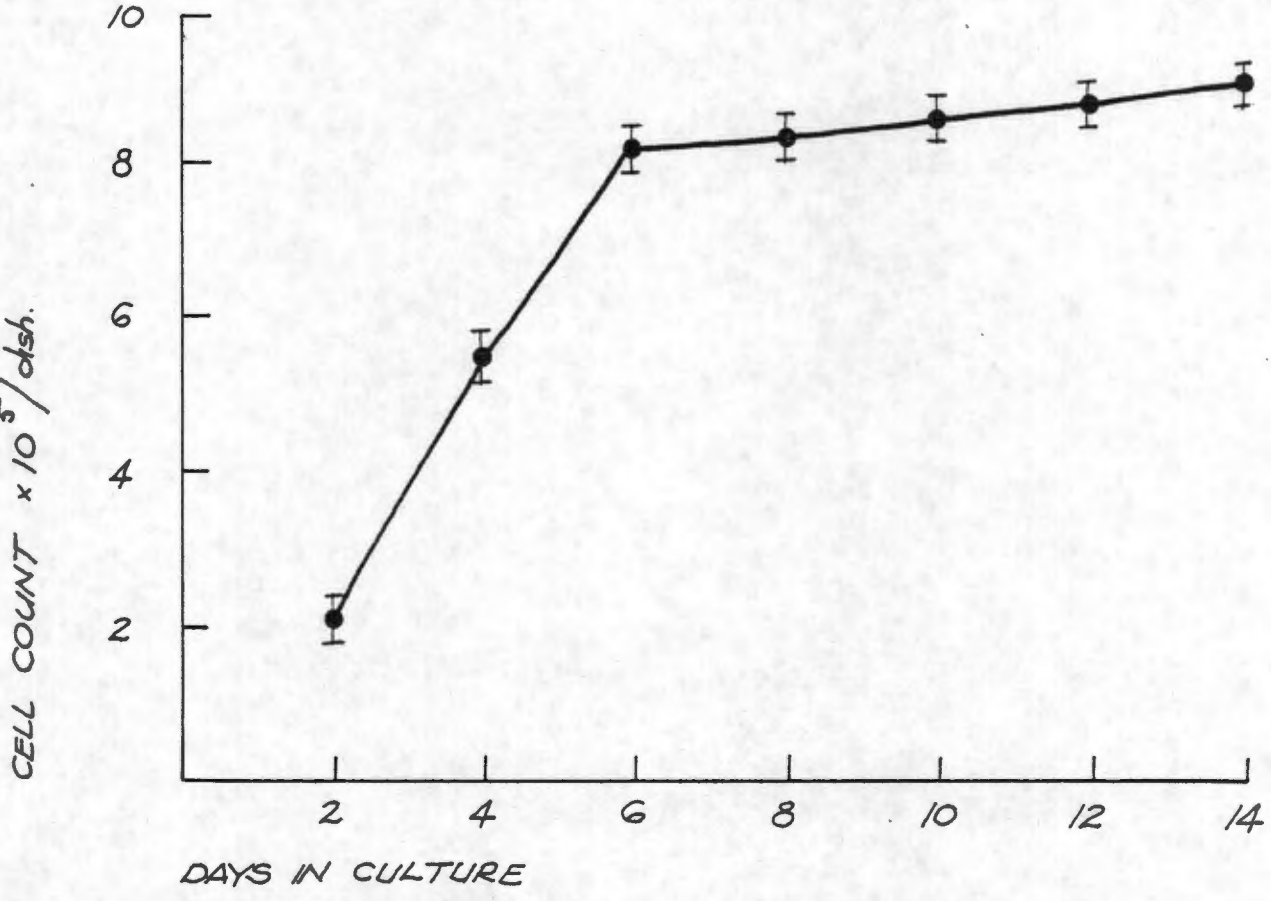


Figure 2.2. GROWTH CURVE OF R22 IN CULTURE.

Smooth muscle cells were plated at 1 x 10^5 / 35 mm dishes and harvested every second day for determination of cell number as described in Section 2.2.2. Each time point represents the mean ± S.D. for 3 cell dishes.

months with regular medium changes and by that stage have formed a thick multi-cellular layer with extracellular matrix material between these layers. Production of cross-linked extracellular matrix protein started early in culture and proceeded steadily throughout the culture period (Fig. 2.3). Cells produced large quantities of matrix proteins such that after 16 days in culture there was over 1 mg of matrix protein laid down on the 35 mm culture dish (Fig. 2.2). The composition of the extracellular matrix proteins is represented on Table 2.1. Although cell growth was unaffected by ascorbate supplementation or deprivation (De Clerck, Y.A. and Jones, P.A., 1980), the matrix composition was altered as shown in Table 2.1. In the presence of ascorbate the culture cells produced 35% trypsin-sensitive material (glycoprotein), 57% elastin and 18% collagen, whilst in the absence of ascorbate the culture cells produce similar amounts of trypsin-sensitive material (30%), but more elastin and only 1% collagen. The total matrix proteins produced were, however, similar in the presence and absence of ascorbate (Table 2.1) (Scott-Burden, T. et al, 1980). The influence of this vitamin on collagen production by these cells is consistent with its accepted physiological role (Prockop, D.J. et al, 1976).

2.3.2. Composition of proteoglycans present in the different culture compartments

Most previous studies using cultured cells had characterized the proteoglycans/glycosaminoglycans present in the culture medium and extractable from the cell layer. This latter fraction consisted of proteoglycans associated with the matrix, cell surface and the intracellular pool. The subfractionation of the cell layer by mild trypsinization results in a fraction of material normally associated with the outside of the cell and the extracellular matrix proteins.

Kraemer, P.M. (1971) demonstrated that the surface material which is released by gentle proteolysis without leakage of intracellular components is rich in heparan sulphate and may represent a metabolically distinct pool (Johnston, L.S. and Keller, J.M., 1979). Glimelius, B.B. et al (1978) started to refer to the trypsin removable fraction as the pericellular compartment and this nomenclature is now accepted

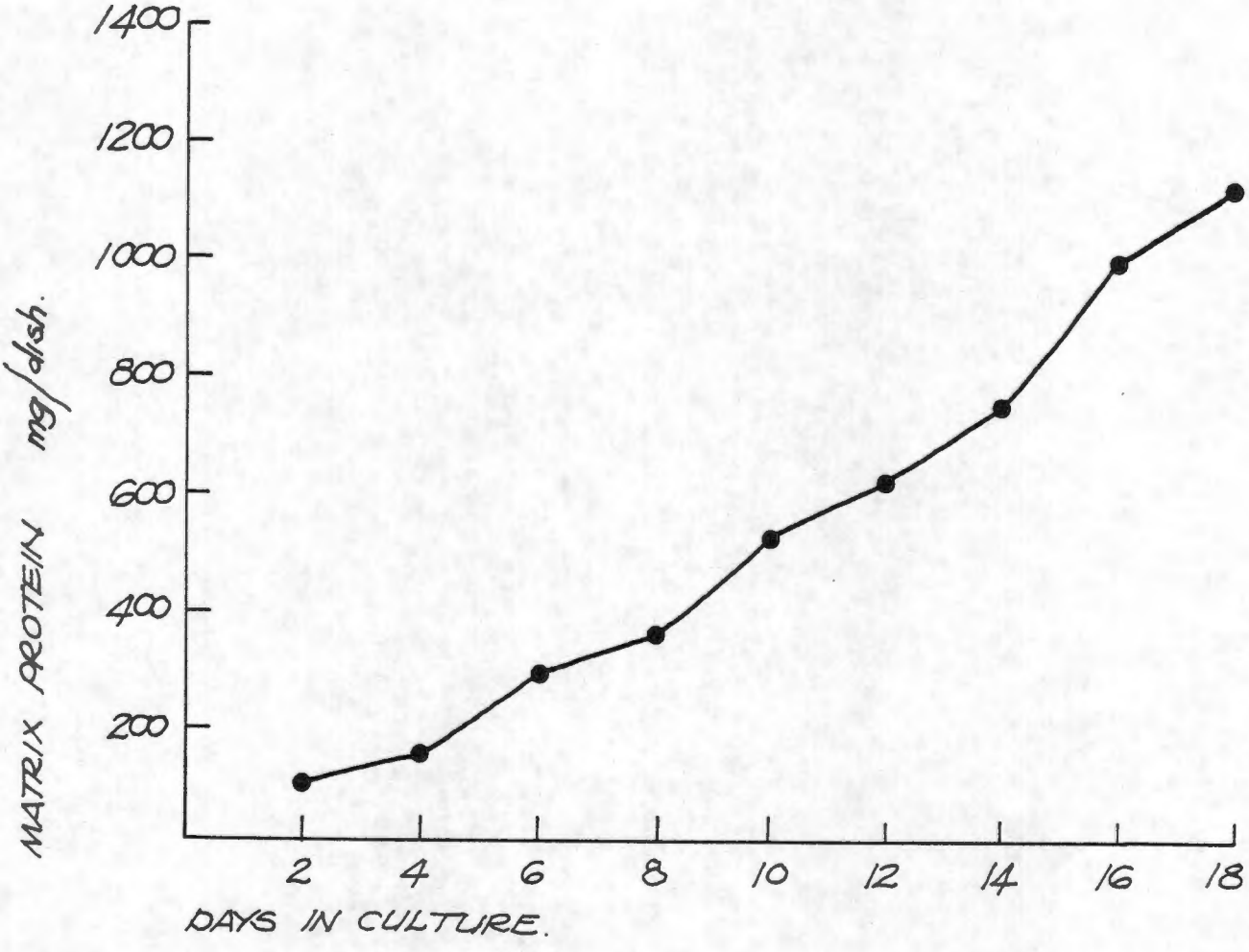


Figure 2.3. MATRIX PROTEIN PRODUCTION OF R22 IN CULTURE.

Smooth muscle cells (R22) were plated at 1 x 10^5/35 mm dishes and harvested every second day for quantitation of matrix proteins as described in Section 2.2.3. The experiment was carried out three times with duplicate dishes for each time point. The data presented represent one such experiment.

TABLE 2.1.

EXTRACELLULAR MATRIX PROTEIN COMPOSITION PRODUCED BY SMOOTH MUSCLE CELLS IN EARLY PASSAGE IN THE PRESENCE OF ASCORBATE AND LATE PASSAGES IN THE PRESENCE AND ABSENCE OF ASCORBIC ACID

Smooth muscle cells were labelled for 24 hrs with 10 μCi/ml [^{3}H]-proline, and extracellular matrix proteins were analysed by sequential enzyme digestion as described in Section 2.2.4. Each value is the mean ± S.D. from 4 cultures.

Ascorbate addition	Total DPM/Dish	DPM released by			Percentage Composition		
		Trypsin	Elastase	Collagenase	Glycoprotein(s)	Elastin	Collagen
Passage 17-25							
+	89277 ±6069	31078 ±1209	41782 ±4006	16417 ± 854	35	47	18
-	79578 ±2452	23783 ±1258	54929 ±1098	865 ±96	30	69	1
Passage 4							
+	53119 ±2792	24179 ±1382	13288 ± 830	15652 ± 580	46	25	29

by most workers in the field.

In this project various agents were used to isolate proteoglycans from the cell surface and/or matrix layer specifically. These included various concentrations of EDTA in phosphate buffered saline solution and heparin. The former had been used by other workers in an attempt to isolate surface proteoglycans from human embryo fibroblasts HLM 18 (Vogel, K., 1978). The latter released 50% of the heparan sulphate from the pericellular compartment according to data reported by Kraemer, P.M., 1977. The results of these studies have been summarized in Table 2.2, and clearly there is some release of radiolabelled material (18%) by heparin at the highest concentration using 5 mg/ml, but EDTA had no effect on either surface or matrix bound proteoglycans (Results not shown). The heparin releasable material represented only heparan sulphate as has been shown for Chinese hamster ovarian cells (Kraemer, P.M., 1977) and did not affect other pericellular associated proteoglycans/glycosaminoglycans such as chondroitin sulphate, dermatan sulphate and hyaluronic acid.

Also shown in Table 2.2 are the results of studies carried out to determine the concentrations of trypsin required to release the pericellular material. Cell viability was observed using the trypan blue exclusion technique and it was assumed that intact viable cells did not release a significant quantity of sulphated proteoglycans to the trypsinate by secretion. In all future experiments cells were routinely treated with 0,1% trypsin for 5 min at 37°C. As a check to confirm that no sulphated material remained associated with the culture dish after removal of cells, dishes were treated with 2 M NaOH for 12 hours and 500 µl of the alkaline suspension was counted after neutralisation with glacial acetic acid. No radioactivity was detected in these samples.

The proteoglycan content of all three culture compartments was assessed over a period of 18 days in culture (Fig. 2.4). Since the data represent the incorporation of $|^{35}S|\ Na_2SO_4$ during the previous 24 hrs at each time point, it is an assessment of the rate of intracellular synthesis and secretion into the two external compartments. For the first 48 hrs there is a sharp increase in the rate of proteoglycan

TABLE 2.2.

RELEASE OF PERICELLULAR PROTEOGLYCANS/GLYCOSAMINOGLYCANS

(a) Release of radiolabelled proteoglycans/glycosaminoglycans by different concentrations of heparin dissolved in PBS was performed on eight-day old cultures (R22) which had been labelled with 10 μCi/ml of radioactive label for the previous 24 hrs. Experiments were performed on ice to minimise secretion from the intracellular compartment, and treatments were for half an hour.

(b) Cells were cultured and labelled for 24 hrs as described above. Trypsin was made up fresh in PBS and incubations were for 5 min at 37°C. In both types of experiment quantitised by determination of proteoglycans, total radioactive sulphate incorporated as described under Section 2.2.5 and cell suspensions were counted, using a coulter counter.

(a)

|HEPARIN| mg/ml	HEPARIN RELEASED $\|^{35}S\|$-Na_2SO_4	TRYPSIN RELEASED $\|^{35}S\|$-Na_2SO_4	TOTAL	PERCENTAGE RELEASED BY HEPARIN
0,05	1666	14704	16371	10%
0,10	1858	13167	15025	12%
1,0	1881	12706	14587	13%
5,0	2696	12539	15135	18%

(b)

|TRYPSIN|	CELL COUNT	$\|^{35}S\|$ RELEASED
0,005%	$1,2 \times 10^5$	7115
0,010%	$1,6 \times 10^5$	8914
0,050%	$4,6 \times 10^5$	14918
0,100%	$5,3 \times 10^5$	13880
0,150%	$5,3 \times 10^5$	13841
0,200%	$5,2 \times 10^5$	13992

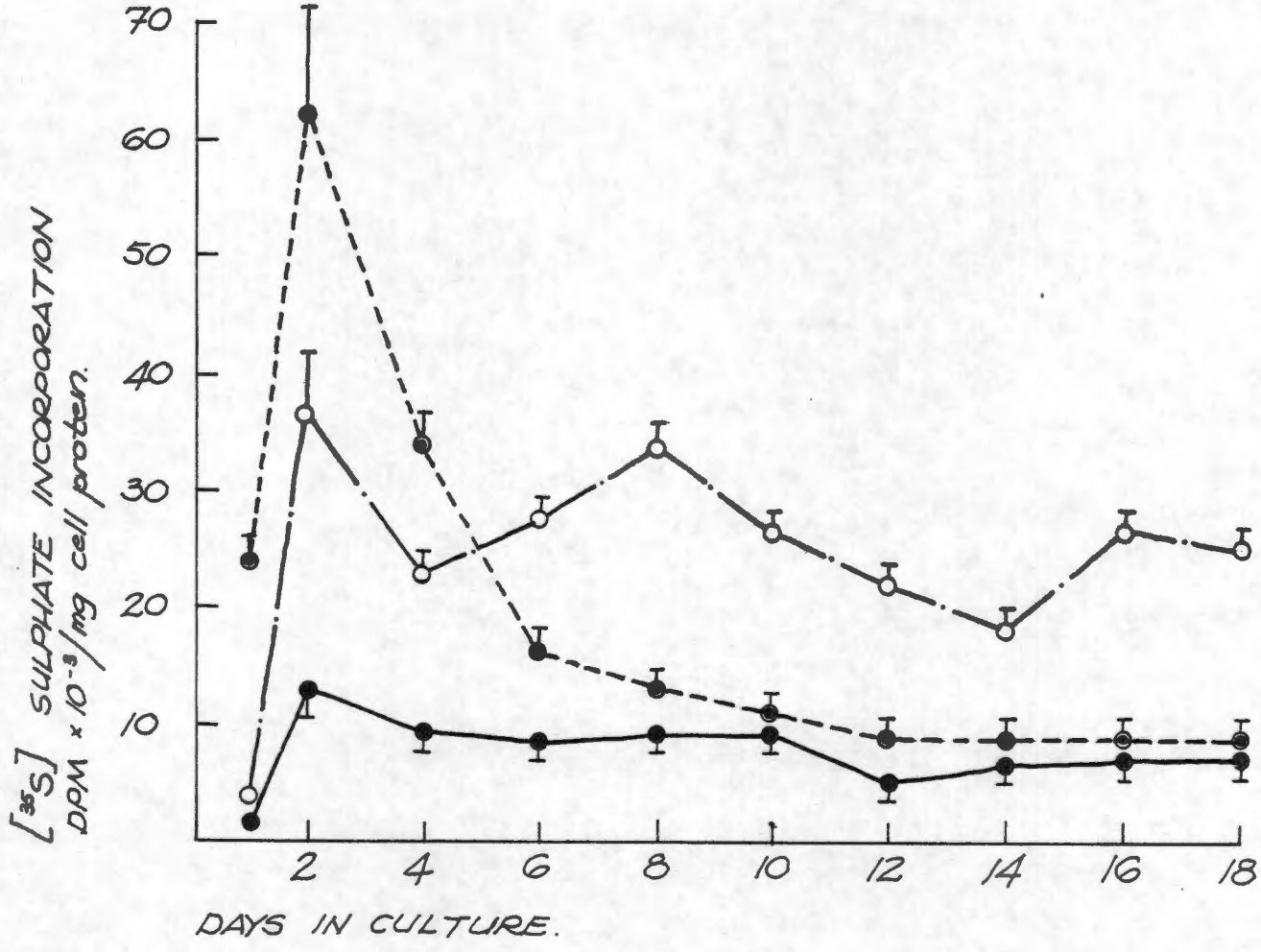


Figure 2.4. INCORPORATION OF $|^{35}S|$-Na_2SO_4 INTO PROTEOGLYCANS.

Smooth muscle cells (R22) were plated 1 x 10^5/35 mm dish, and labelled for 24 hrs with 10 µCi/ml of $|^{35}S|$-Na_2SO_4. At each time as represented on the figure the three culture compartments (extracellular -⊖-, pericellular ··O·· and intracellular —●—) were collected and assayed for their total incorporated radioactive sulphate as described under Section 2.2.5. Each point represents the mean ± S.D. for 3 cell dishes.

production. This takes place at a time when the freshly plated cells are undergoing attachment to their substratum and are busy in the synthesis of proteoglycans involved in the conditioning of their media. In this regard it has now been established that sulphated proteoglycans are components of cell surface and play a role in cell substrate adhesion (Culp, L.A. et al, 1978). Thus the rapid rates of synthesis and secretion in the first 48 hrs may be the direct result of events discussed above.

Thereafter (Fig. 2.4) the specific activity of the extracellular compartment decreases during the culture period to reach an equilibrium level by the twelfth day of culture. This drop in macromolecular sulphate specific activity of this compartment may be related to the action of ecto-sulphatase enzyme(s) discussed in Chapter 4, or it may be just a reflection of the increase in cell protein during progressive culture of cells.

The intracellular specific activity remained fairly constant throughout the culture period, indicating that the rate of synthesis of sulphated proteoglycans remains constant during the culture period but as may be seen from the figure 2.4, the extracellular (pericellular and culture medium) distribution of macromolecules varies over this time.

The rates of synthesis in the pericellular compartment suggests a biphasic pattern. After the initial high rates seen after 24 hrs in culture, there is a decrease in the specific activity of the pericellular pool which then gradually increases again to peak at day 8. This pattern is repeated again with a further peak apparent at day 16. This variation in specific activity was reproducible and not related to the nutritional status of the culture, since during these experiments fresh medium was given to all cells every 48 hrs. In other words, at day 2 dishes were taken for analysis, and the remainder given fresh medium with the same taking place at day 4, etc. Since one of the principal areas of interest of this study related to the pericellular peoteoglycans, cultures were routinely grown up to 8 days for experiments. Treatment and experiments were designed to take place such

that the eighth day represented the mid-point of the experimental period.

Analysis of the size heterogeneity of proteoglycans, synthesised by smooth muscle cells at two different times during the eighteen days culture period, was carried out using Sepharose 4B chromatography (Fig. 2.5). The results of these analyses indicated that at least two main size classes of proteoglycan monomer existed, namely a large species that eluted in the void volume of the column and a smaller molecular weight species. The large-sized monomer (V_o) that was isolated from both early (day 10) and late (day 18) cultures was present in both the culture medium (extracellular compartment) and guanidine extract of the cell layer (intracellular and pericellular compartment). The proportions of V_o proteoglycan monomer, however, varied in relation to the amount of included small proteoglycan monomer that was present. Also, the small proteoglycan monomer in the culture medium was larger (K_{av} 0,38) than the guanidine-extracted cell-associated small proteoglycan monomer, K_{av} 0,58 (Fig. 2.6). The monomers extracted at the end of the culture period showed an increase in the relative amount of the small proteoglycan monomer in both compartments. This may be due to increased degradation of proteoglycans by the long cultured cells as a result of their normal turnover pathways.

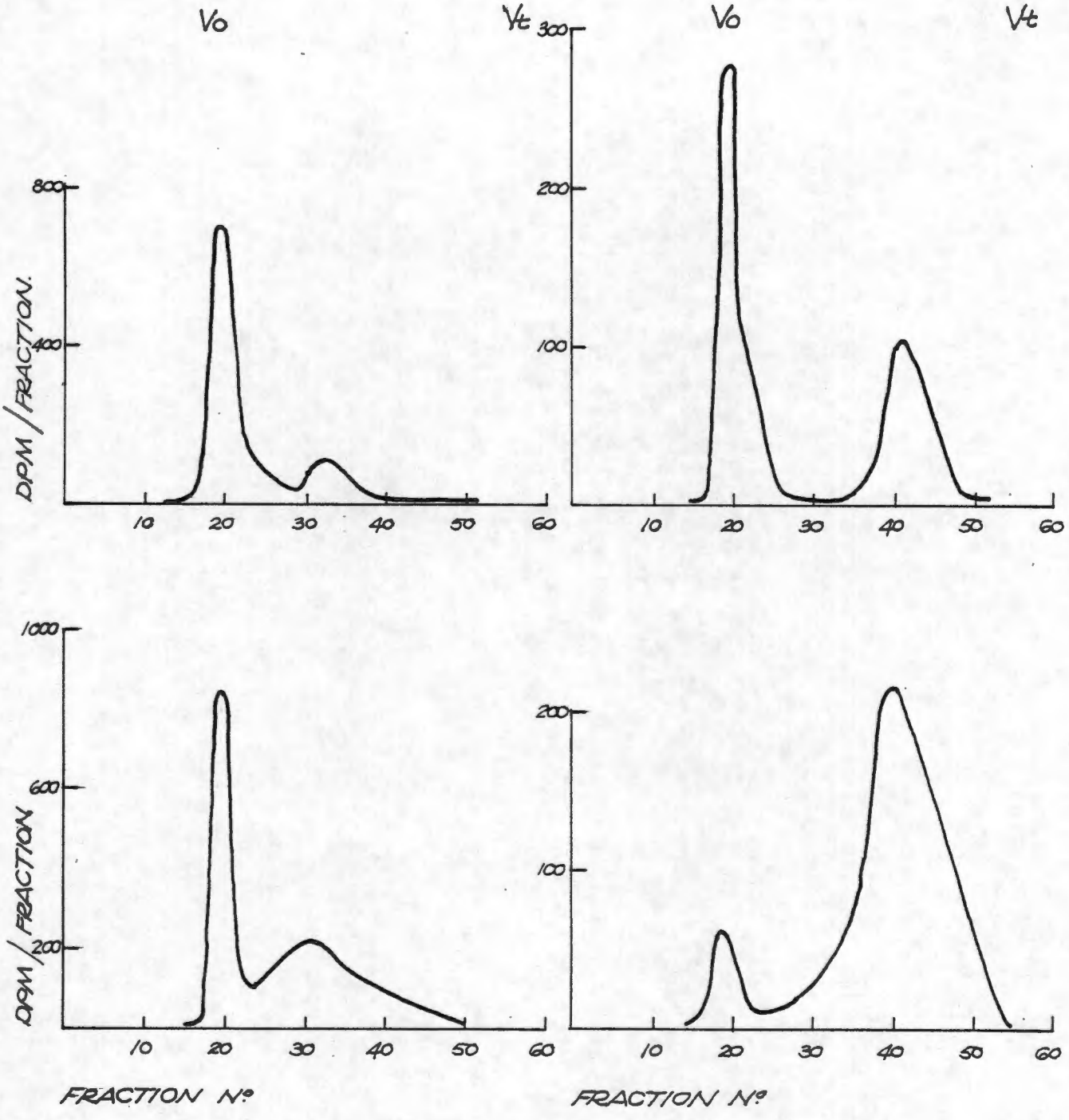


Figure 2.6. MOLECULAR EXCLUSION CHROMATOGRAPHY OF THE PROTEOGLYCAN MONOMER(S) FROM THE CULTURE MEDIUM AND CELL LAYER EXTRACT.

Cells were grown as described in Section 2.2.1 and labelled with 50 µCi $|^{35}S|$-Na_2SO_4 for 72 hrs. Culture medium and cell layers were treated with 4 M guanidinum chloride mixed with CsCl and centrifuged for 48 hrs. The bottom 3 ml were pooled, dialysed and freeze-dried. Samples (5 mg) were dissolved in 0,5 M Na acetate buffer pH 6,8 and applied to the Sepharose 4 CLB column as described in Section 2.2.6.

2.4. SUMMARY

On reaching monolayer, cultured rat smooth muscle cells showed a decrease in the rate of proliferation. However, growth continued with cells piling upon one another and surrounded by an extensive extracellular matrix of collagen, elastin and glycoproteins. In the presence of ascorbate these cells producedmore collagen at the expense of elastin, whilst in scorbutic cultures the converse was found. Proteoglycans were synthesized by these smooth muscle cells at all stages of culture and secreted into two distinct extracellular compartments, namely the culture medium (extracellular) and the trypsin-sensitive, pericellular compartment. This compartment showed a biphasic pattern of synthesis with two reproducible peaks of activity on the eighth and sixteenth days of culture. Two sizes of proteoglycan monomer were synthesized by the smooth muscle cells and they were associated with both the culture medium and the cell layer. There were differences in the size of the small proteoglycan monomer associated with the two culture compartments, and furthermore, the proportion of the small species increased with time in culture.

CHAPTER 3

SMOOTH MUSCLE CELL CULTURE AT DYNAMIC EQUILIBRIUM: THE EFFECTS OF VARIOUS EXOGENOUS AGENTS ON THIS EQUILIBRIUM

3.1. INTRODUCTION

In Chapter Two, smooth muscle cells were shown to have reached a dynamic equilibrium after 6-8 days in culture in respect of their proteoglycan synthesis, turnover and growth rate. There was a slight increase in the rate of deposition of sulphated proteoglycans into the matrix on the eighth day of culture (Fig. 2.3) which coincided with the visible appearance of cross-linked matrix proteins. Since the biosynthesis of matrix-associated proteoglycans was of particular interest to the project, it was decided to characterize the rat smooth muscle system in terms of the amount and the types of proteoglycans synthesized by cells during the 8 days in culture. These findings would have a bearing on the latter studies in regard to turnover of the pericellular proteoglycans.

It was also important to assay the effect of a number of exogenous agents on the biosynthetic capacity of our system, and once again it was considered, for the reasons set out above, that this should be carried out once cells had reached a dynamic equilibrium in terms of growth and metabolism.

In chondrocyte culture systems, it had been noted that addition of hyaluronate to the culture medium inhibited the biosynthesis of sulphated proteoglycan molecules up to 50% in 24 hrs (Handley, C.J. and Lowther, D.A., 1976). However, fibroblasts were unaffected by hyaluronate addition to the culture medium (Wiebkin, O.W. et al, 1975). The mode of action of hyaluronic acid in the chondrocyte cultures has not as yet been elucidated. The effect of added hyaluronate to culture medium of rat smooth muscle cells is discussed in this chapter.

Tunicamycin, an antibiotic, has been shown to block dolichol-diphospho-N-acetylglucosamine synthesis, resulting in the inhibition of transfer of short oligosaccharides to the asparagine residues during glycoprotein synthesis (Kuo, S.C. and Lampen, J.O., 1974; Tkacz, J.S. and Lampen, J.O.,

1975). The antibiotic has been successfully used to suppress synthesis of extracellular glycoproteins like fibronectin (Olden, K. et al, 1978) which contain N-linked carbohydrate chains. It does not, however, affect synthesis of glycoproteins such as collagen containing predominantly O-linked polysaccharide moeities (Olden, K. et al, 1978; Duksin, D. and Bornstein, P., 1977). Incubations of rat cultures (R22) with tunicamycin were performed to investigate the role of N-linked polysaccharides in the structure and synthesis of proteoglycans by this system, as has been done for fibroblasts and chondrocyte cultures (Pratt, M.R. et al, 1979).

The discovery that several β-D-xyloside derivatives are able to substitute in vivo for the protein core in the initiation of chondroitin sulphate polysaccharide synthesis has prompted much speculation on the rate-limiting steps of proteoglycan synthesis (Schwartz, N.R. et al, 1974; Gibson, K.D. and Segen, B.J., 1977, inter alia). It has been shown that added β-D-xyloside derivatives enhance synthesis of chondroitin sulphate found in the medium up to 10-fold (Schwartz, N.R. et al, 1974). Studies using rat serosal mast cells have shown that β-D-xyloside treatment resulted in increased chondroitin sulphate synthesis at the expense of heparan sulphate synthesis, the normal predominant species of glycosaminoglycan chain found associated with the mast cell proteoglycans. This suggests that β-D-xyloside favoured the polymerization of chondroitin sulphate moieties rather than heparan sulphate. The implications here in terms of the specificity of the protein core are important (Stevens, R.L. and Austen, K.F., 1982). Furthermore, since the xyloside has no effect on the pericellular proteoglycans (Johnston, L.S. and Keller, J.M., 1979), which in many systems are made up of a large proportion of heparan sulphate, at least two intracellular sites of synthesis for peptido heparan sulphate may exist: one amenable to xyloside manipulation that becomes associated with the culture medium, and one that is inaccessible to xyloside pretreatment (Johnston, L.S. & Keller, J.M., 1979) and associated with the plasma membrane (Kramer, P.M., 1971, inter alia). The effect of p-nitrophenyl-β-D-xyloside on the synthesis of proteoglycans by rat smooth muscle cells is also reported in this chapter.

3.2. METHODS

3.2.1. Determination of total accumulated macromolecular $|^{35}S|$-Na_2SO_4 and uronic acid in cell culture compartments after eight days.

Smooth muscle cells were plated at 10^6 cells/75 cm³ tissue flask in 10 ml of culture medium. After 24 hrs when all viable cells were attached, the medium was removed and replaced with 10 ml fresh medium containing 50 µCi $|^{35}S|$-Na_2SO_4. Flasks, either supplemented daily with ascorbic acid (final concentration of 50 µg/ml culture medium) or maintained under scorbutic conditions, were incubated in a humidified CO_2 incubator at 37°C as described in previous Section 2.2.1. After four days, medium was removed, measured (Medium I) and retained for analysis. Cultures received fresh medium containing radioactive sulphate as above and were maintained for a further 3 days at which stage medium was removed as described above. Cell layers were rinsed 3 times with 4 ml of phosphate buffered saline and the washes added to the batches of cultured media. A solution of 0,1% trypsin (4 ml) containing 0,004% w/v EDTA was added for 10 min at 37°C. Trypsinates were removed and cell suspensions so obtained centrifuged at 3000 rpm for 2 min. Cell pellets were washed by centrifugation with 2 ml of phosphate buffered saline and washings were added to the trypsin supernatants. Washed cell pellets were lysed by sonication with a "Sonifer" (Branson Sonic Company) sonicator after suspension in 2 ml of distilled-deionized water. The determination of total accumulated macromolecular $|^{35}S|$-Na_2SO_4 was carried out on aliquots of exhaustively dialysed media, trypsinate and cell lysate fractions using the methods described under Section 2.2.5.

3.2.2. Determination of total accumulated uronic acid.

Uronic acid was determined by the procedure of Bitter, T. and Muir, H., 1962, which had been adapted for use with the Technicon autoanalyser. Samples of fresh dialysed medium were analysed to determine the background uronic acid content of this fraction, values obtained ($\pm$ 60 µg/ml) were subtracted from the values determined on medium samples from cell cultures.

3.2.3. Determination of intracellular protein concentrations.

The protein concentration of intracellular samples obtained as described above was performed on aliquots (25 µl) of cell lysates using the method of Lowry, O.H. et al (1951) using bovine serum albumin as standard.

3.2.4. Characterization of glycosaminoglycans accumulated by cultures over eight days incubation.

Aliquots (2 ml) from each sample obtained as described under Section 3.2.1. were digested for 6 hrs with pronase (1 mg/ml) to ensure total degradation of the protein core and release of free glycosaminoglycan chains. After digestion, the samples were boiled for 10 min to denature the enzyme, and then were dialysed for 48 hrs against 10 litres of distilled-deionized water to remove free, unincorporated radioactive sulphate. Dialysed samples were made 0,1 M with respect to sodium acetate at pH 6.8, and aliquots (400 µl) removed for treatment with either chondroitinase ABC or AC. Further aliquots were removed for treatment with nitrous acid (Vogel, K.G. and Kendall, V.F., 1980). Following chemical or enzymatic treatments, samples (pericellular and extracellular) were chromatographed on a Sephadex G-50 column (0,5 x 100 cm) which was equilibrated with pyridine acetate pH 5,0 (Kapoor, R. et al, 1981). Samples were eluted with the same buffer and fractions collected and counted as described under Section 2.2.4. Columns had been calibrated for V_o and V_t using bovine nasal septum cartilage proteoglycan monomer and radioactive sulphate respectively. Untreated samples were also analysed on the same column to establish the total material eluting in the V_o fraction.

Treated samples derived from the intracellular compartment were analysed on commercially prepared cellulose thin layer chromatography plates (see appendix). After streaking 20 µl aliquots at the origin, plates were developed by ascending chromatography using n-butanol:ethanol:water (52:32:16) as the first solvent. This procedure removed unincorporated free radioactive sulphate from the origin of the plates. Thereafter, the plates were dried and a second solvent was used, consisting of n-butanol:acetic acid:1 N NH_4OH (2:3:1), to separate the disaccharides and small oligosaccharides generated by the chemical and enzymatic procedures from macromolecular material at the origin. After development

with the second solvent, plates were again dried and stained, using a solution of 0,2% (w/v) toluidine blue in 95% (v/v) ethanol. Macromolecular material at the origin was removed by scrapping and counted as described earlier (Section 2.2.4).

To assay the hyaluronic acid content of the various samples, aliquots of the intracellular, pericellular and extracellular samples (Section 3.2.1.) obtained from cultures maintained for 8 days in the presence of $|^{3}H|$-glucosamine (25 µCi/ml) were analysed as follows: After treatment with pronase (1 mg/ml) for 6 hours as described above in Section 3.2.1, aliquots were made to 0.1 M with respect to Na acetate pH 6.0 and 0,65 M with respect to sodium chloride and were digested with hyaluronidase for 3 hours at 37°C. Digested samples were analysed on Sephadex G-50 columns as described above in Section 3.2.4.

3.2.5. The effect of addition of either hyaluronic acid or Tunicamycin on the biosynthesis of sulphated proteoglycans.

Smooth muscle cells (R22) were grown for 8 days with regular medium changes as described in Section 2.2.1. On the 8th day, culture medium was replaced with 2 ml of fresh medium containing 100 µg ascorbic acid, 20 µCi $|^{35}S|$-Na_2SO_4 and either hyaluronic acid or tunicamycin at concentrations designated in Table 3.3 or 3.4. Cells were returned to the incubator and maintained for a further 24 or 48 hrs prior to the fractionation of cultures into the intracellular, pericellular and extracellular compartments, and quantitation of radioactively labelled macromolecules was performed as described under Section 2.2.5.

3.2.6. The effect of p-nitrophenol-β-D-xyloside on the biosynthesis of sulphated proteoglycans.

These experiments were performed essentially as described under Section 3.2.5., except that all cultures received 0.5 mM p-nitrophenyl-β-D-xylopyranoside dissolved in dimethylsulfoxide instead of tunicamycin or hyaluronic acid. Following incubation for 24 hrs the same analytical procedures were performed on the cultures as described under Section 3.2.5. Control cultures were treated with dimethyl sulphoxide alone and it never exceeded 1% v/v.

3.3. RESULTS AND DISCUSSION

3.3.1. Determination of the total accumulated $|^{35}S|$-Na_2SO_4 and uronic acid in cell culture compartments after eight days in culture.

When smooth muscle cells have reached the end of their rapid proliferative phase in culture, which occurs once they have reached confluency, they showed an increase in their secretory synthetic capacity (Chamley-Campbell, J. et al, 1979).

Rat smooth muscle cells (R22) appear to show the same features as those described above such that by day 8 of culture they have attained a dynamic equilibrium in respect to their synthetic capacity. Biosynthesis and turnover of matrix components will have reached a "steady state". The characterization of the sulphated proteoglycan production at this stage was carried out as described under Methods Section 3.2.1. and 3.2.2.

The total amount of uronic acid and radioactively-labelled sulphated macromolecules associated with each compartment are presented in Table 3.1. The distribution of radiolabelled sulphate (into macromolecules) showed that, when assessed on a radioactivity basis alone, the extracellular compartment contained the highest levels, with scorbutic cultures accumulating some 44% more radioactivity in this compartment than cultures grown in the presence of ascorbic acid. This is balanced by their respective pericellular compartments which accumulated less radiolabel under scorbutic conditions. A truer reflection of the synthesis of sulphated proteoglycans is however obtained when the specific activity of each culture compartment is assessed. This datum clearly showed that the pericellular compartment has a significant increase in specific activity of radioactive sulphated proteoglycans synthesized in the presence of ascorbic acid, whether based on the total cell protein or uronate content of the respective compartments.

This increase suggests that ascorbic acid may be involved in the sulphation of proteoglycans either directly or as a cofactor. Controversy surrounds this type of observation, since it has been suggested that ascorbic acid-2-sulphate plays a role in biological sulphation

TABLE 3.1.

SULPHATE AND URONIC ACID CONTENT AT "STEADY STATE"

Cells (R22) were cultured and labelled with 50 µCi $|^{35}S|$-Na_2SO_4 for 8 days as described under Section 3.2.1. Quantitation of radioactively labelled macromolecules and uronic acid in the three culture compartments was performed as described in Section 3.2.1. and 3.2.2. The values in the Table are the means of quadruplicate (DPM) or duplicate (µg uronate or protein) determinations within one experiment. Experiments were performed twice and the results were in good agreement with each other.

	Ascorbate presence	DPM	Uronic Acid (µg)	DPM/ Uronic Acid (µg)	DPM/Cell Protein (µg)	Uronic Acid (µg)/ Cell Protein (µg)
Extracellular	+	234658	320	733	183	0,17
	-	362208	480	756	221	0,23
Pericellular	+	129275	420	308	101	0,22
	-	96803	558	174	59	0,27
Intracellular	+	18442	42	439	16	0,02
	-	20456	53	386	11	0,02

pathways (Hatanaka, H. and Egami, F., 1976). This appears to have been disproved by Shapiro, S.S. and Poon, J.P. (1975). It has been shown that the presence of ascorbic acid increased deposition of collagen (Table 2.1), which has the capacity to interact with sulphated glycosaminoglycan chains on proteoglycans. Thus the initial observations of increased radioactive sulphate incorporation into the pericellular compartment in the presence of ascorbic acid could be related to the increased collagen present there. However, as the values illustrate in Table 2.1, there is no concomitant increase in uronic acid content of the pericellular compartment under the same conditions. An important observation from this Table is that the specific activity expressed as uronic acid/cell protein differs only marginally within the culture compartments from ascorbic acid-supplemented or deprived cultures.

Therefore, the conclusion mentioned above, namely ascorbic acid influencing sulphation levels of proteoglycans, seems the most likely from the data presented in Table 3.1.

When the types of glycosaminoglycans found associated with each culture compartment after eight days of culture were analysed, it was apparent that these smooth muscle cells synthesize heparan sulphate, chondroitin sulphate and dermatan sulphate, but no detectable hyaluronic acid (Table 3.2).

Heparan sulphate was found to be the main glycosaminoglycan present in the pericellular and the intracellular compartments, whilst very little heparan sulphate was associated with the extracellular compartment. These findings are in agreement with findings of Kraemer, P.M. (1971) and Vogel, K.G. and Kendall, V.F. (1980) for cultured Chinese hamster ovarian cells and fibroblasts.

Chondroitin sulphate was found predominantly in the extracellular compartment both in the absence and presence of ascorbate.

In the presence of ascorbic acid there was an increase in the amount of dermatan sulphate associated with the pericellular compartment, which agrees with the current theories relating to the former's association

TABLE 3.2.

COMPOSITIONAL ANALYSIS OF SULPHATED GLYCOSAMINOGLYCANS

Compositional analysis of glycosaminoglycans was performed as described in Section 3.2.4 using specific enzymatic digestions or nitrous acid treatment. The values represent the DPM associated with each class of glycosaminoglycan as analysed in a 1 ml sample with the figures in parentheses representing the percentage of the total of each specific glycosaminoglycan analysed. Abbreviations used in the Table were as follows: HA, hyaluronate; HS, heparan sulphate; CS, chondroitin sulphate and DS, dermatan sulphate. The analysis represents the mean of duplicate determinations from one experiment.

Compartment	Ascorbate Presence	Total DPM in each Glycosaminoglycan class			
		HA	HS	CS	DS
Extracellular	+	0	1073(19)	4348(77)	225(4)
	-	0	1785(20)	5540(62)	1607(18)
Pericellular	+	0	5717(62)	2028(22)	1475(16)
	-	0	4786(74)	1164(18)	517(8)
Intracellular	+	0	3360(57)	2040(34)	540(9)
	-	0	5200(68)	1223(16)	1223(16)

with collagen. In the absence of ascorbic acid there is some evidence of dermatan sulphate accumulation intracellularly prior to its secretion into the culture medium (extracellular compartment).

In the absence of ascorbic acid more heparan sulphate is present in the pericellular and intracellular compartments. From Table 2.1. it may be seen that these smooth muscle cells produce more elastin in the absence of the vitamin supplementation, and this could account for the increased heparan sulphate content of the pericellular fraction under scorbutic conditions since the former is apparently associated preferentially with elastin (Radhakrishnamurthy, B. et al, 1977).

In Chapter 2 (Section 2.3.2) the fact that 18% of the heparan sulphate in the pericellular compartment could be displaced by heparin was discussed. That observation may be due to there being two populations of peptido-heparan sulphate having different binding affinities for pericellular molecules; the low affinity binding species being readily displaced by heparin, whilst the high affinity species require more severe treatment, e.g. enzymatic digestion, for removal. This is consistent with the current thinking about the pericellular heparan sulphate proteoglycans, since it has been suggested that in some systems peptido-heparan sulphate is inculcated into the plasma membrane, whilst other species are merely associated with the pericellular matrix components or free in the culture medium under tissue culture conditions. The heparan sulphate associated with the cell surface may be a metabolically distinct pool (Johnston, L.S. and Keller, J.M., 1979).

3.3.2. The effect of hyaluronic acid on the biosynthesis of sulphated proteoglycans by rat smooth muscle cells.

When smooth muscle cells (R22) were incubated in the presence of hyaluronic acid (K_{av} 0,43), as described under Section 3.2.4., the incorporation of radioactive sulphate was unaffected (Table 3.3). This is diametrically opposite to what has been found using cultured chondrocytes (Handley, C.J. and Lowther, D.A., 1976). In all three culture compartments the levels of radioactive macromolecular sulphate was within 10% of one another (Table 3.3). This ineffectiveness of hyaluronate in the rat smooth

TABLE 3.3

THE EFFECT OF HYALURONIC ACID ON SULPHATED PROTEOGLYCAN SYNTHESIS

Cells (R22) were cultured for 8 days and labelled for 24 hrs with 20 µCi $|^{35}S|$-Na_2SO_4. Radioactively labelled macromolecules were quantitized in all three compartments as described in Section 3.2.5.

	|HYALURONIC ACID| (µg/ml)		
	0	100	300	400
Extracellular	54996 ±1556	57343 ±1687	55450 ± 776	55934 ± 933
Pericellular	135706 ± 2778	138638 ± 1039	130690 ± 3712	131113 ± 1648
Intracellular	23113 ± 325	23047 ±1831	21074 ± 616	21326 ± 510

muscle system may be due to the fact that these smooth muscle cells do not synthesize hyaluronic acid and are thus unable to recognize it as an effector or regulator as reported for chondrocytes (Handley, C.J. and Lowther, D.A., 1976). However, it has also been reported that hyaluronic acid does not effect sulphated proteoglycan synthesis in cultured fibroblasts which do, however, synthesise hyaluronic acid as one of their glycosaminoglycan chains. It has not been shown yet whether hyaluronic acid is able to enter cells (Wiebkin, O.W. et al, 1975) and thus exert an effect on the synthetic mechanisms for proteoglycans. It has been proposed that there are receptors on the cell surface that can recongize hyaluronic acid, and an intracellular effector that can specifically repress either synthesis of the protein core or xylosyl-transferase activity at the level of transcription or translation. These receptors may be absent in the rat smooth muscle cells (R22).

3.3.3. The effect of Tunicamycin on the biosynthesis of sulphated proteoglycans by rat smooth muscle cells.

When smooth muscle cells (R22) were cultured in the presence of tunicamycin as described under Section 3.2.5, their rates of biosynthesis of radioactive sulphated proteoglycans were unaffected as compared to control cultures without the antibiotic (Table 3.4).

In some instances, at the lowest dose used (0,05 µg/ml) there was some stimulation of $|^{35}S|$-Na_2CO_4 incorporation over the first 24 hrs of incubation (Table 3.4). However, this stimulation was not seen reproducibly and was absent at later times in culture (48 hrs). At the same concentration of the antibiotic (0,05 µg/ml), Pratt, R.M. et al, 1979, reported a 60-75% inhibition of radioactive sulphate incorporation by chick embryo fibroblasts. At a higher concentration of antibiotic (0,5 µg/mg), R22 cells exhibited a 60% inhibition of $|^{3}H|$-glucosamine incorporation into the intracellular compartment (results not shown), but there was still no decrease in incorporation of $|^{35}S|$-Na_2SO_4 at this concentration (Table 3.4). It may be concluded from the data that tunicamycin inhibits glycoprotein synthesis but not sulphated proteoglycan biosynthesis in the rat smooth muscle cell system.

The explanation of the inhibition of proteoglycan synthesis observed by

TABLE 3.4

THE EFFECT OF TUNICAMYCIN ON THE BIOSYNTHESIS OF SULPHATED PROTEOGLYCANS

Rat smooth muscle cells (R22) were cultured and treated with doses of tunicamycin as indicated below (Dose) and as described under Section 3.2.5. After the requisite incubation period, cultures were fractionated into the three culture compartments designated below and samples were quantitised as previously described in Section 2.2.5. All values represent the mean ± S.D. of triplicate dishes. Experiments were performed twice and the data represent one such experiment.

Incubation Time	Dose	INCORPORATION OF $\lvert^{35}S\rvert$-Na_2SO_4 (DPM/mg Cell Protein) Intracellular	Pericellular	Extracellular
24 hrs	0 ng/ml	6891±293	26705±1500	9472±900
	50 ng/ml	100054±579	32027±457	11456±1070
	500 ng/ml	10516±900	28581±511	11002±519
48 hrs	0 ng/ml	11883±200	46976±200	19992±1059
	50 ng/ml	12964±356	45426±4715	18009±1012
	500 ng/ml	11010±1084	43696±361	18688±2437

other workers by tunicamycin (Schwartz, N.B. and Dorfman, A., 1975) may be related to the fact that the enzyme xylose transferase is a glycoprotein. Tunicamycin could cause the inhibition of synthesis of this enzyme which is essential for glycosaminoglycan chain initiation. It has also been suggested that there may be decreased amounts of enzyme available since the unglycosylated enzyme is more susceptible to proteolytic degradation (Olden, K. et al, 1978).

3.3.4. The effect of β-xyloside on the biosynthesis of sulphated proteoglycans by rat smooth muscle cells.

Rat smooth muscle cells cultured in the presence of p-nitro-phenyl β-D-xyloside showed 10-fold increase in the level of radioactive sulphated macromolecules associated with the extracellular compartment (medium) (Table 3.5). Conversely, the levels of radioactive sulphated molecules in the pericellular and intracellular compartments were reduced in the presence of xyloside (Table 3.5). Findings such as these have been observed by other workers (Schwartz, N.B. et al, 1974) and suggest a large increase in synthesis and rapid secretion of sulphated glycosaminoglycan chains to the extracellular pool.

The specific activity (DPM/μg uronic acid) of the extracellular compartment material shows that a large increase in sulphation has taken place during the biosynthesis of glycosaminoglycans in the presence of xyloside. This result has not been reported previously by other workers, and is the more interesting in the light of the previous findings relating to increased sulphation levels of proteoglycans when synthesized in the presence of ascorbic acid (Section 3.3.1). These experiments were also carried out in the presence of ascorbic acid.

From the work of others using xyloside derivatives (Johnston, L.S. and Keller, J.M., 1979), it has been shown that xyloside-glycosaminoglycan complexes (xyl-GAG) are secreted directly into the medium and, as mentioned in Section 3.1, often consist of predominantly chondroitinase ABC sensitive material. This even when the cultured cells used were normally synthesizing high levels of peptido-heparan sulphate in the absence of xyloside (Stevens, R.L. and Austin, K.G., 1982). The composition data for the glycosaminoglycans found in culture medium in the

TABLE 3.5.

DATA RELATED TO THE EFFECT OF β-D-XYLOSIDE ON THE BIOSYNTHESIS OF SULPHATED PROTEOGLYCANS BY SMOOTH MUSCLE CELLS

Rat smooth muscle cells (R22) were cultured and treated with p-nitrophenol-β-D-xyloside (final concentration 0.5 mM) as described under Section 3.2.6. Analysis of incorporation of $|^{35}S|$-Na_2SO_4 into culture compartments was performed as described in Section 2.2.5. The quantitation of uronic acid present in the extracellular compartment was carried out as described under Section 3.2.2. and the analysis of the different glycosaminoglycan chains was performed as described in Section 3.2.4. The data under (A), Incorporation of radioactive sulphate into macromolecules associated with the different culture compartments, represents the mean ± S.D. for 3 culture dishes, and experiments were repeated once. The determination of uronic acid content shown under (B), Specific activity of culture medium, was performed in duplicate for each experiment and represents the mean of the two values. Analysis of glycosaminoglycan chains shown under (C) was carried out in duplicate for each experiment.

	In the absence of Xyloside	In the presence of Xyloside
A. Incorporation (DPM/mg Cell Protein) into		
Extracellular compartments	61594±782	528786±22185
Pericellular compartments	147870±4499	98920±2336
Intracellular compartments	43158±1306	31327±1602
B. Specific activity of culture medium DPM/μg uronic acid	35	243
C. Percentage composition of glycosaminoglycan chains		
Heparan sulphate	19%	5%
Chondroitin sulphate	77%	55%
Dermatan sulphate	4%	40%

presence and absence of β-D-xyloside confirms these findings (Table 3.5).

In Chapter 4 it is shown that the pathway of secreted proteoglycans synthesized by R22 cells is via the pericellular compartment to the medium. Furthermore, these molecules spend some finite time in the pericellular compartment where they appear to be exposed to ecto sulphatase enzymes. However, as shown by others, if the xyl-GAGs travel directly to the extracellular (medium) compartment, they may avoid the disulphation step which occurs in the pericellular compartment and thus be more highly sulphated.

The explanation of the high sulphation levels found with medium-associated xyl-GAG may also be that in some way xyloside and ascorbic acid stimulate the mechanisms of intracellular sulphation of nascent glycosaminoglycan chains. What sort of mechanisms(s) would be involved in this stimulation process is as yet not known; it will require a further investigation to ascertain the way in which the number of sulphate residues present per uronic acid residue may be so greatly increased.

3.4. SUMMARY

The total accumulated synthesis of sulphated proteoglycans and their secretion into the different culture compartments was assessed after eight days in culture. The total uronic acid content of each culture compartment was also determined and the specific activity of macromolecules synthesized was calculated both on a uronic acid and cell protein basis. It was seen that ascorbate supplemented cultures showed an increase in the levels of sulphation of proteoglycans associated with the pericellular pool.

Smooth muscle cells synthesized heparan sulphate, chondroitin sulphate and dermatan sulphate, but no detectable hyaluronic acid. Heparan sulphate was associated predominantly with the pericellular and intracellular compartments, whilst chondroitin sulphate was found mainly in the extracellular compartment. Increased levels of dermatan sulphate were present in the pericellular compartment of cells cultured in the presence of ascorbic acid. Scorbutic cultures showed an increased level of heparan sulphate associated with the same compartment, at the expense of dermatan sulphate levels.

Neither Tunicamycin nor hyaluronic acid affected the biosynthesis of sulphated proteoglycans in the rat culture system. However, p-nitrophenyl-β-D-xyloside added to the culture medium resulted in a ten-fold increase in radioactive sulphate associated with macromolecules in that compartment. This increase was due to an increase in sulphation levels of the medium associated material, since there was little concomitant increase in uronic acid levels in the presence of the xyloside.

CHAPTER 4

THE KINETICS OF SULPHATE INCORPORATION AND TURNOVER DURING PROTEOGLYCAN SYNTHESIS AND DEGRADATION

4.1. INTRODUCTION

The intracellular site of proteoglycan sulphation has been discussed in detail in Chapter One. Sulphation occurs rapidly on preformed nascent glycosaminoglycan chains prior to secretion of the proteoglycan molecule to the extracellular space. As in the preceding chapters, I have separated this compartment into two subcompartments, namely, the pericellular (or trypsin releasable) compartment and the culture medium.

A number of laboratories have studied the distribution of sulphated proteoglycans between the extracellular, pericellular and intracellular compartments, using various different types of cultured cells (Vogel, K.G. and Kendall, V.F., 1980; *inter alia*).

From studies like these it has been shown that there is an unequal distribution of proteoglycans between compartments, with the extracellular (medium) compartment always having the largest portion of the total sulphated proteoglycans produced. It has been observed that with progressive subculture there is decreased incorporation of macromolecular sulphate into the extracellular compartment but not into the pericellular compartment (Vogel, K.G. *et al*, 1981).

Frantantoni, J.C. *et al*, 1968, have suggested that there are several different intracellular pools of proteoglycans associated with synthesis, secretion and degradation. This is supported by data accumulated on proteoglycan biosynthesis and secretion by cultured chondrocytes (Kim, J.J. and Conrad, E., 1980; Hascall, V.C. *et al*, 1976, *inter alia*) and fibroblasts (Vogel, K.G. and Kendall, V.F., 1980, *inter alia*). This has also led to the suggestion that there is a simultaneous bidirectional secretion of proteoglycans into the culture medium and to the cell surface. Cell surface or pericellular proteoglycans may subsequently migrate to the medium as a result of many processes, among them degradation. However, this bidirectional concept has not yet been clearly established. In

cultured cells some newly synthesized proteoglycans may be inserted directly into the plasma membrane prior to subsequent release into the surrounding medium as part of a membrane-shedding process (Doljansky, F. and Kapeller, M., 1976). Proteoglycans secreted directly into the culture medium may combine with cell surface receptors at some later stage in their life and be reinternalised and degraded. Pulse-chase experiments have shown that there is rapid movement between the intracellular compartment(s), the cell surface and the culture medium (Vogel, K.G. and Kendall, V.F., 1980). It has also been suggested that some of the newly synthesised proteoglycans in both the intracellular and pericellular compartments are rapidly degraded, the latter by reinternalisation after desulphation. It is known that proteoglycans are internalised by receptor-mediated endocytosis prior to intracellular degradation by lysosomal enzymes (Prinz, R. et al, 1978; Truppe, W. and Kresse, H., 1978).

The high and low specificity membrane receptors appear to interact with the protein core of the proteoglycan, since protein-free glycosaminoglycan chains cannot bind to these receptors (Prinz, R. et al, 1978). It has also been suggested that sulphated proteoglycans impede endocytosis (Prinz, R. et al, 1977), thus desulphation of proteoglycans acts as a stimulus for their uptake. In previous work it has been shown that only some 3% of the sulphated proteoglycans offered in the medium to cultured fibroblasts are internalized over 24 hrs (Truppe, W. and Kresse, H., 1978). However, these same workers have proven that this low uptake is not due to a selective internalization since the composition of the remaining material is constant. Studies on the mode of action of lysosomal enzymes during proteoglycan degradation have shown that there is desulphation before sequential enzyme digestion in the lysosomes (Buermann, C.W. et al, 1979).

It has been observed that, both in human embryo fibroblasts (IMR-90) (Vogel and Kendall, 1980) and in pig medial smooth muscle cell cultures (Deudon, E. et al, 1980), the pericellular compartment contributes to the degradative pool, since the amount of sulphated proteoglycans involved in the catabolic pathway exceeded the amount of labelled material initially in the intracellular compartment. This suggested that there

was some mechanism in the pericellular compartment that contributes to the catabolic process.

In this chapter the work reported was carried out to investigate the nature of the biosynthetic pathway in an attempt to ascertain if there was simultaneous bidirectional secretion taking place as reported by others. Also the contribution of the pericellular compartment to the overall degradation of sulphated proteoglycans was investigated.

4.2. METHODS

4.2.1. $|^{35}S|$-Na_2SO_4 Incorporation studies

The smooth muscle cells were grown for 8 days with regular medium changes and ascorbic acid supplementation where required, as outlined in Section 2.2.1. After 8 days in culture, cells received 2 ml of fresh culture medium containing 100 µg ascorbic acid, and radioactive sulphate (10 µCi/ml culture medium) was added, as described under the legends to the relevant figures. Cells were incubated for time periods as indicated in the relevant figures at 37°C, except where indicated to the contrary. Isolation and quantitation of radiolabelled macromolecular sulphated proteoglycans present in each culture compartment was carried out as detailed under Section 2.2.5.

In experimental procedures involving the use of "pulse-chase" techniques, cells were labelled for the relevant times shown in the legends to the respective figures. At the end of the pulse labelling period, the culture medium was removed, cells were washed 3 times with fresh culture medium and finally 2 ml "cold" medium at 37°C was added to each dish. Cells were returned to the incubator for the required chase times at 37°C (see legends to figures).

4.2.2. Gel chromatography of material from pulse chase experiments.

Culture medium from "pulse-chase" experiments was analysed by Sephacryl S300 column chromatography to assess the sizes of the labelled species present at different experimental times. Aliquots of 1.5 ml were applied to a 15 x 750 mm column equilibrated with sterile phosphate

buffered saline pH 7.4 containing 60 µg/ml penicillin G and 100 µg/ml streptomycin sulphate. Samples were eluted with the same buffer, fractions were collected and counted to determine elution profiles. Columns were calibrated with respect to V_o and V_t using bovine nasal septum proteoglycan monomer and radiolabelled sulphate respectively.

4.2.3. Inhibition of intracellular lysosomal degradation.

Cultured cells labelled and treated as described for "pulse-chase" experiments (Section 4.2.1.) were treated with lysomotropic inhibitors of lysosomal degradation in the following manner. Two ml of fresh medium containing either chloroquine at a final concentration of 70 µM or ammonium chloride (10 µM final concentration) were added to cultures of pulse labelled cells. Since the solutions of chloroquine were temperature- and light-sensitive, fresh, sterile solutions were always used. Quantitation of macromolecular, radioactive sulphate-labelled proteoglycans was then carried out as described under Section 2.2.5.

4.3. RESULTS AND DISCUSSION

4.3.1. The distribution of $|^{35}S|$-Na_2SO_4 between compartments of the culture system during continuous radioactive incorporation.

The incorporation of $|^{35}S|$-Na_2SO_4 into the three culture compartments during 48 hrs resulted in the profiles shown in Figure 4.1.A. All three compartments showed a rapid incorporation of radioactive sulphate over the first 6 hours of the incubation period. However, there were clear differences between compartments even at this early time, with the rate of incorporation into the pericellular compartment being much higher than that into the medium (extracellular) and intracellular compartments (Fig. 4.1.A.). The pericellular compartment continued to accumulate sulphated macromolecules at a rapid rate over the full forty-eight hour incubation period, whereas the intracellular incorporation slowed up as it approached equilibrium, which would be consistent with its role as the initial site for sulphate incorporation. The incorporation into the extracellular compartment showed a steady slow increase which suggests that either it was the end point for the incorporated molecules that had already accumulated in the pericellular compartment

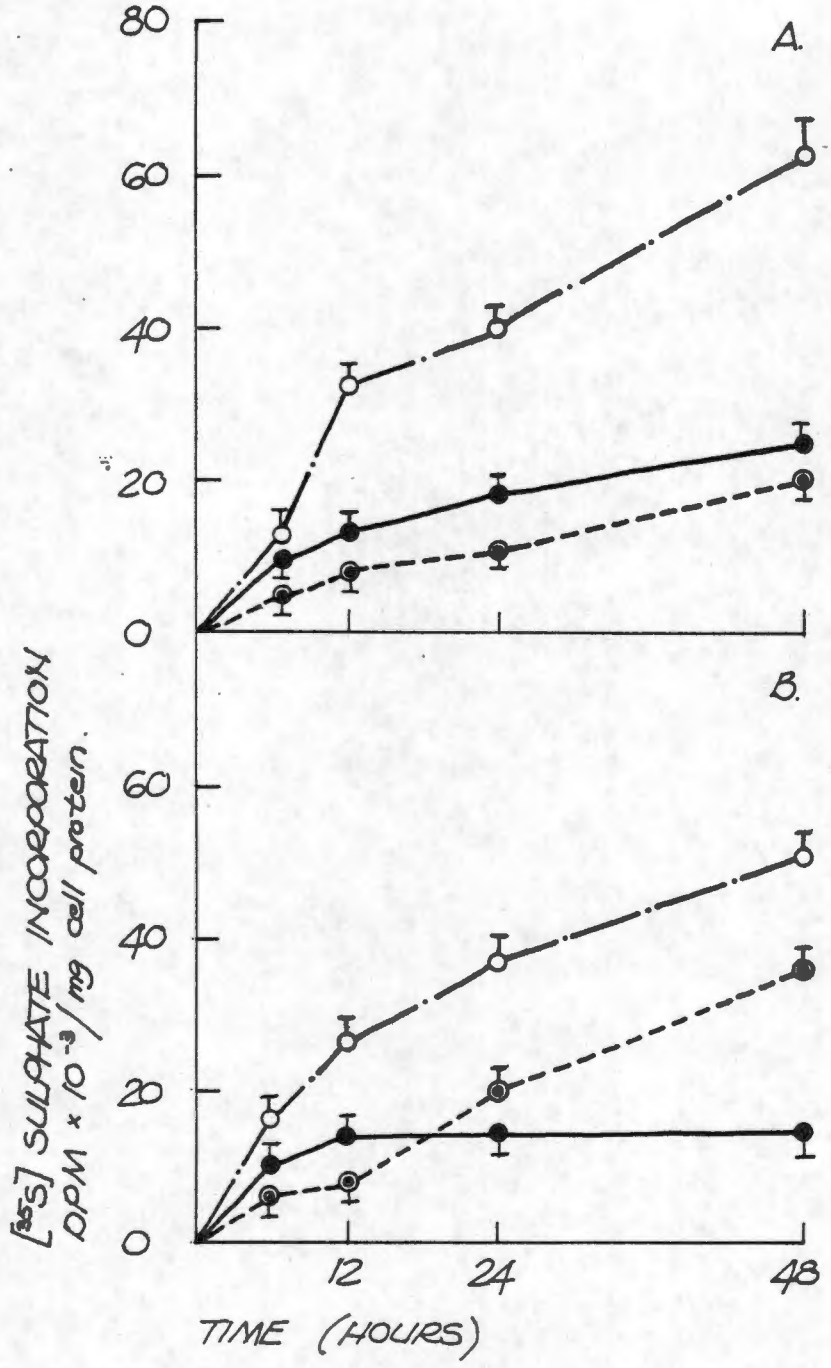


Figure 4.1.A & B. THE KINETICS OF RADIOACTIVE SULPHATE INCORPORATION DURING 48HRS INCUBATION.

Cells (R22) were cultured in the presence (A) and absence (B) of ascorbic acid for 7 days. $|^{35}S|$-Na_2SO_4 (10 µCi/ml) was added to cultures from day 7 to 9 (48 hrs) and incorporation of radioactivity into macromolecules was quantitised by the procedures described under Section 4.2.1 and Section 2.2.5. Each time point represents the mean ± S.D. for 3 dishes and the experiment was performed twice. The data represent one such experiment and the symbols represent incorporation into the extracellular -⊙-, the pericellular ··O·· and intracellular —●— compartments.

or that there is a bidirectional secretion of sulphated molecules from the intracellular compartment, and secretion into the extracellular (medium) occurs at a slower rate than that into the pericellular compartment.

To investigate further the kinetics of synthesis, shorter labelling periods were employed, with shorter time intervals between analysis (Fig. 4.2.A and B). In figure 4.2.A it can be seen that, although there is again a very rapid rise in the incorporation of sulphate into the pericellular compartment, there is a definite lag in the time of appearance of radioactively labelled macromolecules in the extracellular compartment. Over the short time periods involved, the intracellular compartment has not yet reached equilibrium and continues to show increased incorporation throughout the incubation period.

The data presented in Fig. 4.2.B illustrate still more clearly that there is a delay (45 min) in the appearance of radiolabelled sulphated material in the extracellular compartment. At the shorter time intervals of incubation used in these experiments, it can also be seen that the rate of incorporation of $|^{35}S|$-Na_2SO_4 by the intracellular compartment is initially higher than the pericellular compartment. These data and the data obtained when cells were incubated at 27°C in the presence of radioactive sulphate (Fig. 4.2.C) indicated that the pathway of secretion of sulphated proteoglycans goes via the pericellular compartment to the extracellular rather than the converse situation. At 27°C there is a much greater time lag in the appearance of sulphated macromolecules in the extracellular compartment, since after 60 min there was still no detectable macromolecular material in this part of the culture system.

The effect of ascorbate deprivation on the incorporation pattern of the smooth muscle cells over the 48 hrs of incubation is shown in Fig. 4.1.B. In comparison with the cultures that have been supplemented with ascorbic acid (Fig. 4.1.A), deprived cultures show decreased incorporation rates into the pericellular compartment and increased sulphate incorporation into the extracellular compartment. The intracellular compartment also reaches equilibrium quicker with ascorbic acid deprivation. These

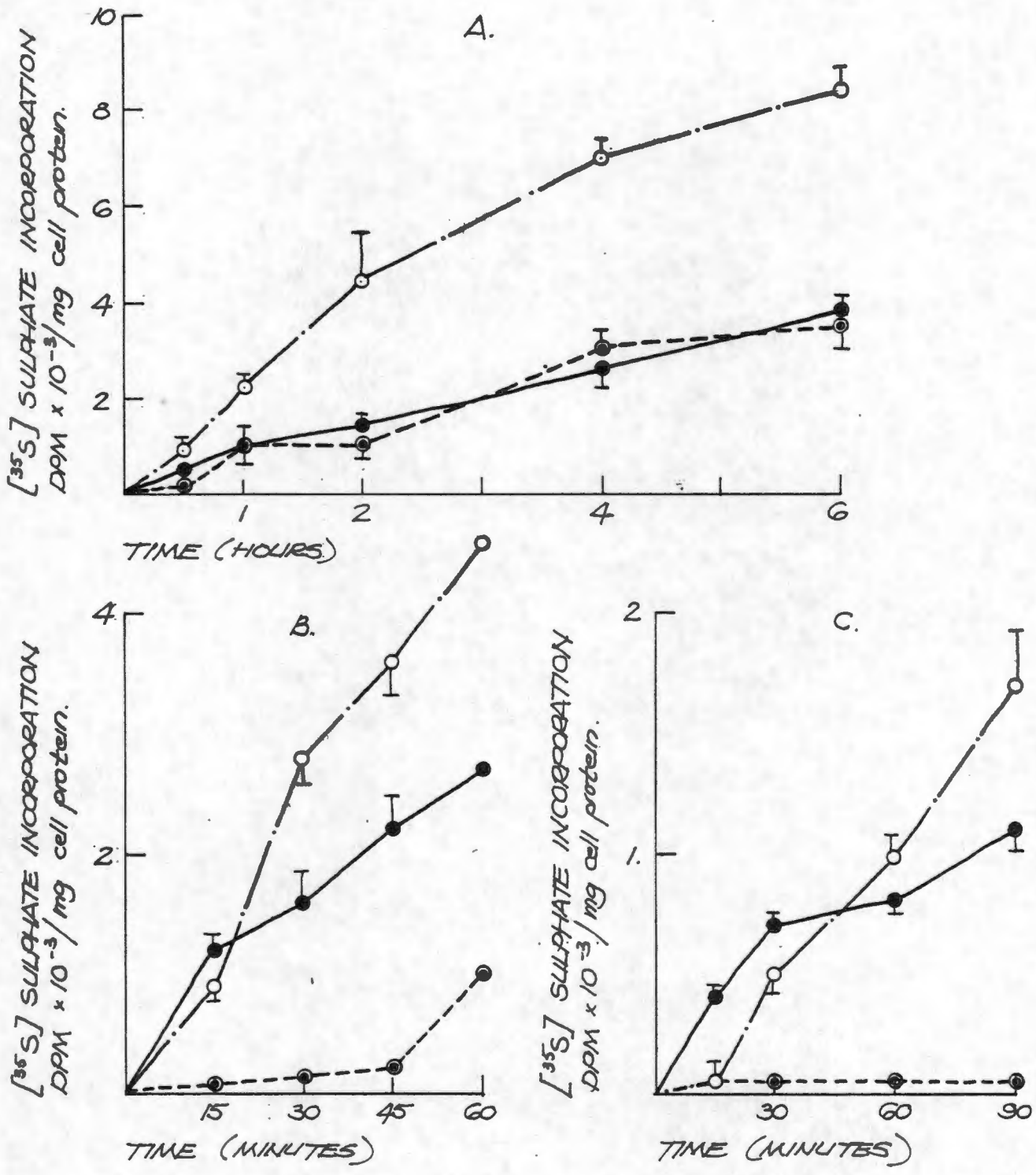


Figure 4.2. A, B and C. THE KINETICS OF RADIOACTIVE SULPHATE INCORPORATION FOR VARIOUS INCUBATION TIMES AT 27°C AND 37°C..

Cells were grown and incubated with $|^{35}S|$-Na_2SO_4 as described under Fig. 4.1, except that incubations were for 6 hrs at 37°C (A), or 1 hr at 37°C (B). Incubations at 25°C (C) were for 90 min. Each point was the mean ± S.D. for 3 dishes and experiments were performed twice. The data represent one such experiment and symbols represent incorporation into extracellular -⊖-, pericellular ··O·· and intracellular —●— compartments.

findings are consistent with the observation made in respect of the proteinaceous composition of the extracellular matrix (pericellular) compartment of cells when cultured in the presence and absence of ascorbic acid. Supplementation with this vitamin results in an increased collagen deposition into the matrix with a resultant increased possibility for sulphated proteoglycan/collagen interaction, and thus delay in their transfer to the extracellular compartment (Fig. 4.1.A and Table 2.1). Furthermore, it has been a consistent observation that the degree of sulphation of macromolecules associated with the pericellular compartment is increased in the presence of ascorbic acid, thus giving rise to the increase in sulphate found associated with this fraction.

Comparing the early passage cultures (Fig. 4.3) over the 48 hr incubation with the later passage cells (Fig. 4.1.A) show a distribution of sulphated macromolecular material which is consistent with findings in other studies (Vogel, K. et al, 1981). The extracellular compartment accumulates most of the sulphated macromolecules secreted by the cells. The pericellular compartment reaches equilibrium after 24 hrs while the intracellular compartment reaches equilibrium already after 6 hrs. Comparing the amount of total sulphate incorporation in the two systems (Fig. 4.4), there seems to be no difference in the first 24 hrs; thereafter the later passage cells show higher sulphate incorporation. An explanation of these differences between early and late passage cells may be found on comparing the total amount of matrix protein produced by the two systems (Table 2.1). The later passage cells lay down nearly twice as much cross-linked matrix proteins as the early passage cells. As discussed above, this will result in much less interaction between sulphated proteoglycans and collagen and/or elastin in the pericellular compartment of the early passage cells, and therefore less accumulation of sulphate macromolecules in this compartment.

The data presented here confirm the findings of others, namely that sulphation of proteoglycans is a rapid process (Hardingham, T.E. and Muir, H., 1972a). These authors have shown that in cultured chondrocytes it takes only a few minutes before sulphated proteoglycans are secreted to the exterior.

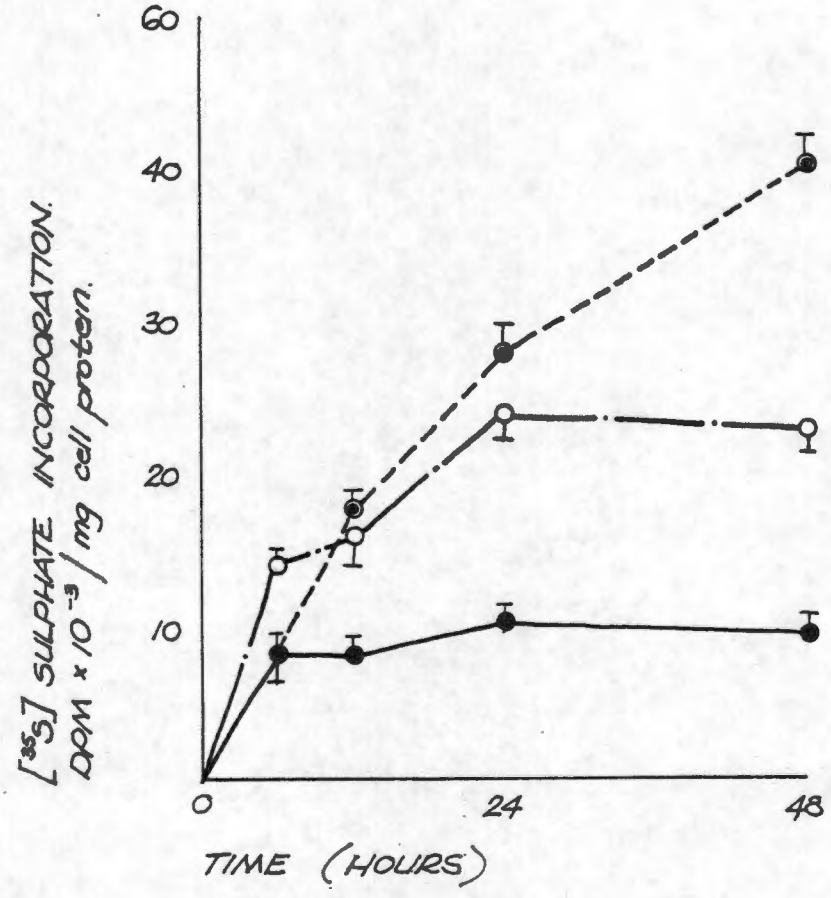


Figure 4.3. KINETICS OF RADIOACTIVE SULPHATE INCORPORATION FOR 48 HRS BY 4TH PASSAGE CELLS.

Cells were cultured and incubated with $|^{35}S|$-Na_2SO_4 as described under Fig. 4.1. Each time point was the mean ± S.D. for 3 culture dishes and experiments were performed twice. The data represent one such experiment and the symbols represent incorporation into the extracellular -⊖-, pericellular ··O·· and intracellular —●— compartments.

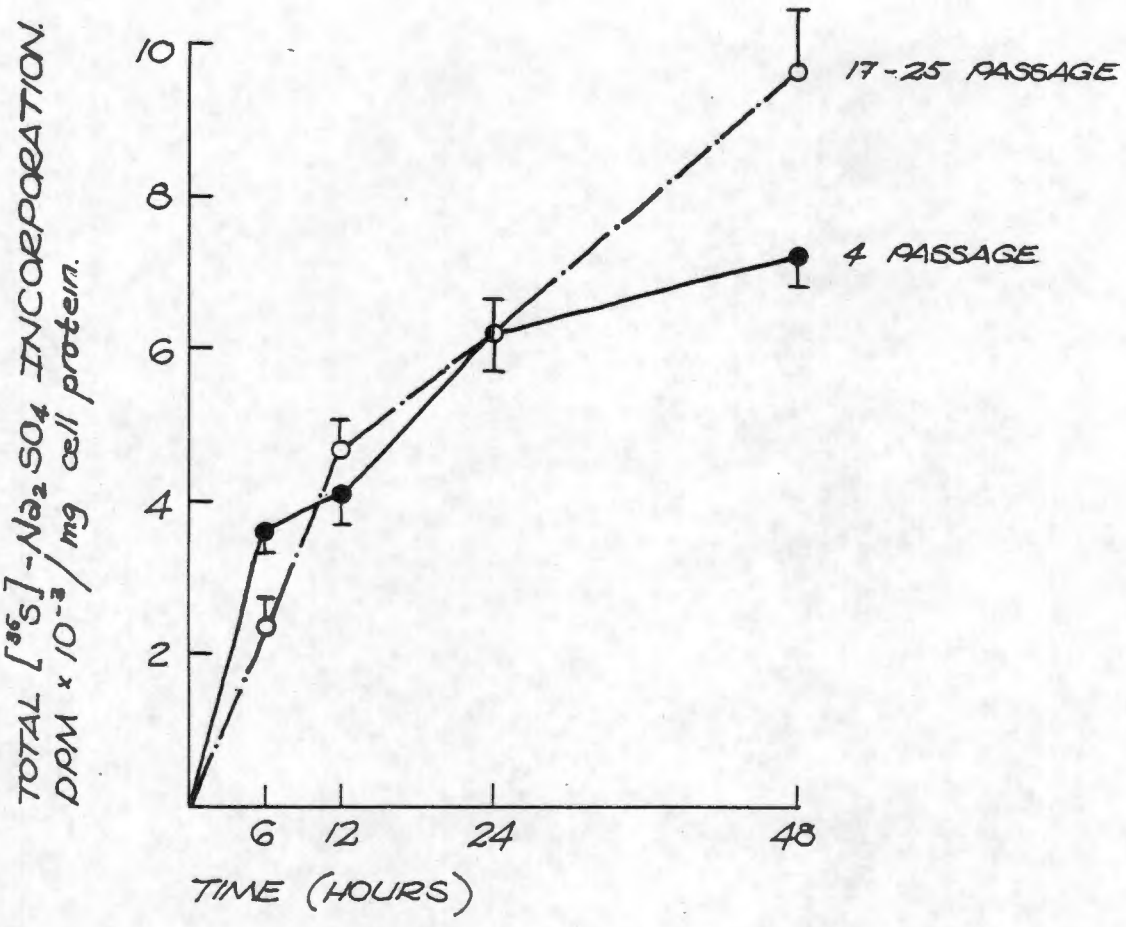


Figure 4.4. COMPARISON OF TOTAL $|^{35}S|$-Na_2SO_4 INCORPORATION BY LOW AND HIGH PASSAGE CELLS.

Smooth muscle cells were cultured and incubated with radioactive sulphate as described under Fig. 4.1. The data represent the total incorporation over the time periods shown and are obtained from additions of the incorporation into the separate extracellular, pericellular and intracellular compartments as shown in Figs. 4.1.A and 4.3.

The data from experiments using a continuous labelling regime do not give a clear indication of the pathway of secretion in spite of the results shown in Fig. 4.2.B and Fig. 4.2.C, and for this reason the biosynthetic pathways were further studied by using "pulse-chase" experimental techniques.

4.3.2. Distribution of $[^{35}S]$-Na_2SO_4 between the culture compartments using pulse-chase labelling techniques.

After a pulse of radioactive sulphate for 24 hrs more than 80% of the sulphated proteoglycans were present in the pericellular compartment (Fig. 4.5). This high level of labelling in the pericellular compartment relative to the intracellular is a result of the length of the pulse period and the large pool size of that compartment. Shorter pulse periods (to be discussed below), although of more value in terms of interpreting the sequence of secretion of sulphated molecules, had the major disadvantage in that the labelling of macromolecules was very low and thus difficult to follow accurately.

In the first 24 hrs of the chase, the amount of radioactive material in the pericellular compartment decreased by approximately 50% and the levels in intracellular compartment also dropped over this period. Both these falls are consistent with transfer of sulphated macromolecules from the intracellular to the pericellular compartments, and thence to the extracellular compartment; this latter compartment being the only one to show an increase in sulphated macromolecules during the chase period.

When the specific activity (DPM/mg Cell Protein) data from Fig. 4.5 are replotted as the percentage of the total specific activity in each compartment at each individual time point, the profile shown in Fig. 4.6 was obtained. From the pattern it may be seen that the apparent biphasic decrease in the pericellular compartment is still present and suggests a rapid transfer (loss) of material from this compartment over the first 24 hrs, with a subsequent slowing up of this process. The loss of radioactive material from the intracellular compartment only occurs during the last 24 hrs of culture, but the rise in the extracellular compartment is most rapid over the first 24 hr period.

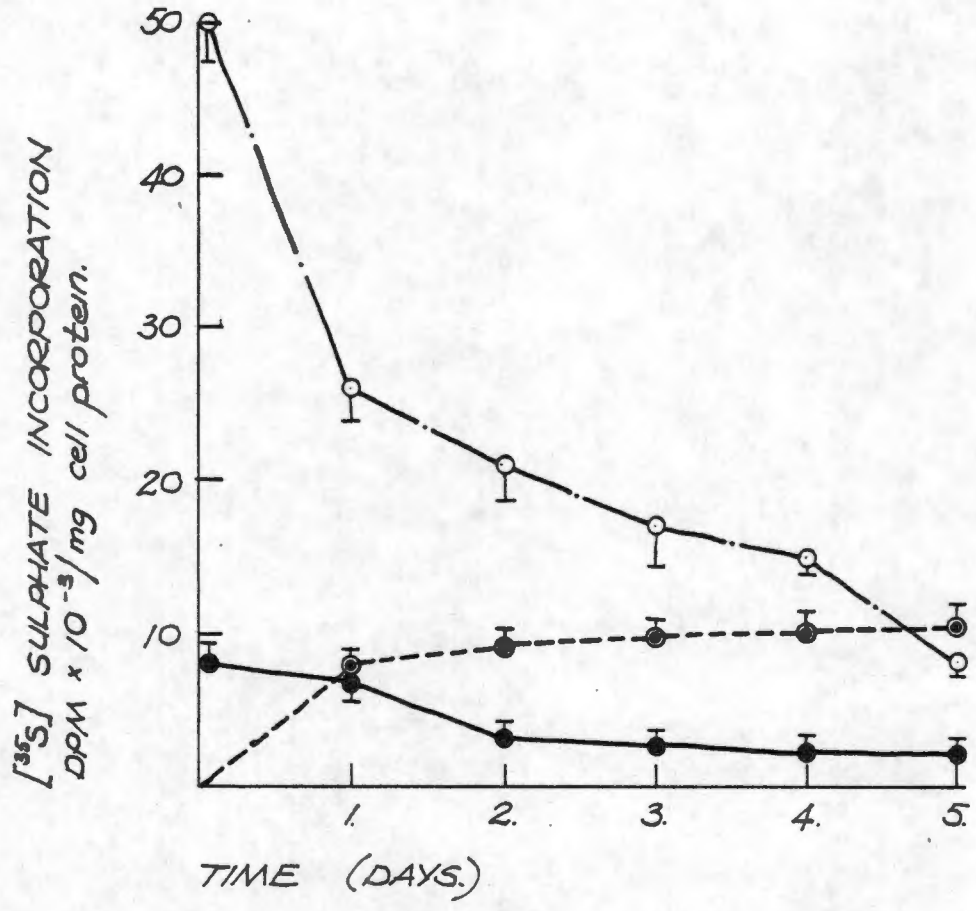


Figure 4.5. KINETICS OF PULSE-CHASE INCORPORATION OF ^{35}S -Na_2SO_4 BY CELLS OVER SIX DAYS.

Smooth muscle cells (R22) were cultured for 8 days and incubated with 5 µCi/ml of $|^{35}S|$-Na_2SO_4 for the final 24 hrs. After replacement of radioactive medium with fresh cold medium, cultures were maintained for a further 5 days as described under Section 4.2.1. Amounts of macromolecular radioactive sulphate in each compartment were assayed as described under Section 2.2.5 and the symbols represent the extracellular -●-, the pericellular ··O·· and intracellular compartments -●- Each point represents the mean ± S.D. for 3 culture dishes and experiments were performed twice with the data representing one such experiment.

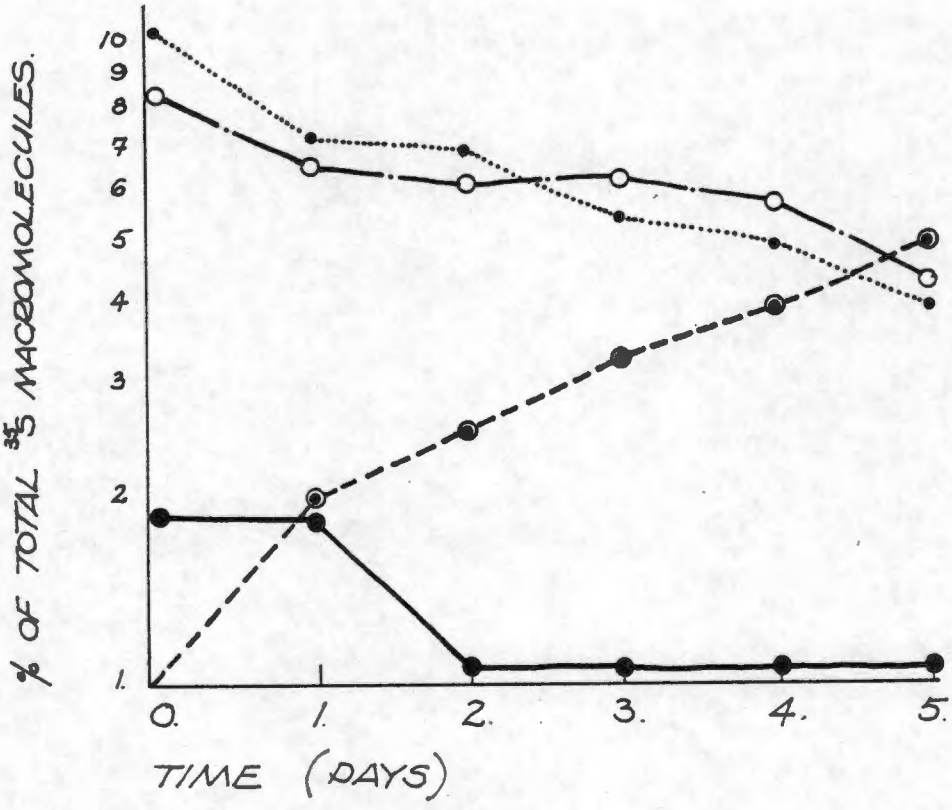


Figure 4.6. SEMI-LOGARITHMIC PLOT OF PERCENTAGE DISTRIBUTION OF RADIOACTIVE SULPHATE DURING PULSE-CHASE INCUBATION.

The data from Fig. 4.5 are represented here as a semi-logarithmic plot of the percentage of the total specific activity associated with each compartment at each experimental time point. The symbols used are the same as for Fig. 4.4, with the addition of - - - which represents the percentage of total macromolecular sulphate present at the start of the chase period (Time 0) at each experimental time point.

Taking these data as a whole, they suggest that the extracellular compartmental rise in the first 24 hrs is due to material being released from the pericellular compartment and not the intracellular fraction. The subsequent slower increase in specific activity of the extracellular pool may be partially due to secretion from the intracellular compartment (24 - 48 hrs).

To confirm these conclusions, and in particular to investigate the rapid decrease in specific activity of the pericellular pool over the first 24 hrs, a 1 hr pulse was undertaken (Fig. 4.7.A and B). In spite of difficulties with low levels of incorporation (see comments above), the results obtained confirmed the previous findings. Namely, an initial rapid fall in the specific activity (Fig. 4.7.A) of the pericellular compartment followed by a slower rate of disappearance of radioactive macromolecular sulphated molecules from this compartment. This was accompanied by an initial rapid increase in specific activity of the extracellular compartment and a slow decrease in specific activity of the intracellular pool.

These findings are consistent with a pathway of secretion of sulphated proteoglycans from the intracellular pool to the extracellular (medium) compartment via the pericellular compartment, where they reside for a certain finite time, due most probably to their individual electrostatic interaction with the extracellular proteins present in this compartment.

Applying pulse-chase techniques to cultures grown in the absence of ascorbic acid (Fig. 4.8.A and B) and comparing the data to that obtained with supplemented cultures (Fig. 4.5 and 4.6), there was a lower incorporation of sulphate into sulphated macromolecules over the 24 hr pulse. The pericellular compartment contained 60% of the labelled material at the start of the chase period and the intracellular compartment contained the other 40% as compared to about 80% and 20% respectively (Fig. 4.6) with ascorbate supplemented cultures.

In the first 24 hrs of the chase the radioactivity in both the intracellular and pericellular compartments fell rapidly (Fig. 4.8.A),

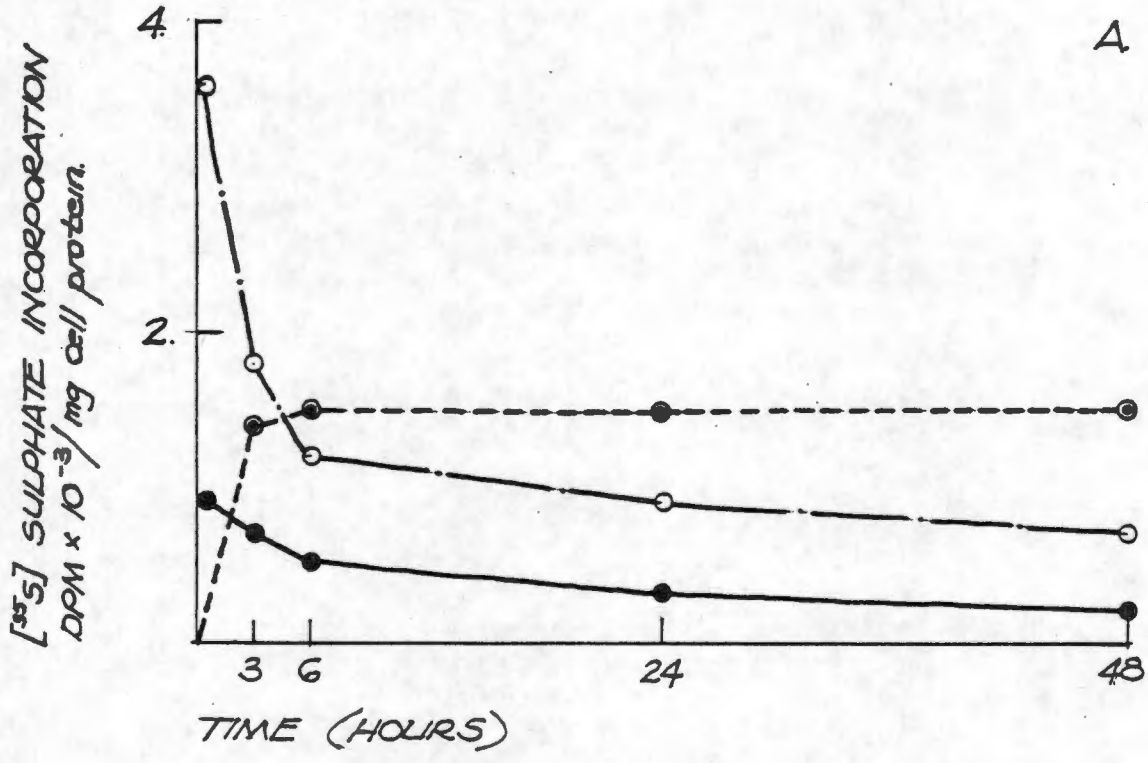


Figure 4.7.A. KINETICS OF PULSE-CHASE INCORPORATION OF $|^{35}S|$-Na_2SO_4 BY CELLS OVER 49 HRS.

Cells were cultured and treated as described under Figure 4.4, except that pulse times were for 1 hr at 37°C and after replenishment with fresh medium, cells were cultured for a further 48 hrs. Determinations of radioactive sulphate incorporation were carried out as described in Section 2.2.5 and the symbols represent incorporation into the extracellular -0-, pericellular --O-- and intracellular —●— compartments. Each point represents the mean ± S.D. for 3 culture dishes and experiments were performed twice, with the data representing one such experiment.

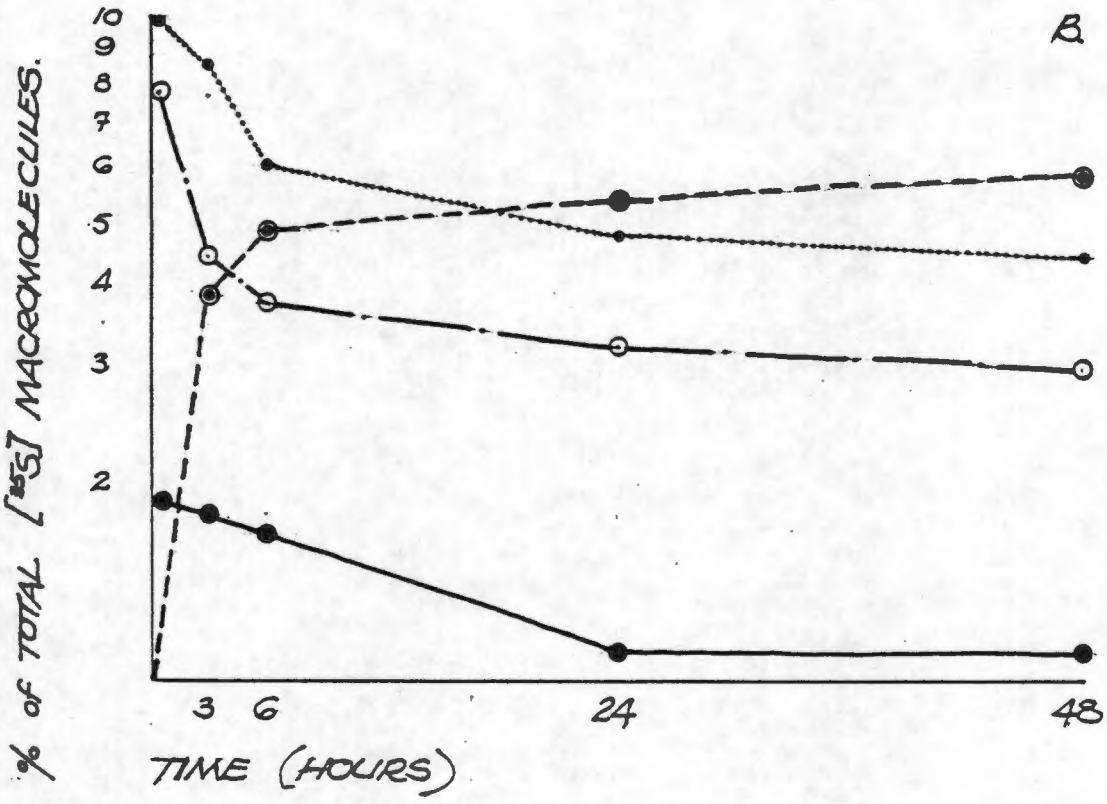


Figure 4.7.B. SEMILOGARITHMIC PLOT OF PERCENTAGE DISTRIBUTION OF RADIOACTIVE SULPHATE DURING PULSE-CHASE INCUBATION.

The data from Fig. 4.7.A have been treated as described under Fig. 4.6. The symbols used are the same as described for Fig. 4.7.A and Fig. 4.6.

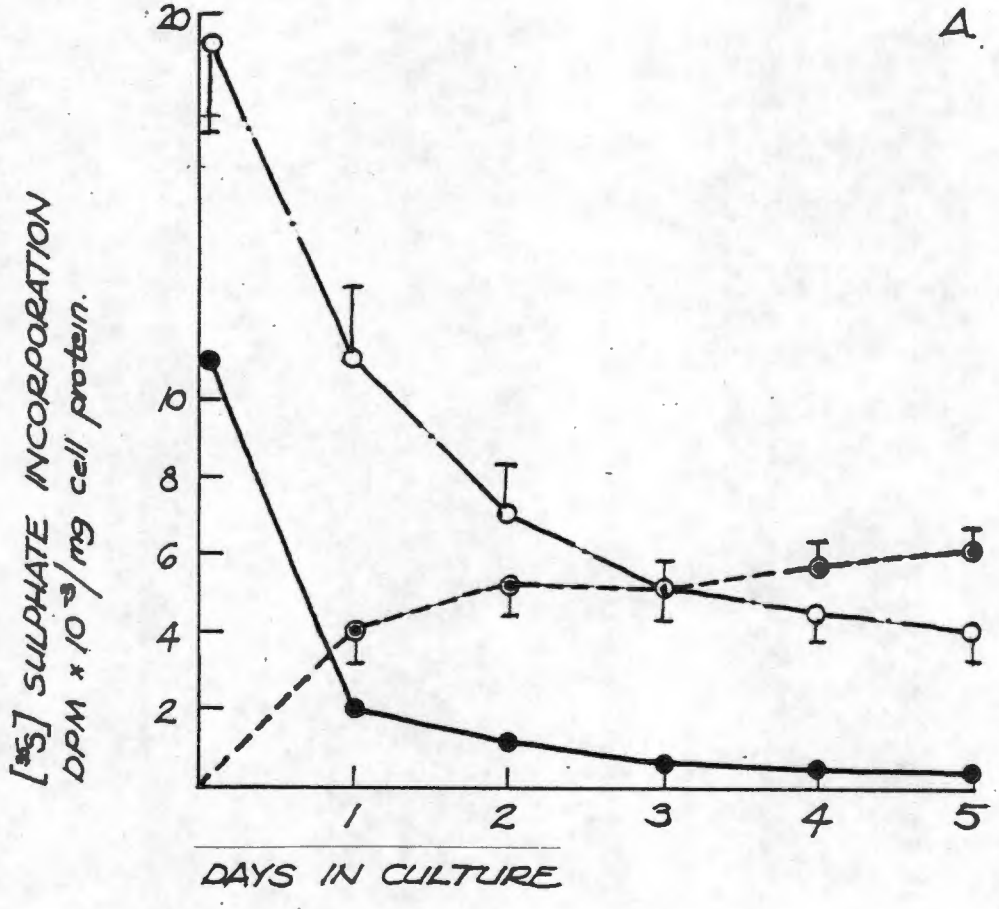


Figure 4.8.A. KINETICS OF PULSE-CHASE INCORPORATION OF $|^{35}S|$-Na_2SO_4 BY CELLS OVER SIX DAYS IN THE ABSENCE OF ASCORBIC ACID.

Cells were cultured and treated as described under Fig. 4.4, but in the absence of ascorbic acid. Determination of $|^{35}S|$-Na_2SO_4 incorporation was carried out as described under Section 2.2.5 and the symbols represent incorporation into the extracellular -⊖-, the pericellular ··O·· and intracellular —●— compartments. Each time point represents the mean ± S.D. from 3 culture dishes, and experiments were performed twice. The data represent one such experiment.

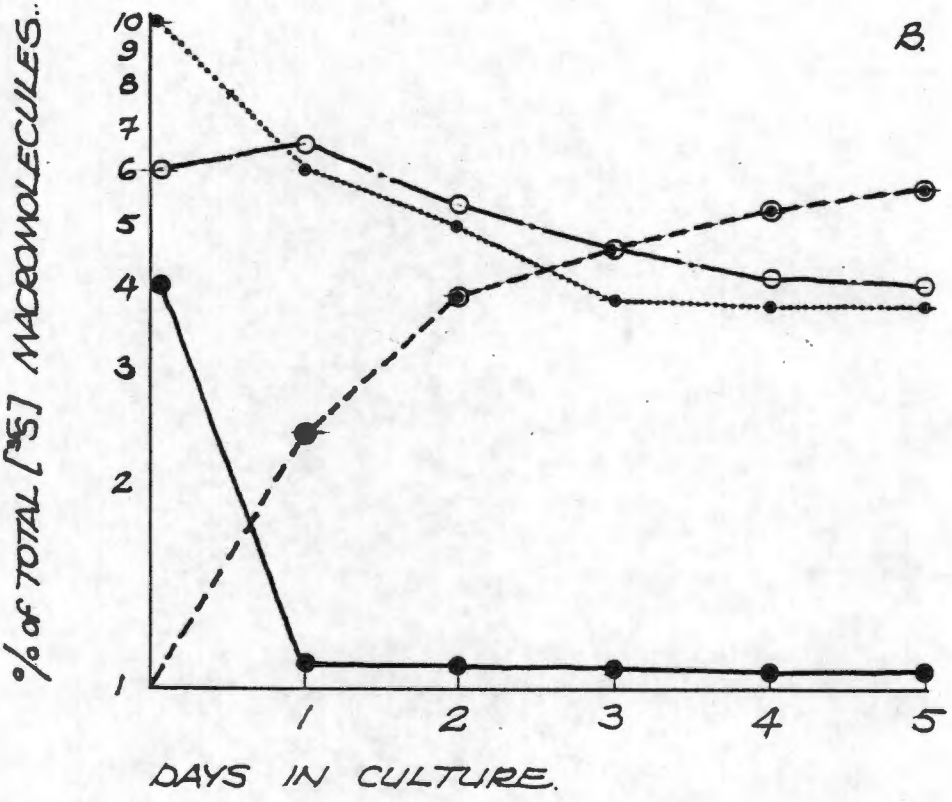


Figure 4.8.B. SEMILOGARITHMIC PLOT OF PERCENTAGE DISTRIBUTION OF $|^{35}S|$-Na_2SO_4 DURING PULSE-CHASE INCUBATION IN THE ABSENCE OF ASCORBIC ACID.

The data from Fig. 4.8.A have been treated as described under Fig. 4.6 and the symbols used are the same as for that Figure.

which was then followed by slower drop in both compartments. From the semi-logarithmic percentage plot (Fig. 4.8.B) there seems to be a slight rise in radioactivity associated with the pericellular compartment during the first 24 hrs with concomitant fall in the intracellular compartment, which suggests that transfer of labelled material has occurred from the intracellular compartment to the pericellular compartment. In the presence of ascorbic acid (Fig. 4.6) this critical increase in radioactivity in the pericellular compartment was never observed.

The explantion for the differences seen between scorbutic and ascorbate-supplemented cultures is speculative. Ascorbic acid may be assisting in the secretory mechanisms of the cell (Boxer, L.A. et al, 1978) or again it may be the result of increase collagen presence in the pericellular compartment that gives rise to these observations (see previous discussion above.).

4.3.3. Desulphation of sulphated proteoglycans.

A consistent observation in the pulse chase experiments carried out and described above was a loss in total macromolecular sulphate present in the system during the chase period. Referring to Figure 4.6, the total macromolecular sulphate present at the end of the full chase period has fallen to 38% of the starting value and in Figure 4.7.B this drop is also of the order of 50%. To account for this observation, it was proposed that turnover of sulphated proteoglycans was occurring and this was investigated in more detail.

It had to be established whether there was a desulphation enzyme present in the culture medium which resulted in loss of macromolecular sulphate from proteoglycans present in this compartment. Medium was therefore removed from cells after a twenty-four hour pulse period and retained at 37°C for the full period of the chase (5 days). The results in Table 4.1 clearly show that there was no loss in macromolecular sulphate, which ruled out the presence of any secreted sulphatase enzyme(s) in the medium. Therefore, the disappearance of macromolecular sulphate is associated with either the pericellular or intracellular compartments.

TABLE 4.1

TOTAL TURNOVER OF MACROMOLECULAR $|^{35}S|$-Na_2SO_4 IN THE CELL CULTURE MEDIUM

Cells were cultured for 8 days in the presence (+) or absence (-) of ascorbic acid, and incubated during the final 24 hrs with 5 µCi/ml $|^{35}S|$-Na_2SO_4. The culture medium was removed and retained at 37°C for five days, in a humidified CO_2 incubator. Aliquots were taken daily and analysed as described in Section 2.2.5.

TIME (DAYS)	DPM $|^{35}S|$-Na_2SO_4 MACROMOLECULES	
	+	-
0	45100 ± 1150	63960 ± 1044
1	39628 ± 1367	61762 ± 1287
2	40081 ± 1276	63668 ± 983
3	44342 ± 183	59561 ± 2000
4	39269 ± 1429	65600 ± 1378
5	42343 ± 1281	63760 ± 1566

To determine whether this loss of macromolecular sulphate was due to the total degradation of glycosaminoglycan chains or merely their desulphation, extracellular sulphated proteoglycans were analysed by gel exclusion chromatography (Fig. 4.9). The only sulphated molecular species present in this fraction were molecules excluded from the column with high molecular weight or included material which eluted at the column V_t. This material showed an increase with time of chase period and is consistent with a process of desulphation giving rise to increasing amounts of free sulphate. The sulphated macromolecular material excluded from the column also increased over the 24 hrs, which agrees with the data shown in Fig. 4.5 and described under the previous Section 4.3.2. The free $|^{35}S|$-Na_2SO_4 in the system increased proportionally to the amount that disappeared from macromolecular sulphated molecules and represents the amount that was turned over (Table 4.2). Most of this free $|^{35}S|$-Na_2SO_4 is associated with the culture medium as shown in Table 4.2.

Since proteoglycans are internalized (Prinz, R. et al, 1978) and degraded intracellularly by lysosomal hydrolases (Buermann, C.W. et al, 1979), it had to be established whether inhibiting this mechanism had any effect on the decrease of sulphated macromolecules seen in this system. It has been shown that ammonium chloride (Seglen, P.O. and Ruth, A., 1976; Seglen, P.O. et al, 1979) and chloroquine (Wibo, M. and Poole, B., 1974; Amenta, J.S. and Brocher, S.C., 1980) accumulate in the lysosomes due to their basic properties and cause inhibition of the lysosomal enzymes due to increased pH. It has been shown that both ammonium chloride and chloroquine inhibit intracellular lysosomal degradation in smooth muscle cells (Bates, P., 1981).

The presence of these lysomotrophic inhibitors had no effect on the decrease of macromolecular sulphate associated with the pericellular compartment (Fig. 4.10). In the presence of ammonium chloride (Fig. 4.10B) the intracellular sulphated material decreased throughout the chase period. Thus even when intracellular degradation was inhibited there was still a loss in radioactive sulphate from the intracellular pool, and this may only be explained by normal secretion of nascent macromolecules. It has also been shown that chloroquine has a deleterious effect on microtubular organisation in cultured cells,

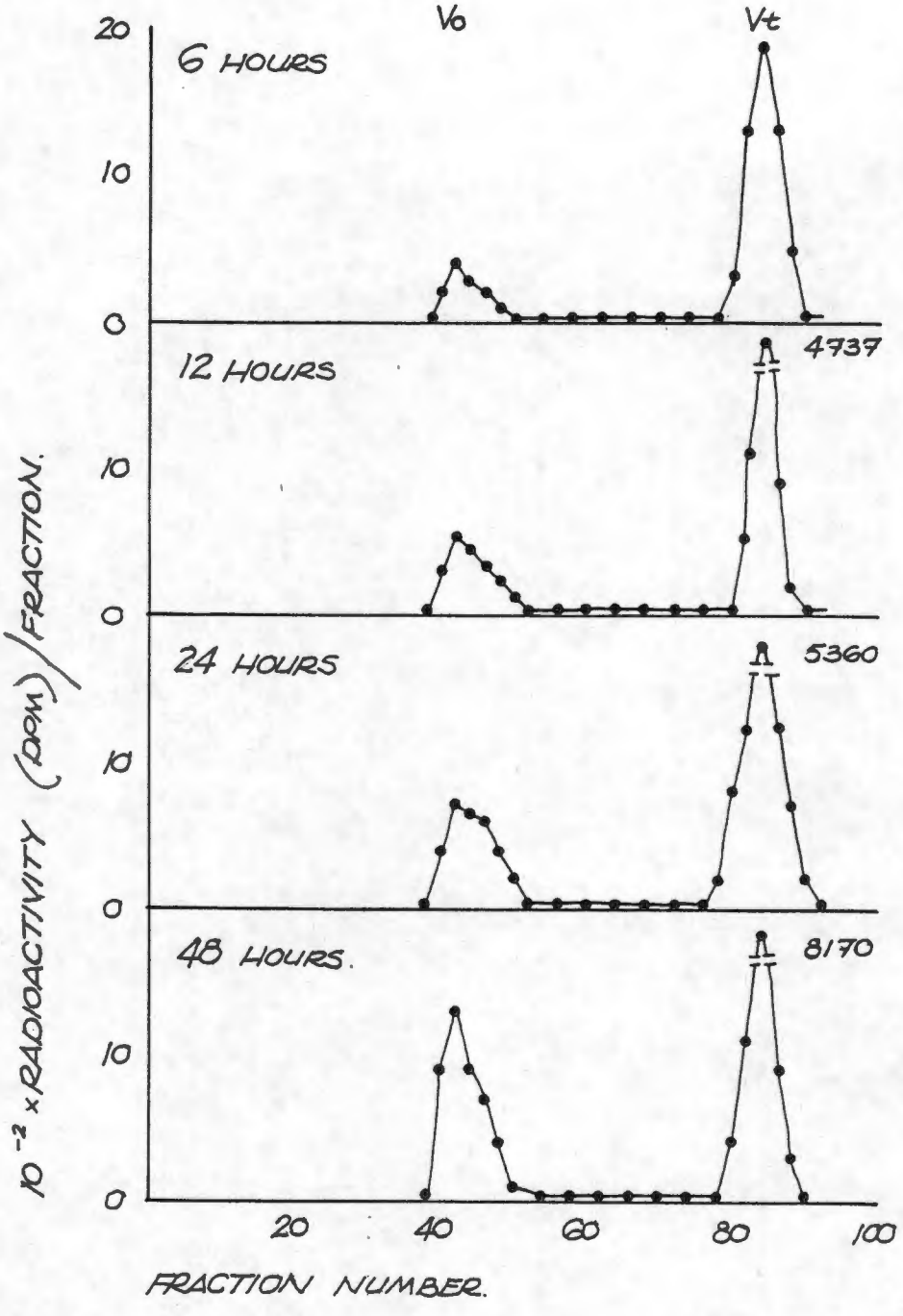


Figure 4.9. MOLECULAR EXCLUSION CHROMATOGRAPHY OF EXTRACELLULAR SULPHATED PROTEOGLYCANS/GLYCOSAMINOGLYCANS.

Cells were cultured and treated as described under Figure 4.6. After replenishment of radioactive medium with fresh cold medium and further incubation of cultures at 37°C, aliquots of medium were removed and chromatographed on a Sephacryl S300 column as described under Section 4.2.2. Samples were eluted using PBS buffer and monitored as described in the same section. The figures on the right of the material eluting at the V_t of the column represent the total counts in the peak fraction.

TABLE 4.2

DISTRIBUTION OF TOTAL $|^{35}S|$-Na_2SO_4 DURING PULSE CHASE EXPERIMENTS

The distribution of total radioactive sulphate present in the intracellular and pericellular compartments as macromolecular sulphate, and in the extracellular compartment as macromolecular and free sulphate was carried out, using procedures described under Sections 2.2.5 and 4.2.2. The data were obtained from experiments described under Figs. 4.10.A and 4.9, and assays for residual free radioactive sulphate associated with the pericellular and intracellular compartments were not performed.

TIME	MACROMOLECULAR RADIOACTIVE SULPHATE IN			FREE
	Pericellular	Intracellular	Extracellular	$\|^{35}S\|$-Na_2SO_4
0	72617 ± 758	24031 ±2027	0	0
6	59886 ±1838	12681 ± 836	3077 ±197	15334 ±1290
12	45904 ±1520	10512 ± 450	3664 ± 94	24528 ±3062
18	43495 ±3076	8149 ±325	3889 ±166	28548 ±3733

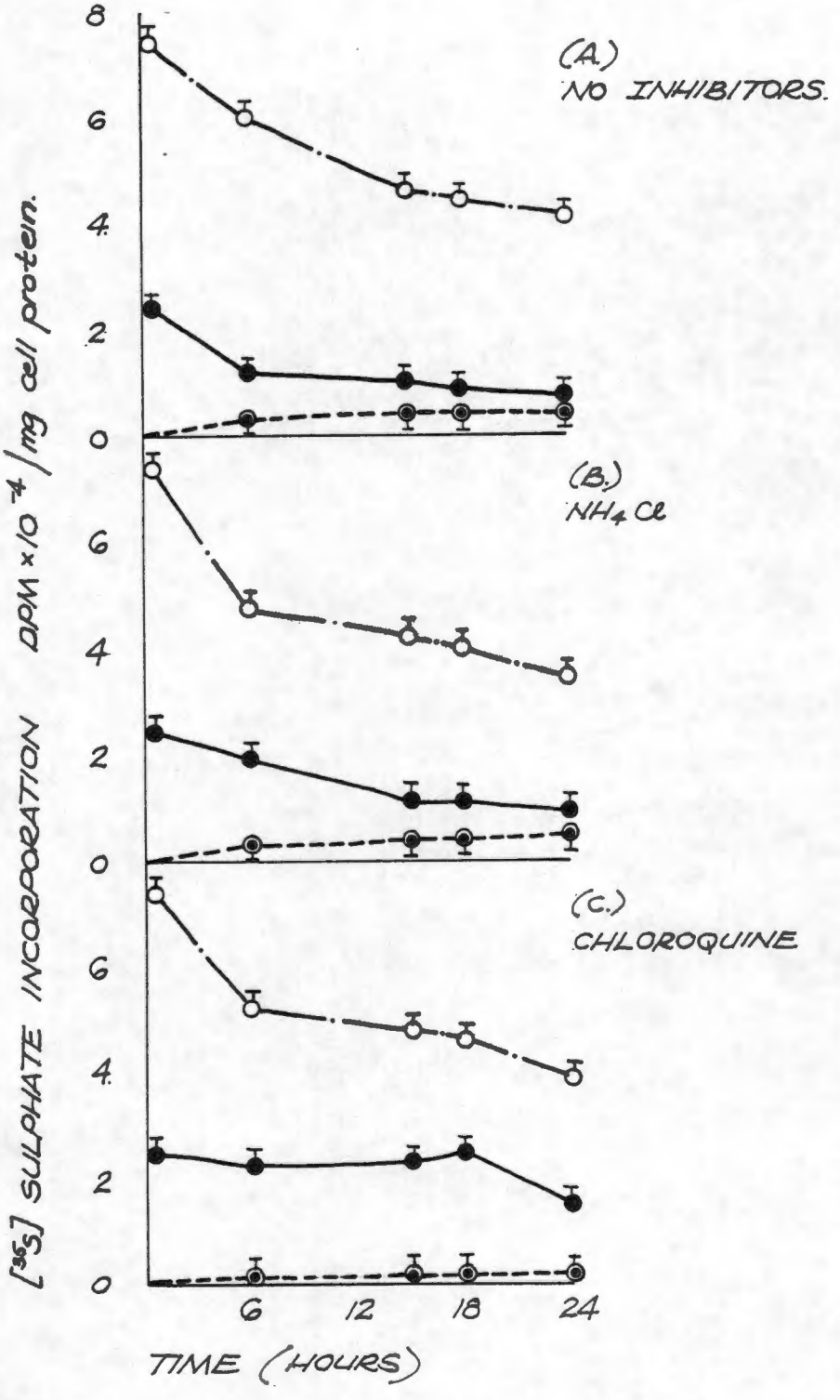


Figure 4.10. THE EFFECT OF LYSOMOTROPHIC INHIBITORS ON THE KINETICS OF INTRACELLULAR MACROMOLECULAR SULPHATE DISAPPEARANCE.

Cells were cultured and treated as described under Figure 4.5, except that pulse labelling was for 24 hours and the chase period for a further 24 hrs. Addition of inhibitors where specified was for the final 24 hour chase period only. Quantitation of macromolecular sulphated material was carried out as already described, Section 2.2.5, and inhibitors were used as described under Section 4.2.3. The symbols represent macromolecular radioactive sulphate associated with the extracellular -●-, pericellular -○- and intracellular —●— compartments. The points represent the mean ± S.D. of three dishes, and the experiment was repeated once and gave very similar data.

resulting in impaired secretion of macromolecules (Fedorko, M.E. et al, 1968a and b). The data (Fig. 4.10.C) shows that in the presence of chloroquine there was no decrease of intracellular sulphated macromolecules during the chase period and this is consistent with there being impaired secretion of sulphated proteoglycans to the exterior. From the data it can be summarised that the loss in radioactivity associated with the intracellular compartment is due to a normal secretion of newly synthesized sulphated proteoglycans and not their degradation.

The conclusion from these findings is that the loss in macromolecular sulphate during the experimental chase period is related to an enzyme or enzymes associated with the pericellular compartment of the culture system.

When medium removed from cultures grown in the presence of $|^{35}S|$-Na_2SO_4 was added to fresh cultures following dialysis to remove any free $|^{35}S|$-Na_2SO_4, there was no generation of free radioactive sulphate over 48 hrs. This observation suggests that sulphated proteoglycans in the culture medium of rat smooth muscle cells have little access to the sulphatases present in the extensive pericellular compartment. Furthermore, since, as has been shown, the medium-associated proteoglycans are the end point of the biosynthetic pathway in the rat culture system, they may be unable to associate with pericellular elements essential for ecto-sulphatase activity, having already in their synthesis transversed that compartment and been processed by the enzyme(s) in question.

4.4. SUMMARY

The biosynthesis pathway for sulphated proteoglycans in rat smooth muscle cells involves transfer from the intracellular pool of synthesis, where rapid sulphation takes place, to the pericellular pool and thence to the culture medium (extracellular). The process is complicated by the fact that during the time that material is associated with the pericellular compartment it may be acted upon by sulphatases associated with this pool. This results in turnover of some of the macromolecular material present in the pericellular compartment, and a loss of macromolecular sylphate to the system. However, reutilization of this sulphate may take place in the culture system described, making interpretation of the data more complex.

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APPENDIX

Chemicals and Materials were obtained from the following suppliers:

Chemicals and Enzymes:

B.D.H. Chemicals, Poole, England	:	Ethylenediamine tetraacetic acid Sodium dodecyl sulphate
Difco Laboratories, Detroit, Michigan, USA	:	Tryptose phosphate, trypsin
Gibco, New York, USA	:	Minimum essential medium
Glasco, Wadeville, Transvaal, SA	:	Penicillin G, streptomycin sulphate
E. Merck, A.G. Darmstadt, Germany	:	Supplied all the normal laboratory chemicals used
Miles Laboratories, Goodwood, SA	:	Hyaluronic acid, pronase, bovine serum albumin
NewEngland Nuclear, Boston, USA	:	L(3,4(n)-Proline)
The Radiochemical Centre, Amersham, Bucks, England	:	$\|^{35}S\|$-Na_2SO_4; $\|^{3}H\|$-glucosamine
Sigma Chemical Co., St Louis MO, USA	:	Elastase (E.C.3.4.21.11) collagenase (E.C.3.4.24.3) (if pure (bacterial) from Worthington, USA), phenylmethylsulphonyl fluoride, heparin, carbozole, chloroquine, p-nitrophenyl-β-D-xylopyranoside, chondroitinase ABC lyase (E.C.4.2.2.4) " AC " (E.C.4.2.2.5)
State Vaccine Institute, Cape Town, SA	:	Foetal calf serum

Materials:

Beckman Instruments Fullerton, USA	:	Polyallomer tubes
Corning Limited, Stafforshire, England	:	Tissue culture flasks and dishes
E. Merck, A.G. Darmstadt Germany	:	Thin layer cellulose plates
Packard Instruments, Downers Grove, USA	:	Scintillation mixture 299
Pharmacia, Uppsala, Sweden	:	Sephacryl S300, Sephadex G50 Sepharose 4CL.B
Whatman Ltd, Kent, England	:	3 MM chromatography paper.