PROLIFERATIVE AND CHEMOTACTIC REPONSES OF CELLS INVOLVED IN WOUND HEALING TO ANIONIC ANIMAL AND PLANT POLYSACCHARIDES

Varrie A. Craig BSc(Hons)

University of Glasgow

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University of Stirling

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A final vote of thanks also goes to Barry for encouraging me to spent countless Sundays writing up while he played Football !

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ABSTRACT

The aim of this study was to investigate the effects of various polysaccharides and their breakdown products on the proliferation and migration of cells involved in wound healing, both *in vitro* and *in vivo*, with the ultimate aim of developing a commercially viable collagen dressing containing an active polysaccharide fragment which would stimulate the wound healing response to such a degree that good quality and significantly faster healing would take place.

Hyaluronic acid (HA), chondroitin sulphate (CS), heparin, Oxidised Regenerated Cellulose (ORC) and pectin were tested in this study. Some HA fragments and CS fragments significantly stimulated ($p<0.05$) the proliferation of Bovine Aortic Endothelial (BAEC) cells, although other HA or CS fragments were without effect. All HA and CS fragments tested also had no effect on the migration of L929 cells in the Boyden Chamber assay. Pectin stimulated the proliferation and migration of L929 cells, whereas, ORC 1 and heparin both suppressed proliferation (25% - 45% inhibition) of these cells but stimulated their migration. When injected subcutaneously into Polyvinyl alcohol sponges in the rat wound model at concentrations of 10 and 50μg/ml, heparin brought about an increased presence of granulation tissue in the wound after 10 days and 7 days respectively.

These results indicate that structurally similar polysaccharides can have profoundly different effects on cell proliferation and migration, and thus have potential therapeutic use in guiding cell movement in wound healing.

LIST OF ABBREVIATIONS

BAEC	Bovine Aortic Endothelial Cell
CS	Chondroitin Sulphate
DMAB	Dimethylaminobenzaldehyde
DMEM	Dulbecco's Modified Eagle's Medium
DS	Dermatan Sulphate
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal Calf Serum
aFGF	Acidic Fibroblast Growth Factor
bFGF	Basic Fibroblast Growth Factor
GAG	Glycosaminoglycan
HA	Hyaluronic Acid
HBSS	Hanks Balanced Salt Solution
HPLC	High Pressure Liquid Chromatography
HS	Heparan Sulphate
HSPG	Heparan Sulphate Proteoglycan
KS	Keratan Sulphate
MW	Molecular Weight
NAG	N-acetylglucosamine
NMR	Nuclear Magnetic Resonance
ORC	Oxidised Regenerated Cellulose

PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PVA	Polyvinyl Alcohol
SEM	Standard Error of the Mean
TBS	Tris Buffered Saline
TEMED	N, N, N', N' - Tetramethylethylenediamine
TGFß	Transforming Growth Factor ß
UA	Uronic Acid
VEGF	Vascular Endothelial Growth Factor

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AIMS OF THE STUDY

Chronic wounds are defined as those which do not follow the expected sequence of repair in terms of healing time, appearance and response to appropriate treatment. They can be divided into 2 distinct categories by their etiology : (i) pressure, ischaemic, venous, neuropathic and diabetic ulcers and (ii) infected, traumatic, and dehisced post-surgical wounds. Their degree of severity can range from epidermal injury to full-thickness skin loss with subsequent tissue necrosis which can affect underlying structures such as muscle, tendon and bone. Treatment of chronic wounds has changed in recent years due to the discovery of specific mechanisms which regulate repair. To date however, there are no products on the market that have proven clinically to reduce the healing time of chronic wounds although many investigators are studying this area. Some of these treatments are discussed below.

The addition of growth factors to chronic wounds (Robson *et al*, in press; Marks *et al*, 1991; Robson *et al*, 1992) has had encouraging results, however, the cost to manufacture these on a large scale may result in a costly product and may not be commercially viable for hospitals who have to treat hundreds of thousands of patients each year. Other treatments have included the addition of extracellular matrix molecules, such as collagen with added glycosaminoglycans (GAGs) which are known to influence the adhesion, proliferation and differentiation of cells (Yannas & Burke, 1980; Hook *et al*, 1982; McPherson *et al*, 1988; Doillon *et al*, 1988; Srivastava *et al*, 1990).

It has previously been reported that Hyaluronic acid (HA) oligosaccharides have the ability to stimulate angiogenesis in the chick chorioallantoic membrane *in vivo* and endothelial cell proliferation *in vitro* (West & Kumar, 1989; Rooney *et al*, 1993). These findings suggest there may be a role for the degradation products of HA and other GAGs in the wound healing process.

Currently, Johnson & Johnson have a number of collagen dressings on the market including Instat (haemostat) and Fibracol (collagen-alginate topical dressing), are keen to expand the product range by incorporating a bioactive polysaccharide(s) in into these dressings in order to stimulate chronic wound healing.

The aims of this project therefore were :

- To investigate the role of certain naturally occurring plant and animal polysaccharides and their oligosaccharides, in addition to one synthetically derived polysaccharide, on their ability to inhibit or stimulate cell types associated with wound healing in order to ascertain their potential to stimulate the wound healing process.

- To ascertain whether these polysaccharides and oligosaccharides stimulate wound healing processes in certain animal models both in combination with collagen and on their own.

- To develop a wound healing dressing prototype that would reduce healing time of chronic wounds significantly.

GENERAL INTRODUCTION

1.1 INTRODUCTION

The first written records documenting wound healing come from Ancient Egypt. They describe the use of fresh meat, fat, honey and castor oil as dressings or adhesive plaster to bring wound edges together. In later years, the Romans irrigated wounds with wine, sea water and oil and laid figs on ulcerating wounds (Cohen *et al*, 1992). By 1346, English soldiers were carrying boxes of spiderwebs to lay across their bleeding wounds and circa 1600, Shakespeare described the use of flax and egg-white for bleeding face wounds in several of his plays. The materials used for dressings were most commonly linen and flax, but, wool, cotton and flannel were also used (Cohen *et al*, 1992).

During the past 25 years the wound dressing market has developed quickly primarily due to the work of George Winter (1962) on the effects of occlusive dressings in pigs. He developed the criteria for the ideal wound dressing and this resulted in the production of many new wound products. He advocated that the ideal dressing should (a) maintain a moist environment, (b) remove excess exudate, (c) permit gaseous exchange, (d) demonstrate impermeability to microorganisms, (e) demonstrate a low level of adherence to the wound and (f) be free from particulate and toxic contaminants. Even today, wound management products have incorporated some of these principles : some devices are intended as a mechanical barrier for compression or absorption of exudates, whereas others are intended to manage the micro-environment of wounds (Collyer, 1994).

1.2 WOUND HEALING

There are several excellent texts on wound healing (Fowler, D. 1989; Morgan & Pledger, 1992; Asmussen & Sollner, 1993). The following section will only attempt to summarise these works and introduce new findings where appropriate.

The healing response can be conveniently separated into 4 major biological components: (1) the vascular response, (2) blood coagulation, (3) inflammation and (4) formation of new tissue. The four components of wound healing do not occur one after the other but overlap to varying degrees and are described in the following sections.

1.2.1 Vascular Response

A cutaneous or deep wound is usually accompanied by bleeding and this has the effect of cleaning the wound by washing out any foreign bodies which may be present. A few seconds later, the blood vessels begin to constrict which prevents further blood loss and a clot is formed (Weksler, 1988). Following vasoconstriction, mast cells release histamine and serotonin that promote vasodilation to allow the recirculation of blood and this results in a local rise in temperature around the wound after about 10 minutes (Westaby, 1985). The mast cell components also cause the endothelial cells in the vessels to move apart, thereby increasing the permeability of the capillary walls to allow erythrocytes, leukocytes, platelets and plasma to move from the blood into the wound (Wahl & Wahl, 1992). Finally, the wound becomes oxygen deficient due to vascular stasis and the high concentration of CO_2 causes the accumulation of fluid in the tissue.

1.2.2 Blood Coagulation

The body creates its own "bandage" by forming a scab. Scab formation is regulated by the blood vessel walls, platelets, damaged connective tissue cells and the coagulation system and begins when damaged cells release suitable mediators to initiate the coagulation cascade (Wahl & Wahl, 1992). When damage occurs to a vessel wall, mediators like von Willebrand factor immediately adhere to the collagen exposed on the vessel wall and this stimulates platelets to change their shape from flat discs to spherical structures with long pseudopods (Sixma & Wester, 1977). With these processes, they make contact with each other and accumulate to form a plug. This is mediated by the release of Thromboxane A_2 which stimulates platelet aggregation (Fowler, 1989). The blood clot itself consists of a fibrin and fibronectin mesh in which other blood cells such as erythrocytes are caught (Adzick & Longaker, 1992). The clot rapidly dries in air thereby protecting the wound from the external environment and preventing further blood loss. The deposition of this fibrin matrix is thought to provide a scaffold on which inflammatory cells can enter the wound site (Weigel *et al*, 1986).

1.2.3 Inflammation

The first cells to appear at the wound site are the neutrophils which have the ability to phagocytose and release enzymes which break down degenerating connective tissue. Their primary role is to create a bacteria free wound and rapidly decrease in number when no infection is present (Fowler, 1989). Soon after, monocytes are attracted into the wound by chemotactic factors such as Platelet Derived Growth Factor (PDGF), Transforming Growth Factor β (TGFß) and C5a (Ross *et al.*, 1986; Wahl *et al.*, 1987;

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Marder *et al.*, 1985). Once in the wound, the monocytes transform into macrophages and reach maximum numbers after 24 hours (Fowler, 1989). The function of the macrophage is as a phagocyte-destroying bacteria and cellular debris, however it also produces or releases many growth factors needed for the formation of granulation tissue (Knighton & Fiegel, 1989).

In addition to the non-specific defence system, the body also has a specific immune response in which antibodies are effective against a specific antigen (Barbul, 1992). Antibodies are produced by B-lymphocytes and are assigned to different immunoglobulin classes: IgG, IgA, IgM, IgE and IgD (Roitt, 1980). The immunoglobulins circulate in the serum and on recognising an antigen, either bind to it or mark it for destruction by phagocytes. In conjunction with B-lymphocytes which produce antibodies, T-lymphocytes are also involved in specific defence. They do not produce antibodies but instead act as "killer cells", identifying and destroying foreign cells, thereby fighting infection and encouraging new tissue formation. The immune response will not be described further.

1.2.4 Formation of New Tissue

To ensure good wound healing, new blood vessels begin to bud from intact vessels and grow into the wound, activated by growth factors and other mediators already present in the wound (Whalen, 1992). Parallel to this vascularisation, fibroblasts migrate along the fibrin matrix, rapidly dividing and producing proteoglycans and collagen fibres essential for the stability of the tissue (Morgan & Pledger, 1992). The new vascularised

tissue forms small, rounded nodes of tissue on the wound bed known as granulation tissue, and appears granular, dark red and shiny.

Following granulation tissue formation, the fibrin matrix is broken down by the enzyme plasmin and fibroblasts transform into fibrocytes or myofibroblasts, the latter being able to draw the wound edges together by contraction (Fowler, 1989). At the end of the wound healing process, the wound is closed by epithelialisation. This is initiated by an increased rate of cell division in the basal layer of the epidermis and migration of the resulting epithelial cells towards each other from the wound margins, resulting in the defect being covered by a fine layer of skin (Chernoff & Robertson, 1990). Repair is not complete until some time after coverage by the epithelium and an increase in the tensile strength has been achieved. Remodelling of the new tissue takes months with the deposition of type 1 collagen fibrils, resulting in the formation of a scar, which lacks some components of normal tissue and it is therefore less functional (Adzick & Longaker, 1992).

1. 3   EXTRACELLULAR MATRIX COMPONENTS IN WOUND HEALING

The extracellular matrix (ECM) has an important role in wound healing. In the past, it was thought to be an inert support material, providing a scaffold that stabilised the physical structure of tissues (Alberts *et al*, 1983) The ECM is now known to influence adhesion, migration, proliferation and differentiation of cells (Hook *et al*, 1982). The ECM is composed of two main classes of extracellular macromolecules: collagen and glycosaminoglycans. The collagen fibres strengthen and help to organise the matrix,

while the gel-like glycosaminoglycans permit the diffusion of nutrients, metabolites and hormones between the blood and the tissue cells. Elastin, proteoglycans, fibronectin and laminin are also part of the ECM (Alberts *et al*, 1983). The relative amounts of the different types of matrix macromolecules and the way they are organised within the ECM vary with the type of matrix.

1.3.1 Collagen

1.3.1.1 Introduction

The collagens are a family of highly characteristic fibrous proteins found in all multicellular animals. They constitute 25% of the total protein in mammals and are therefore extremely abundant. The polypeptide chains have the repeating structure of Gly-X-Y, where X is usually proline and Y hydroxyproline. This sequence does not apply to the non-helical domains at each of the collagen molecule. The triple helix consists of 3 alpha chains wrapped around each other with each chain containing about 1000 amino acids, right-handed and rod-shaped (Bailey, 1985). Collagen is responsible for many of the essential characteristics of connective tissue, particularly their mechanical and biological properties (Bailey & Light, 1989; Gorham S.D., 1991). To date, 16 different genetic forms have been isolated and identified. Some of these types and their structures are listed in Table 1.1 (Bailey & Light, 1989 ; Martin *et al*, 1985 ; Summers *et al*, 1988). Type I is the major component of most collagen based prosthetic materials.

Type	Molecular Configuration	Tissue Location
I	$[\alpha 1(I)]_2\ \alpha 2(I)$	Bone, cornea, skin, tendon
II	$[\alpha 1(I)]_3$ trimer	Tumours, skin
III	$[\alpha 1(III)]_3$	Cartilage, vitreous
IV	$[\alpha 1(IV)]_2\ \alpha 2(IV)$, plus $\alpha 3(IV)$, $\alpha 4(IV)$, $\alpha 5(IV)$ chains	Basement membranes
V	$[\alpha 1(V)]_2\ \alpha 2(V)]$ $[\alpha 1(V)]2\ \alpha 2(V)\ \alpha 3(V)]$ $[\alpha 1(V)]_3$	Placental tissue, bone, skin
VI	$[\alpha 1(VI)\ \alpha 2(VI)\ \alpha 3(VI)]$	Uterus, skin, cornea, cartilage
VII	$[\alpha 1(VII)]_3$	Amniotic membrane, skin, oesphagus
VIII	$[\alpha 1(VIII)_2\ \alpha 2(VIII)]$	Endothelial cells
IX	$[\alpha 1(IX)\ \alpha 2(IX)\ \alpha 3(IX)]$	Cartilage, vitreous
X	$[\alpha 1(X)]_3$	Calcifying cartilage
XI	$[\alpha 1(XI)\ \alpha 2(XI)\ \alpha 3(XI)]$	Cartilage, invertebral disc
XII	$[\alpha 1(XII)]_3$	Skin, tendon, cartilage
XIII	$\alpha 1(XIII)$	Endothelial cells, epidermis
XIV	$[\alpha 1(XIV)]_3$	Skin, tendon, cartilage

Table 1.1 : The Collagen Family

1.3.1.2   Synthesis of Collagen

Synthesis of collagen has been described in many reviews (Duance & Bailey, 1981 ;

Last & Reiser, 1984; Burgeson & Nimni, 1992), and therefore only a brief account will

be given here.

The precursor chains of the collagen molecule are synthesised on ribosomes, transferred

into the endoplasmic reticulum where the signal sequences are removed, then secreted

in secretory granules from the Golgi apparatus in its triple helical form (Duance &

Bailey, 1981; Weinstock & Leblond, 1974). The individual collagen molecules then

spontaneously aggregate to form a "quarter-stagger" arrangement to form fibrils. The

heads of the collagen molecules are staggered along the length of the fibril resulting in

the characteristic 64 nm spacing of cross-striations (Duance & Bailey, 1981). Following

formation of the fibrils, intermolecular cross-linking takes place which stabilises the

collagen (Eyre, 1985).

1.3.1.3   Degradation of Collagen

Collagen degradation is important in the repair or replacement of damaged tissue in

wound healing. There are three groups of enzymes which are responsible for collagen

degradation: the metalloproteinases, neutral proteinases and lysosomal cathepsins

(Duance & Bailey, 1981 ; Sellers & Murphy, 1981). During degradation, collagen

fragments are generated from the fibre which are then capable of being phagocytosed

and degraded in lysosomes by acid-proteinases (Bailey & Etherington, 1985)

Collagen and collagen-derived peptides have been found to serve as chemoattractants

for fibroblasts and neutrophils *in vitro* and therefore may function as chemotactic

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stimuli to effect cell migration to sites of inflammation *in vivo* (Laskin *et al*, 1986; Postlethwaite *et al.*, 1978; Albini & Adelmann-Grill, 1985). Such a role for collagen peptides may be important in the development of biological dressings.

1.3.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) are high molecular weight carbohydrate polymers and are important structural elements of the extracellular matrix of animal connective tissue. They consist of polymers of repeating disaccharide units that contain a hexosamine and either a carboxylate or sulphate ester, or both, which provide the chains with linear arrays of anionic groups. The following GAGs are found in mammalian tissues : hyaluronic acid, chondroitin 6-sulphate, chondroitin-4-sulphate, dermatan sulphate, heparin, heparan sulphate and keratan sulphate and the principal repeating units of the individual GAGs are shown in Fig 1.1

GAGs normally exist covalently bound to core proteins in the form of proteoglycans. Table 1.2 shows the five different classes of proteoglycan each differing in GAG composition, core protein size or source (Templeton, 1992). **Family a** proteoglycans consist of multiple glycosaminoglycan chains. This family of proteoglycans associate with hyaluronic acid to form large aggregates, and reside in the extracellular compartment. Aggrecan belongs to this family and has a 220kDa core protein with over 100 chondroitin sulphate chains and a keratan sulphate-rich region also (Doege *et al*, 1987). **Family b** proteoglycans are small molecules containing only 1 or 2 GAG chains and are found in the connective tissue (Templeton, 1992). Decorin belongs to this

(a)

(b)

R' = H, R = SO₃H for Chondroitin -4-sulphate
R = H, R' = SO₃H for Chondroitin-6-sulphate

(c)
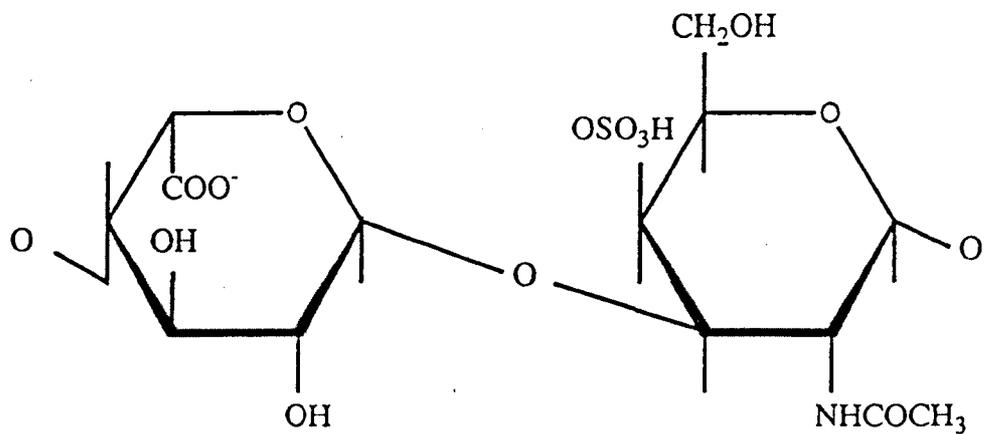
Figure 1.1 :   The repeating units of (a) Hyaluronic acid, (b) Chondroitin sulphate
and (c) Dermatan sulphate

(d)

(e)

$$Y = COCH_3 \text{ or } SO_3^-$$

(f)
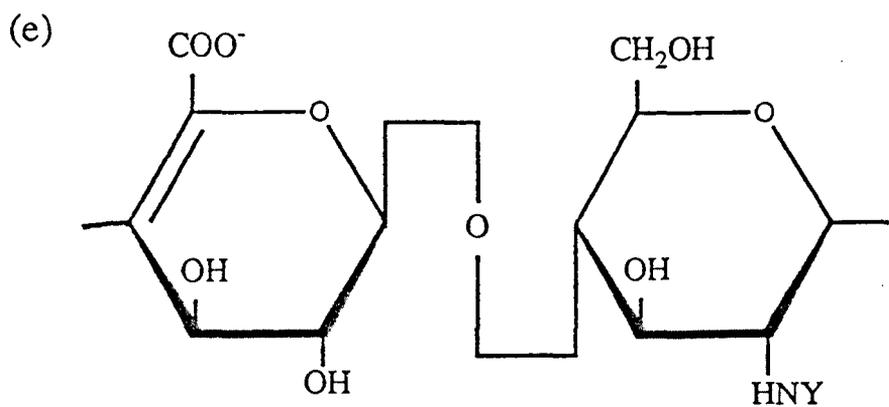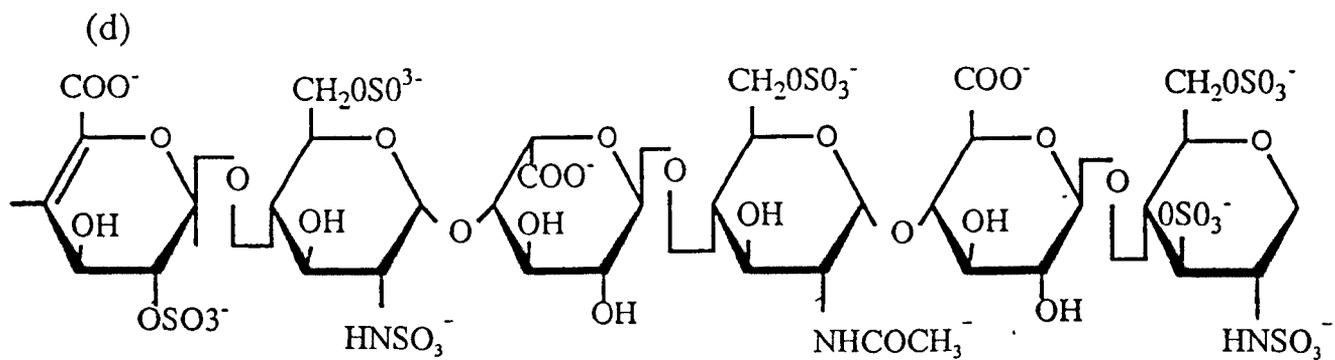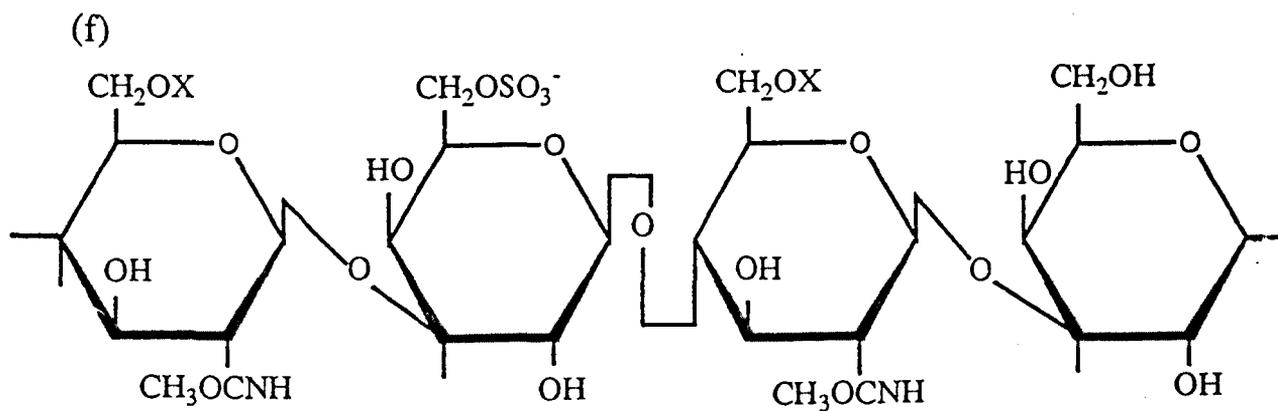
$$X = SO_3^- \text{ or } H$$

Figure 1.1 :   The repeating units of (d) Heparin (e) Heparan sulphate and (f) Keratan sulphate

NAME	SOURCE	CORE PROTEIN SIZE (kDa)	GAG CHAINS
Family a	**Large extracellular proteoglycans**		
Aggrecan	Cartilage	220	>1000CS, >10KS
Versican	Fibroblasts	265	12-15 CS
Family b	**Small connective tissue proteoglycans**		
Decorin	Many cells	36	1DS
Biglycan	Many cells	38	2DS
Fibromodulin	Many cells	41	1KS
Family c	**Basement membrane heparan sulphates**		
	EHS tumour	400	2-3 HS
	Glomerular basement membrane	30-400	2-6 HS
Family d	**Cell surface proteoglycans**		
Syndecan	Mammary epithelium	31	1-2 CS, 1-2 HS
Betaglycan	Fibroblasts	110	CS, HS
	Hepatocytes	35-77	HS
Thrombomodulin	Endothelial cells	60	1 CS
Family e	**Intracellular proteoglycans**		
Serglycin	Many cells	17-19	Heparin, CS, DS, HS

Table 1.2   :   Classification of Proteoglycans

family and has a core protein of only 40kDa (Krusius & Ruoslahti, 1986). **Family c** proteoglycans are the basement membrane heparan sulphate proteoglycans which have large core proteins (400kDa), but only have 2 to 4 heparan sulphate chains (Templeton, 1992). **Family d** are the cell surface proteoglycans such as the mouse mammary epithelial proteoglycan, syndecan. Syndecan consists of a 30kDa core protein with a hydrophobic transmembrane domain and has chondroitin and heparan sulphate chains in the extracellular region (Saunders *et al*, 1989). The last family, **Family e**, are the intracellular proteoglycans which include serglycin, a 15-20kDa proteoglycan. Serglycin contains either chondroitin, dermatan or heparan sulphates and can be shed from the surface of the cell during development (Templeton, 1992).

Each GAG chain is attached by one of three linkages to the core protein of proteoglycans. Type 1 linkages were first isolated from chondroitin sulphate and consist of an O-glycosidic linkage between D-xylose and the hydroxyl group of a serine residue (Roden & Smith, 1966), whereas Type 2 linkages are recognised by an N-glycosylamine linkage between N-acetyl glucosamine and the amide group of asparagine and are seen in corneal keratan sulphate (De Luca *et al.*, 1980). Skeletal keratan sulphate exhibits the type 3 linkage which is an O-glycosidic linkage between N-acetyl galactosamine and the hydroxyl groups of threonine or serine (Bray *et al.*, 1967).

1.3.3   Hyaluronic Acid

Hyaluronic acid (HA) glycosaminoglycan, is a linear polysaccharide composed of two

alternately linked sugars, D-glucuronic acid and N-acetylglucosamine (Fig 1.1a). The molecular weight of HA varies from an average low value of 70,000 for bovine vitreous humour HA, to 12×10^7 for HA isolated from bovine synovial fluid (Varga, 1955; Silpananta et al., 1968). HA occurs in different concentrations and in different forms in tissues, for example, in cartilage, the concentration of free HA is relatively small as it is associated with proteoglycans, whereas in synovial fluid it occurs mainly in solution (Hardingham & Muir, 1972; Swann, 1978). HA is well known for its viscoelastic and joint lubricating properties and has had medical applications as a vitreous substitute in retina-reattachment surgery and in the treatment of osteoarthritis (Balazs et al, 1972; Peyron & Balazs, 1974; Derlinger & Balazs, 1980). HA is known to have various effects on cell proliferation which may reflect its role in the wound healing process. West & Kumar demonstrated that angiogenic HA oligosaccharides (between 3-16 disaccharides) in vivo, stimulated endothelial cell proliferation and collagen production in vitro, whereas bigger oligosaccharides had no effect or inhibited endothelial cell proliferation (West & Kumar, 1989; Rooney et al., 1993). Native HA has also been reported to have no effect on the proliferation of bovine adrenal capillary cells, epithelial cells, smooth muscle cells, fibroblasts and pericytes (Orlidge & D'Amore, 1986), although size independent inhibition has been shown with rabbit synovial cells and 3T3 fibroblasts by Goldberg & Toole, (1987). The difference in findings between the latter and those of Orlidge & D'Amore (1986) may be due to the fact that different sources of cells were used and were cultured under different conditions, however there may be more fundamental reasons for these findings which will need further research to elaborate. HA exists in concentrations between 0.05 and 3mg/ml in adult tissues, but in

GENERAL INTRODUCTION

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Following granulation tissue formation, the fibrin matrix is broken down by the enzyme plasmin and fibroblasts transform into fibrocytes or myofibroblasts, the latter being able to draw the wound edges together by contraction (Fowler, 1989). At the end of the wound healing process, the wound is closed by epithelialisation. This is initiated by an increased rate of cell division in the basal layer of the epidermis and migration of the resulting epithelial cells towards each other from the wound margins, resulting in the defect being covered by a fine layer of skin (Chernoff & Robertson, 1990). Repair is not complete until some time after coverage by the epithelium and an increase in the tensile strength has been achieved. Remodelling of the new tissue takes months with the deposition of type 1 collagen fibrils, resulting in the formation of a scar, which lacks some components of normal tissue and it is therefore less functional (Adzick & Longaker, 1992).

1. 3   EXTRACELLULAR MATRIX COMPONENTS IN WOUND HEALING

The extracellular matrix (ECM) has an important role in wound healing. In the past, it was thought to be an inert support material, providing a scaffold that stabilised the physical structure of tissues (Alberts *et al*, 1983) The ECM is now known to influence adhesion, migration, proliferation and differentiation of cells (Hook *et al*, 1982). The ECM is composed of two main classes of extracellular macromolecules: collagen and glycosaminoglycans. The collagen fibres strengthen and help to organise the matrix,

while the gel-like glycosaminoglycans permit the diffusion of nutrients, metabolites and hormones between the blood and the tissue cells. Elastin, proteoglycans, fibronectin and laminin are also part of the ECM (Alberts *et al*, 1983). The relative amounts of the different types of matrix macromolecules and the way they are organised within the ECM vary with the type of matrix.

1.3.1   Collagen

1.3.1.1   Introduction

The collagens are a family of highly characteristic fibrous proteins found in all multicellular animals. They constitute 25% of the total protein in mammals and are therefore extremely abundant. The polypeptide chains have the repeating structure of Gly-X-Y, where X is usually proline and Y hydroxyproline. This sequence does not apply to the non-helical domains at each of the collagen molecule. The triple helix consists of 3 alpha chains wrapped around each other with each chain containing about 1000 amino acids, right-handed and rod-shaped (Bailey, 1985). Collagen is responsible for many of the essential characteristics of connective tissue, particularly their mechanical and biological properties (Bailey & Light, 1989; Gorham S.D., 1991). To date, 16 different genetic forms have been isolated and identified. Some of these types and their structures are listed in Table 1.1 (Bailey & Light, 1989 ; Martin *et al*, 1985 ; Summers *et al*, 1988). Type I is the major component of most collagen based prosthetic materials.

1.3.1.2   Synthesis of Collagen

Synthesis of collagen has been described in many reviews (Duance & Bailey, 1981 ;
Last & Reiser, 1984; Burgeson & Nimni, 1992), and therefore only a brief account will
be given here.

The precursor chains of the collagen molecule are synthesised on ribosomes, transferred
into the endoplasmic reticulum where the signal sequences are removed, then secreted
in secretory granules from the Golgi apparatus in its triple helical form (Duance &
Bailey, 1981; Weinstock & Leblond, 1974). The individual collagen molecules then
spontaneously aggregate to form a "quarter-stagger" arrangement to form fibrils. The
heads of the collagen molecules are staggered along the length of the fibril resulting in
the characteristic 64 nm spacing of cross-striations (Duance & Bailey, 1981). Following
formation of the fibrils, intermolecular cross-linking takes place which stabilises the
collagen (Eyre, 1985).

1.3.1.3   Degradation of Collagen

Collagen degradation is important in the repair or replacement of damaged tissue in
wound healing. There are three groups of enzymes which are responsible for collagen
degradation: the metalloproteinases, neutral proteinases and lysosomal cathepsins
(Duance & Bailey, 1981 ; Sellers & Murphy, 1981). During degradation, collagen
fragments are generated from the fibre which are then capable of being phagocytosed
and degraded in lysosomes by acid-proteinases (Bailey & Etherington, 1985)

Collagen and collagen-derived peptides have been found to serve as chemoattractants
for fibroblasts and neutrophils *in vitro* and therefore may function as chemotactic

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stimuli to effect cell migration to sites of inflammation *in vivo* (Laskin *et al*, 1986; Postlethwaite *et al.*, 1978; Albini & Adelmann-Grill, 1985). Such a role for collagen peptides may be important in the development of biological dressings.

1.3.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) are high molecular weight carbohydrate polymers and are important structural elements of the extracellular matrix of animal connective tissue. They consist of polymers of repeating disaccharide units that contain a hexosamine and either a carboxylate or sulphate ester, or both, which provide the chains with linear arrays of anionic groups. The following GAGs are found in mammalian tissues : hyaluronic acid, chondroitin 6-sulphate, chondroitin-4-sulphate, dermatan sulphate, heparin, heparan sulphate and keratan sulphate and the principal repeating units of the individual GAGs are shown in Fig 1.1

GAGs normally exist covalently bound to core proteins in the form of proteoglycans. Table 1.2 shows the five different classes of proteoglycan each differing in GAG composition, core protein size or source (Templeton, 1992). **Family a** proteoglycans consist of multiple glycosaminoglycan chains. This family of proteoglycans associate with hyaluronic acid to form large aggregates, and reside in the extracellular compartment. Aggrecan belongs to this family and has a 220kDa core protein with over 100 chondroitin sulphate chains and a keratan sulphate-rich region also (Doege *et al*, 1987). **Family b** proteoglycans are small molecules containing only 1 or 2 GAG chains and are found in the connective tissue (Templeton, 1992). Decorin belongs to this

family and has a core protein of only 40kDa (Krusius & Ruoslahti, 1986). **Family c** proteoglycans are the basement membrane heparan sulphate proteoglycans which have large core proteins (400kDa), but only have 2 to 4 heparan sulphate chains (Templeton, 1992). **Family d** are the cell surface proteoglycans such as the mouse mammary epithelial proteoglycan, syndecan. Syndecan consists of a 30kDa core protein with a hydrophobic transmembrane domain and has chondroitin and heparan sulphate chains in the extracellular region (Saunders *et al*, 1989). The last family, **Family e**, are the intracellular proteoglycans which include serglycin, a 15-20kDa proteoglycan. Serglycin contains either chondroitin, dermatan or heparan sulphates and can be shed from the surface of the cell during development (Templeton, 1992).

Each GAG chain is attached by one of three linkages to the core protein of proteoglycans. Type 1 linkages were first isolated from chondroitin sulphate and consist of an O-glycosidic linkage between D-xylose and the hydroxyl group of a serine residue (Roden & Smith, 1966), whereas Type 2 linkages are recognised by an N-glycosylamine linkage between N-acetyl glucosamine and the amide group of asparagine and are seen in corneal keratan sulphate (De Luca *et al.*, 1980). Skeletal keratan sulphate exhibits the type 3 linkage which is an O-glycosidic linkage between N-acetyl galactosamine and the hydroxyl groups of threonine or serine (Bray *et al.*, 1967).

1.3.3 Hyaluronic Acid

Hyaluronic acid (HA) glycosaminoglycan, is a linear polysaccharide composed of two

alternately linked sugars, D-glucuronic acid and N-acetylglucosamine (Fig 1.1a). The molecular weight of HA varies from an average low value of 70,000 for bovine vitreous humour HA, to 12×10^7 for HA isolated from bovine synovial fluid (Varga, 1955; Silpananta *et al.*, 1968). HA occurs in different concentrations and in different forms in tissues, for example, in cartilage, the concentration of free HA is relatively small as it is associated with proteoglycans, whereas in synovial fluid it occurs mainly in solution (Hardingham & Muir, 1972; Swann, 1978). HA is well known for its viscoelastic and joint lubricating properties and has had medical applications as a vitreous substitute in retina-reattachment surgery and in the treatment of osteoarthritis (Balazs *et al*, 1972; Peyron & Balazs, 1974; Derlinger & Balazs, 1980). HA is known to have various effects on cell proliferation which may reflect its role in the wound healing process. West & Kumar demonstrated that angiogenic HA oligosaccharides (between 3-16 disaccharides) *in vivo*, stimulated endothelial cell proliferation and collagen production *in vitro*, whereas bigger oligosaccharides had no effect or inhibited endothelial cell proliferation (West & Kumar, 1989; Rooney *et al.*, 1993). Native HA has also been reported to have no effect on the proliferation of bovine adrenal capillary cells, epithelial cells, smooth muscle cells, fibroblasts and pericytes (Orlidge & D'Amore, 1986), although size independent inhibition has been shown with rabbit synovial cells and 3T3 fibroblasts by Goldberg & Toole, (1987). The difference in findings between the latter and those of Orlidge & D'Amore (1986) may be due to the fact that different sources of cells were used and were cultured under different conditions, however there may be more fundamental reasons for these findings which will need further research to elaborate. HA exists in concentrations between 0.05 and 3mg/ml in adult tissues, but in

healing tissues, this rises to between 4 and 10mg/ml. After injury therefore, there is an influx of HA to the wound site. It was postulated by Weigel *et al* (1986), that HA interacts with fibrin to provide a matrix for cellular infiltration and regulates many functions of blood cells in the inflammatory reponse. They also suggested that the matrix is degraded as new collagen and glycosaminoglycans are secreted to make a more differentiated matrix (Weigel *et al*, 1986). Exogenously added HA is known to inhibit leukocyte locomotion (Forrester & Wilkinson, 1981), and this may have relevance in healing wounds, as after the inflammatory response, the concentration of HA rises thereby restricting further leukocyte invasion. This concept can be clearly seen *in vitro* with tumour cells which have been shown to be protected from attack by cytolytic immune lymphocytes by the presence of a cell-surface layer of HA (McBride & Bard, 1979). Subsequently, neutrophil adhesion is also inhibited by the presence of HA, thereby preventing locomotion of the cells (Forrester & Lackie, 1981). It would appear therefore that HA provides a regulatory role in the inflammatory response. The potential role of HA and its fragments in wound healing are investigated in this study and the results compared with those previously published.

1.3.4 Chondroitin Sulphate

The chondroitin sulphates glycosaminoglycans are a family of glucuronic acid containing GAGs that include chondroitin sulphate A, C, D, E, H & K which have disaccharide units with different sulphation patterns (Figure 1.1b). In the 1950's, its detailed structure was determined by Meyer and his collaborators (Meyer & Rapport, 1950; Meyer *et al*, 1956) and the repeating unit of chondroitin sulphate was found to be

very similar to that of hyaluronic acid. It has a glucuronic acid residue linked to a N-acetylgalactosamine residue, but in contrast to HA, chondroitin sulphate can have O-sulphated groups especially at the 4- or 6- position of the N-acetylated residue.

Chondroitin Sulphate (CS) is a main component of connective tissue and has been isolated from many sources, including shark skin and hagfish skin (Akiyama & Seno, 1981; Seno *et al*, 1972). The chains of the chondroitin sulphates are of relatively low molecular weight (10,000-20,000) in contrast to HA. However, all exist as proteoglycan complexes with an average of 80 chondroitin sulphate chains per proteoglycan complex. As an ECM molecule, CS plays a role in wound healing. *In vitro*, chondroitin sulphate appears to have no effect on the proliferation of fibroblasts, however, it has been shown to reduce the clotting time of fibrinogen and increase the rate of fibrin polymer formation (Westergren-Thorsson *et al*, 1991; LeBoeuf *et al.,* 1987). When incorporated into a collagen sponge and implanted in the rat lumbar muscle, the sponge containing the CS has been shown to have an improved infiltration of fibroblasts at day 14 when compared to any other GAG combination and had less inflammatory cells present. These findings and the structural similarity with HA may suggest a role for CS and its fragments (oligosaccharides) in wound healing and are therefore the subject of research reported in this thesis.

1.3.5 Dermatan Sulphate

Dermatan sulphates (DS) glycosaminoglycans are composed of alternating galactosamine 4-or 6- sulphate and uronate residues (see Figure 1.1c). The uronate residues can either be glucuronic acid or iduronic acid and the latter can be ester-

sulphated at C-2 (Lindahl & Hook, 1978). DS exists as proteoglycans in tissues such as skin and articular cartilage (Coster *et al*, 1975; Rosenberg *et al*, 1985) and is known to interact with collagen and fibronectin in the ECM (Scott & Haigh, 1985; Lewandowska *et al*, 1987). DS oligosaccharides have been found to interact with plasma heparin co-factor II which results in a dramatic increase in the rate of thrombin inhibition (Tollefsen *et al*, 1986). Dermatan sulphate has also been shown to inhibit the proliferation of fibroblasts *in vitro* (Westergren-Thorsson *et al*, 1991), however, the potential role of DS and its oligosaccharides in wound healing is not investigated in this thesis due to time constraints.

1.3.6 Heparin

Heparin is also a member of the GAG family. The repeating unit is predominantly L-iduronic acid, although D-glucuronic acid is present as a minor constituent, and it is linked to the N-sulphated hexosamine by an a ß(1,4) linkage (Figure 1.1d). Like other GAGs, heparin is synthesised as a proteoglycan and is stored in mast cell granules in many animal tissues, including connective tissues. The most widely known biological properties of heparin are its anticoagulant and anticomplement activities (Barrowcliffe *et al*, 1978; Almeda *et al*, 1983). Heparin has also been reported to have *in vivo* effects on wound healing (McPherson *et al*, 1988) and this will be discussed further in Chapters 4, 5 & 6. These findings suggest a role for heparin in wound healing and are therefore the subject of research reported in this thesis.

1.3.7 Heparan Sulphate

Heparan sulphates are structurally related to heparin, but are the most hetergeneous of the GAGs. They contain both glucuronic acid linked to N-sulphated glucosamine and iduronic acid linked to N-acetylglucosamine (Figure 1.1e). Heparan sulphates (HS) in the form of proteoglycans are found on cell surfaces or in the ECM of all mammalian organs and tissues (Klagsbrun & Baird, 1991; Gallagher & Turnbull, 1992). The function of proteoglycans are numerous due to the range of binding properties of the heparan sulphate chains and variations in structure of the core protein. It is common to find 2 or 3 HS chains close to each other attached to serine residues of the polypeptide core. The 2 main classes of membrane Heparan Sulphate Proteoglycans (HSPG) are the syndecans which are positioned across the membrane and the GPI-anchored glypicans. There are no reports in the literature to date of any *in vivo* effects of heparan sulphate on wound healing, however *in vitro*, it has been shown, like heparin, to inhibit the proliferation of fibroblasts (Ferrao & Mason, 1993). Due to time constraints, heparan sulphate was not investigated for its wound healing potential in this thesis.

1.3.8   Heparan Sulphate & Growth Factor Interactions

Heparan sulphates are known to be important in the activation of many growth factors including the FGFs (fibroblast growth factors), VEGF (vascular endothelial growth factor) & EGF (epidermal growth factor) (Jayson & Gallagher, 1997). bFGF and VEGF will be discussed here due to their angiogenic role in wound healing and bFGF's ability to effect fibroblast proliferation & migration.

1.3.8.1 bFGF and HS Interactions

HS chains bound covalently to membrane proteins are the receptors or co-receptors for

FGF. The high affinity FGF receptors consist of four closely related gene families

(FGFR1-4) and there is 72% amino acid homology across the family.

In cultured capillary endothelial cells, 70% of bFGF is bound to the extracellular matrix

HSPG, perlecan, which also has 2-3 HS chains. Perlecan stores growth factors and

prevent proteolysis and regulates delivery to cells (Aviezer *et al*, 1994). It has also been

found to be the most effective proteoglycan at promoting bFGF action.

The importance of HS in bFGF activation was seen in Chinese hamster ovary cells

defective in HS synthesis or when HS synthesis was inhibited in 3T3 fibroblasts with

chlorate (Stringer & Gallagher, 1996). In all these studies, the response to bFGF was

restored by adding heparin.

The minimal binding sequence for HS in bFGF appears to accommodate a

pentasaccharide sequence [hexuronic acid-GlcNSO$_3$-hexuronic acid-GlcNSO$_3$-IdoA,2S]

(Salmivirta & Jalkanen, 1995), however high affinity binding and bFGF activation

requires longer sequences based on the structure (IdoA,2S-GlcNSO$_3$)$_n$ where n has a

minimum of 5 (Gallagher & Turnbull, 1992). The requirement for longer sequences to

activate bFGF suggests that the saccharide must accommodate 2 bFGF molecules to

create a dimeric form of the growth factor. The dimer is assumed to be necessary to

them dimerise the receptor and promote cell signalling (Coutts & Gallagher, 1995).

However, it has been suggested that the longer sequences may act as a template to

which the bFGF and receptor bind to promote growth factor function by proximity (Kan *et al*, 1993; McKeehan *et al*, 1994).

1.3.8.2 VEGF & HS Interactions

VEGF is a dimeric glycoprotein (34-45kDa) that stimulates endothelial cells, induces angiogenesis and increases vascular permeability and it shares similarities in amino acid sequence to PDGF and placenta growth factor (Toi, 1998). There have been 4 different VEGF transcripts discovered to date : 2 which are largely free to diffuse in tissues because of their absent or reduced heparin binding affinity and 2 with high affinity heparin binding activity which are cell-associated (Toi, 1998). Flt-1 and KDR are the 2 receptors identified for VEGF at present, and they have been found to be essential for the formation of normal vasculature (Tanaka *et al*, 1997). They both contain intracellular tyrosine kinase domains, however, Flt-1 is also known to bind to placenta growth factor, unlike KDR.

As the name suggests, VEGF leads to mitogenesis by endothelial cells, however it also is involved in chemotaxis and expression of plasminogen activators and collagenases, all of which facilitate the migration of new endothelial cells (Ferrara *et al*, 1991). VEGF is 50,000 times more potent than histamine in inducing vascular permeability (Dvorak *et al*, 1995) and it has also been shown to have effects on the migration of monocytes and fetal bovine osteoblasts.

VEGF expression is induced in non-malignant conditions such as in wound repair and its expression can be upregulated by other factors such as PDGF, EGF and TNF-alpha (Toi, 1998). It is also expressed highly in many tumour types specifically within the blood vessels (Berse et al, 1992). Blocking VEGF activity using monoclonal antibodies or antisense constructs can inhibit the growth of tumors in vivo (Kondo et al, 1993).

1.3.9 Keratan Sulphate

Keratan sulphate (KS) was first isolated from bovine cornea by Meyer et al, (1953) and since then, has been found in many other tissues including bovine and human skeletal cartilage (Meyer et al, 1958; Cockin et al, 1986). Skeletal KS chains occur covalently attached to protein in the form of large aggregating proteoglycans, together with chondroitin sulphate and numerous O-linked and N-linked oligosaccharides (Figure 1.1f).

Type I is corneal KS which is N-linked via glucosamine to asparagine (Baker et al, 1969 ; Bray et al, 1967). Type II is skeletal in which there is an O-glycosidic linkage from an N-acetyl glucosamine residue to either serine or threonine (Bray et al, 1967). A possible third type has been isolated from brain tissue (Krusius et al, 1986), which has an O-glycosidic bond between mannose and threonine or serine. All three forms share the same repeating disaccharide sequence of galactose linked $\beta(1,4)$ to N-acetyl glucosamine, these disaccharides being linked by $\beta(1,3)$ linkage to one another. Most of the N-acetyl glucosamine residues are sulphated at C6, as well as some galactose residues (Bhavanandan & Meyer, 1968). Mannose and fucose have also been identified

as components of KS. Recently, monoclonal antibodies to skeletal KS have been manufactured and used in clinical assays as markers for osteoarthritis. As yet there are no reported claims of keratan sulphates being important in wound healing in either *in vitro* or animal studies and are therefore not investigated in this thesis.

1.3.10 Fibronectin

Fibronectin is a widely distributed glycoprotein which is found in high concentrations in the extracellular matrix and in plasma (Ayad *et al*, 1994). The structure of fibronectin consists of two non-identical subunits covalently linked near the COOH-terminal by a pair of disulphide bonds and has a molecular weight of 5.5×10^5 Da (Fig 1.2). In wound healing, fibronectin promotes the spreading of platelets at the site of injury, the adhesion and migration of neutrophils, monocytes, fibroblasts and endothelial cells into the wound area, and the migration of epidermal cells through the granulation tissue. Fibronectin also has a role in tissue remodelling by activating phagocytosis of wound debris by macrophages, fibroblasts and keratinocytes to phagocytose debris (Grinnell, 1984). As fibronectin contains heparin, it may also have a potential role in wound healing with the GAGs role. Fibronectin is not subject to investigation in this thesis.

1.4   CLINICAL APPLICATIONS OF ECM MOLECULES

1.4.1   Collagen

Collagen has many unique features that make it suitable as a biomaterial. These include, high tensile strength, controllable biodegradation and haemostatic properties
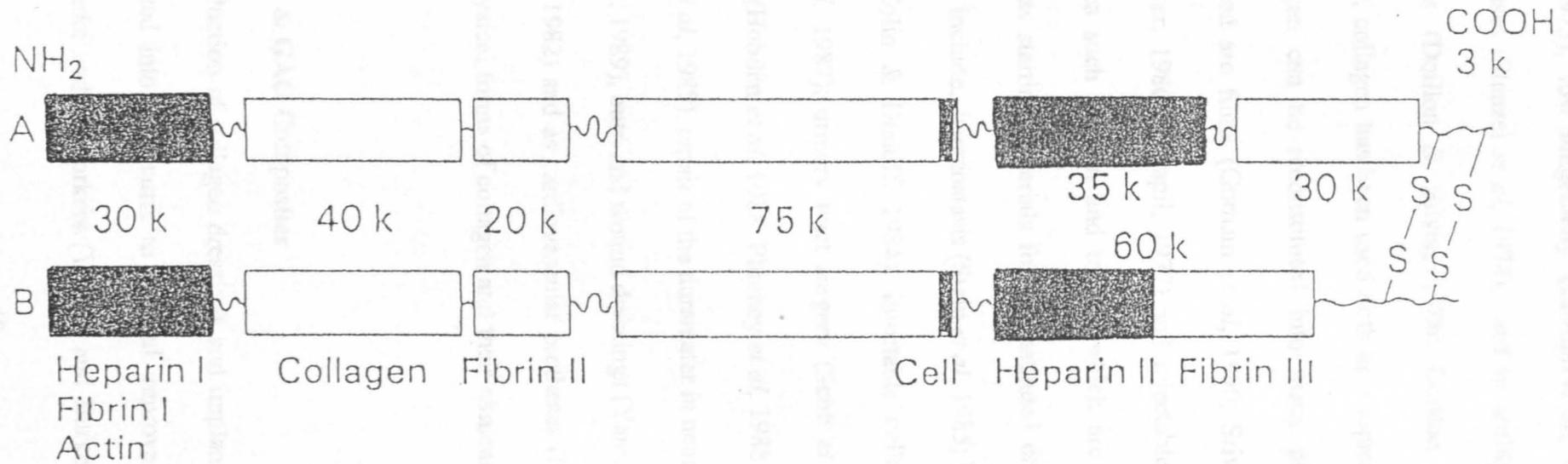
Figure 1.2 :   The structure of fibronectin
Taken from Molecular Cell Biology, Darnell *et al*, Scientific American Books p862

(Chvapil *et al*, 1973), low antigenicity (Chvapil *et al*, 1973), low inflammatory and cytotoxic responses (Stenzel *et al*, 1974), and its ability to promote attachment and growth of cells (Doillon & Silver, 1986; Doillon *et al.*, 1988). Due to its biocompatibility, collagen has been used both as a topical dressing and as an implant material. Collagen can be reconstituted into many physical forms and the most frequently applied are films (Gorham *et al*, 1990; Srivastava *et al*, 1990), sponges (Doillon & Silver, 1986; Chvapil, 1977) and injectable solutions (McPherson *et al*, 1986ab). Tissues such as skin and tendon which are rich in fibrous collagen are generally used as starting materials for reconstituted collagen preparations. Clinical uses of collagen include, haemostasis (Stein *et al*, 1985; Voormolen *et al*, 1987), nerve regeneration (Colin & Donoff, 1984), injectable collagen for tissue augmentation (DeLustro *et al*, 1987), urinary tract surgery (Scott *et al*, 1986; Scott *et al*, 1988), ophthalmology (Hobden *et al*, 1988; Phinney *et al*, 1988) periodontology (Pitaru *et al*, 1988; Minabe *et al*, 1989), repair of the duramater in neurosurgery (Jannetta & Whayne, 1965; San-Galli, 1989), burn and wound dressings (Yannas & Burke, 1980; Burke *et al*, 1981; Chvapil, 1982) and as cardiovascular protheses (Nimmi, 1988). A summary of the different physical forms of collagen and their clinical applications is given in Table 1.3.

1.4.2 Collagen & GAG Composites

Since the introduction of collagen dressings and implants, other ECM molecules have been incorporated into the matrix to try and improve the efficacy of the dressing. Yannas and Burke and co-workers (Yannas and Burke, 1980; Burke *et al*, 1981;

FORM OF COLLAGEN	EXAMPLES OF APPLICATION	REFERENCES
Solution	Injectable	McPherson *et al*, 1988
Gel	Vitreous Body Injectable	Hobden *et al* 1988; Phinney *et al*, 1988 DeLustro *et al*, 1987
Powder	Haemostat	Stein et al, 1985; Voormolen *et al*, 1987
Film	Corneal replacement, corneal shield Wound dressing Patches (aneurysm, bladder) Periodontal treatment	Hobden *et al* 1988; Phinney *et al*, 1988 Srivastava et al, 1990 Scott *et al* 1986 Pitaru *et al*, 1988; Minabe *et al*, 1989
Sponge	Wound dressing Burn dressing Haemostat	Srivastava et al, 1990 Yannas & Burke, 1980: Burke et al, 1981; Chvapil, 1982
Tube	Vessel prostheses	Nimmi, 1988

Table 1.3 :   Physical forms of collagen and their clinical applications

Dagalakis *et al*, 1980) developed a burn dressing from collagen and C6S which functioned like artificial skin. It had a bilaminar structure : an outer silastic membrane layer and an inner porous layer of a collagen/chondroitin-6-sulphate composite which was dehydrothermally crosslinked and then treated with glutaraldehyde. The outer layer prevented fluid loss and acted as a barrier to bacteria as well as giving the dressing mechanical strength. The inner layer, which was in direct contact with the wound bed, had a pore structure which was optimal for cell invasion. Fibroblasts invaded the dressing and degraded the collagen/chondroitin-6-sulphate matrix while simultaneously laying down new collagen. There appeared to be no inflammatory or foreign body response to the dressing and the graft was not rejected in the 10 human patients. A similar dermal substitute containing C6S and collagen was designed by Boyce *et al*, (1988). Their findings showed that when the dermal substitute was grafted onto full thickness burns in human patients, wound contracture was significantly reduced, and more rapid and more complete wound healing took place. Wound healing was determined by the regeneration of connective tissue from the wound bed and the formation of fully stratified and differentiated epidermis. Collagen dressings containing hyaluronic acid, up to 2.5%, and fibronectin have been shown to increase L929 mouse fibroblast proliferation *in vitro*, as well as acting as support structures for cells in culture (Doillon *et al*, 1987 & 1988; Srivastava *et al*, 1990). *In vivo* observations have confirmed these results (Srivastava *et al.*, 1990). This study investigated a series of collagen-based films and sponges containing 10% CS and 20% HA which were implanted into the lumbar muscle of the rat. After 7 days implantation, all materials elicited an inflammatory response, however, by 14 days a marked infiltration by

neutrophils was apparent with subsequent degradation of existing collagen material. The collagen/HA composites evoked a greater inflammatory response than native collagen, whereas the collagen/CS composites elicited the least inflammatory cell response at day 7 and infiltration by fibroblasts was seen after 14 days implantation.

The influence of heparin on the wound healing response to collagen implants has been investigated by McPherson *et al*, (1988). They utilised the rat subcutaneous and guinea pig dermal wound models to observe the effect of adding heparin to reconstituted bovine dermal collagen. The addition of heparin to these implants resulted in significant dose-dependent increase in the degree and extent of fibroblast invasion, but appeared to have no effect on vascularisation. The inclusion of heparin in the guinea pig model did result in a significantly different pattern of wound healing, as histologically, the implants appeared more porous, thereby allowing a greater penetration of granulation tissue into the implant. These effects were not observed with chondroitin sulphate or hyaluronic acid (McPherson *et al*, 1988). Heparan sulphate has also been used to augment wound healing. A recent patent application (Bononi, 1992) suggests that a topical preparation of collagen and heparan sulphate, in the form of a cream, will significantly accelerate the re-epithelialisation process of chronic ulcers experimentally induced in the animal and promote the recovery of chronic phlebostatic ulcers in humans. Glycagen® is a commercially available collagen dressing containing heparan sulphate and chondroitin-4-sulphate. Used as a dressing for gingival wounds, it was shown that the GAGs combined with the patient's glycoproteins (mainly fibronectin), and resulted in the production of fibrinleukocytic tissue followed by acceleration of tissue organisation. The dressing was resorbed by the body and appeared to have no

inflammatory effect (Roussille & Barthet, 1991).

1.4.3   Collagen And Mammalian Cell Composites

Since the introduction of their first artificial skin, Yannas and Burke have since demonstrated the seeding of autologous epithelial cells into their collagen matrices before grafting in order to provide a complete skin substitute (Yannas *et al*, 1982). These grafts were applied to full thickness defects in guinea-pigs. Neovascularisation was observed after 10 days, and during the first year post-grafting, collagen deposition was determined as being randomly orientated, a feature of normal dermis. A skin graft kit has recently been patented which contains epithelial cells and a cross-linked collagen sheet carrier (Barlow, 1992). This dressing was developed for use on full and partial thickness wounds and deep wounds, where it may restore skin profile. Current work has focused on the delivery of fibroblasts to the wound site by seeding into a collagen matrix. The fibroblasts, cultured from the wounded patient, should stimulate *de novo* collagen deposition, and granulation tissue formation without initiating a foreign body response. Bertolami *et al*, (1988) introduced autogenous fibroblasts into a collagen/glycosaminoglycan/silastic membrane and applied it to cutaneous wound sites in rabbits. This dressing inhibited wound contracture and increased infiltration of fibroblasts and peripheral epithelium into the matrix . Doillon *et al*, (1988) observed the synergistic effects of growing fibroblasts and epithelial cells in a collagen sponge, *in vitro*. Their results demonstrated that epidermal cell replication was enhanced in the presence of collagen sponge seeded with fibroblasts. They concluded that this sponge may be convenient for clinical applications.

1.5 PLANT POLYSACCHARIDES AND CLINICAL APPLICATIONS OF WOUND HEALING

Plants have been used for many years as natural remedies for wound healing but have in the main been used in the food industry as thickeners and stabilisers (McDowell, 1977). Several plant polysaccharides are structurally similar to the GAG family and therefore have been focused on in the study reported here to try and ascertain if they have any similar biological activity to GAGs and therefore any advantageous effects on wound healing. The polysaccharides which will be discussed are both natural plant polysaccharides such as pectin and alginate, and synthetic polysaccharides derived from cellulose, for example, oxidised regenerated cellulose.

1.5.1 Pectin

Pectin is universally found in the cell walls of green land plants, especially in citrus fruits, and has a MW of about 50,000 to 180,000. D-galacturonic acid is the principal constituent, but some neutral sugars are also present. Small amounts of D-xylose, L-arabinose, and D-galactose may be part of the side chains or of accompanying neutral polysaccharides. Pectin can be methoxylated to varying degrees and the degree of this influences the properties of the molecule, especially the solubility and gelation requirements. The structure of pectin can be seen in Figure 1.3(c) and has similarities to other polysaccharides such as hyaluronic acid. Pectin is mainly used in the food industry as a gelling agent, however it has also found uses as an anti-diarroheal agent (Vahouny *et al*, 1978), lowering blood serum cholesterol (Johnson & Chang, 1978) and
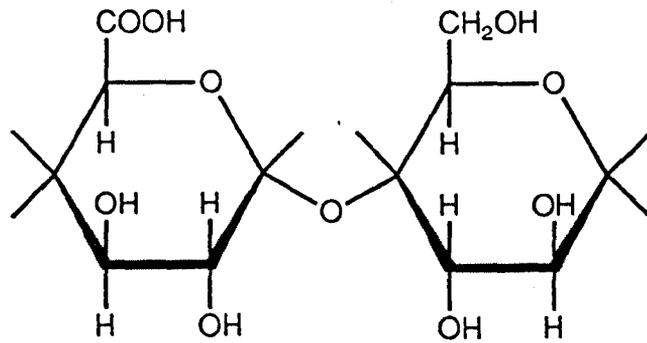
Figure 1.3(a) :   Oxidised Regenerated Cellulose
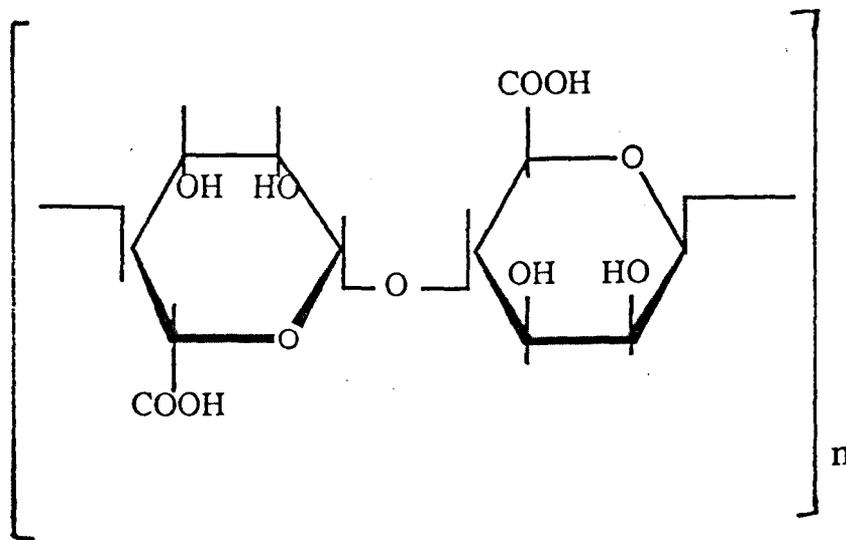
Figure 1.3(b) :   Alginate
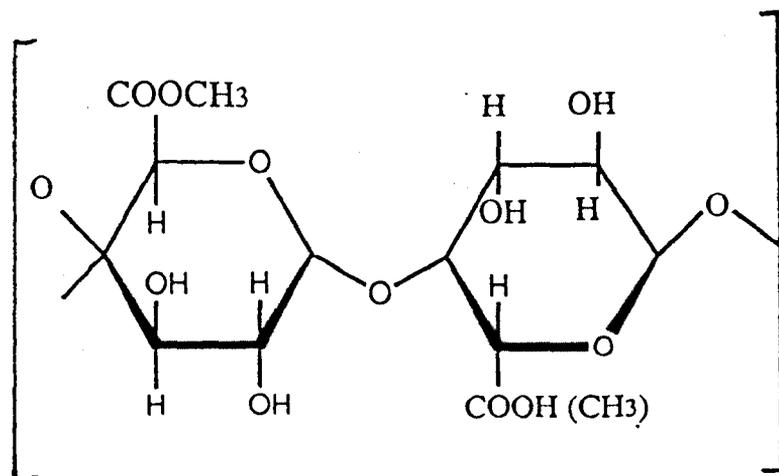
Figure 1.3(c) :   Pectin

also as an ingredient in an absorbant and curative wound dressing (US Patent 4341207). The structural similarities suggest a role for pectin in wound healing and are therefore the subject of research reported in this thesis.

1.5.2   Oxidised Regenerated Cellulose

Oxidised Regenerated Cellulose (ORC) is a synthetic polysaccharide and is produced from cellulose by oxidation with nitrogen tetraoxide in a chlorofluorocarbon solvent (Saferstein & Boardman, 1992). The resulting structure can be seen in Figure 1.3(a). The oxidation results in around 83% of the D-glucose units converting to D-glucuronic acid residues, giving a structure resembling that of several GAGs. The unconverted glucose units are randomly scattered throughout the chain. A secondary oxidation pathway exists in which one in ten repeating units carries a ketone carbonyl group on the C-2 or C-3 atom. ORC is insoluble in water, but has been found to progressively break down in alkaline solutions to oligosaccharide and monosaccharide components (Dimitijevich, 1990). Structurally ORC is most closely related to Alginic acid and chondroitin-6-sulphate and HA (Fig 1.1b).

Oxidised cellulose was first developed in 1943 by Eastman Kodak and first introduced as an absorbable haemostat by Parke-Davis as Oxycel in 1944. Oxidised regenerated cellulose was first marketed by Johnson & Johnson in 1950 as Surgicel® absorbable haemostat. Since then, another haemostat, NuKnit® and an adhesion barrier, Interceed® have been launched by Johnson & Johnson. As described previously in section 1.4.2, GAGs have been shown to influence wound healing. Since ORC is structurally similar to certain GAGs, the role of ORC and its oligosaccharides form part of the study

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reported here. Further support for a potential role of ORC oligosaccharides have come from findings at Johnson & Johnson which have shown that ORC oligosaccharides bind growth factors *in vitro* (PDGF) and mannose-6-phosphate (personal communication, Paul Watt, Johnson & Johnson).

1.5.3 Alginate

Alginates are naturally occurring polysaccharides which are found in certain species of brown seaweed. They are polymers of ß-D mannuronic acid and α-L guluronic acid (Fig 1.3b). Alginates are used extensively in the food industry as thickening and stabilising agents, but are also widely used in wound management due to their haemostatic and absorbant qualities (Odugbesan & Barnett, 1987: Groves & Lawrence, 1986). Alginates have been used as haemostats and wound dressings since the 1940's. Blaine described the benefits of using alginate as haemostats and wound dressings in several papers, including their apparent lack of toxicity and ability to be broken down by the body (Blaine, 1947; Blaine, 1951). Many commercial alginate products are now available and a summary is shown in Table 1.4. Sorbsan™, made from pure calcium alginate, has been used in the treatment of burns and donor sites. It was found that the alginate dressings allowed half as much blood loss from fresh split-thickness grafts than gauze alone, indicating its ability to act as a haemostat and was useful in absorbing fluid from heavily exuding wounds. Kaltostat™ contains 20% sodium alginate : 80% calcium alginate and can be used on heavily or lightly exuding wounds. Tegagel™ is a calcium alginate dressing, but due to the manufacturing process, the fibres are unable to expand freely and this limits its absorbancy. A relatively new dressing currently on

PRODUCT	COMPANY	USE
Algosteril	Johnson & Johnson	Heavily exudating wounds
Sorbsan™	Steriseal	Packing deeper wounds
Sorbsan Plus™	Steriseal	Heavily exudating wounds
Sorbsan SA™	Steriseal	Lightly exudating wounds
Kaltostat™	Brticair	Dry or lightly exudating wounds
Tegagel™	3M	Exudating wounds
Stop Hemo™	Windsor	First aid dressing
Fibracol*	Johnson & Johnson Medical	Topical dressing for leg ulcers

Table 1.4 :   Commercial Alginate Preparations

the market in the U.K. is Fibracol™, a freeze-dried collagen-calcium alginate sponge dressing manufactured by Johnson & Johnson Medical. This dressing combines the properties of collagen with calcium alginate (90 : 10 w/w) to provide a scaffold for the deposition and organisation of new collagen fibres in the wound (Doillon & Silver, 1986). The collagen-alginate dressing performed as well as Kaltostat™ in the treatment of leg ulcers in clinical trials, however it was preferred for its ease of removal and non-adherence to the wound (Personal communication, Johnson & Johnson Ltd, 1996). Alginates are not fully biodegradable and this has been demonstrated in some studies which have noted immune responses occurring in their presence, such as encapsulation and giant cell formation (Blaine, 1951) as well as granuloma formation (Personal communication, Johnson & Johnson Ltd., 1996). Alginates are not part of the investigation in this study as they are marketed by Johnson & Johnson as wound healing products.

1.5.4   Other Plant Derived Polysaccharides

Many other plant polysaccharides are recognised for their biological activity. Lentinan, a polysaccharide from a Japanese mushroom has been reported to have strong anti-tumour activity (Chirara et al, 1970), whereas immunostimulating activity has been shown by the polysaccharide AIP, extracted from the roots of Angelica acutiloba and is used as a potent adjuvant (Zhu et al, 1982). Sulphated polysaccharides such as those extracted from marine algae show anticoagulant activity (Forwell, 1956). Johnson & Johnson Ltd. have published a patent on sulphated ORC (sORC) and have shown that sulphated ORC oligosaccharides can bind growth factors, have anti-coagulant properties

and exhibit chemotactic effects which are very similar to the properties of heparan sulphate (Harvey, Light & Watt, UK Patent No.9613683.3). Anti-inflammatory activity has been demonstrated by polysaccharides extracted from *Aloe vera* as well as polysaccharides 101 and 103, isolated from medicinal plants of the *Asteraceae* and *Tiliaceae* families. No. 101 stimulated the healing of the gastric mucosa in rats with experimental ulcers (Venkata Rao, 1992).

1.6 Other Animal & Microbial Polysaccharides And Their Clinical Applications

1.6.1 Introduction

Many reports exist in the literature regarding the uses of other naturally occurring polysaccharides as well as those produced by bacteria. The following sections give a brief account of their uses only as they will not form of part of the investigation reported in this thesis.

1.6.2 Chitin & Chitosan

Chitin is a natural structural component of shellfish and is extracted from the shells of crab, lobster and shrimp (Klokkevold *et al*, 1991). Next to cellulose, chitin is the most plentiful natural polymer and is both biodegradable and non-toxic as its breakdown products are the natural metabolites glucosamine and N-acetyl glucosamine (Fig 1.4a & 1.4b; Crescenzi, 1994)). The main use of chitin is in the production of chitosan, which has a similar structure to cellulose. The molecular weight of chitosan is between 0.8 and
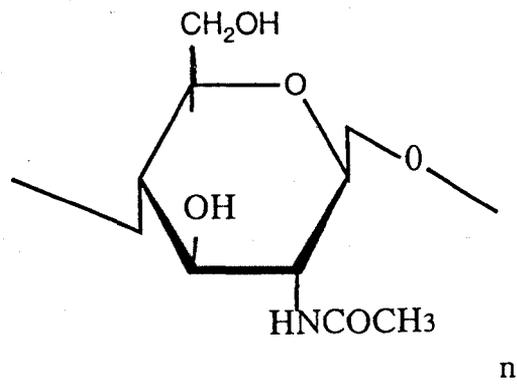
Figure 1.4(a) : Chitin
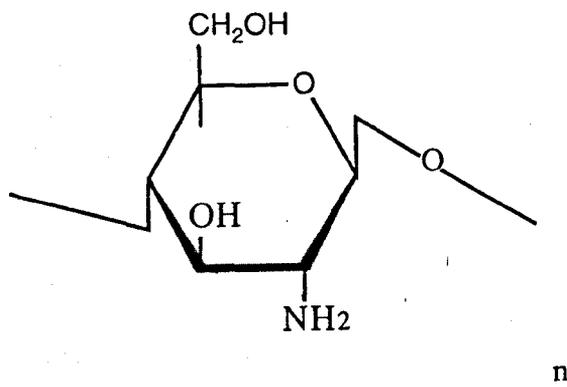
Figure 1.4(b) : Chitosan

1.5 x 10^6 Da and is a linear polymer of N-acetyl-D-glucosamine (Klokkevold *et al*, 1991). Chitin and chitosan have many applications in the food, cosmetic, water treatment and pharmaceutical fields and most interestingly, the medical field. The biomedical applications of chitosan include its uses to deliver drugs (Thanoo *et al*, 1992; Chandy & Sharma, 1991)), manufacture non-allergenic contact lenses (Protan Ltd.), and accelerate wound healing (Kyocera Corp., 1992; Saintigny *et al*, 1993)

1.6.3 Microbial Polysaccharides

Microbial polysaccharides exhibit a wide range of primary structures, some of which are exclusive to the microbial world while some exhibit structures similar to biopolymers from human, animal or vegetal sources. Large amounts of exocellular polysaccharides can be produced by microbial cell lines and are therefore cost effective and have potential relevance in industry (Crescenzi, 1994; Minami, 1993; Tanioca 1993). The principal industrial microbial polysaccharides and their uses are shown in Table 1.5 and are listed in order of decreasing industrial production.

1.7   GROWTH FACTORS IN WOUND HEALING

Although growth factors are not the subject of this study, it is important to understand this area as the role of these polysaccharides in wound healing might be via binding to growth factors. Exogenous additions of oligosaccharides may play a key role in protecting and/or regulating the role of growth factors in wound healing and these are discussed in the following sections.

Cohen & Levi-Montalcini isolated factors from tumour cells, snake venom and mouse

BIOPOLYMER	COMMON MICROBIAL SOURCE	APPLICATION
Dextran	*Leuconostoc spp.*	Pharmaceuticals, chemical gels
Xanthan	*Xanthomonas campestris*	Tertiary oil recovery, food additives, textiles
Gellan	*Pseudomonas elodea*	Food additives, physical gel fluids
Scleroglucan	*Sclerotium spp.*	Drilling fluids, rheology modifier
Succinoglycan	*Pseudomonas spp.*	Drilling fluids, rheology modifier
Curdlan	*Agrobacter spp.*	Physical gel fluids, food additives
Pullulan	*Aureobasidium pullulans*	Films for food uses
Welan	*Alcaligenes spp.*	Rheology modifier, drilling fluids

Table 1.5 :   Industrial Microbial Polysaccharides.
Taken from: Polysaccharide Science and Technology:
Development & Trends, Crescenzi, V. (1994), *TRIP*, **2**, March Issue.

submandibular salivary gland, which exhibited growth and differentiation-related activities in neural ganglia and ectodermal epithelium (Levi-Montalcini, 1987). To be classed as a growth factor, a biological molecule must be (i) a polypeptide that has the ability to bind to the specific receptors on the target cell surface (ii) be able to elicit a specific response induced by its binding to the receptor and (iii) be removed along with the receptor from the cell surface (Pittelkow, 1991). Several growth factors have been identified in tissue extracts or fluid from various types of wounds, such as TGFß, FGF and PDGF, and their functions are discussed below.

1.7.1   Platelet Derived Growth Factor (PDGF)

When platelets aggregate in the wound clot, they release PDGF which is stored in the alpha granules of the platelets. PDGF has been found to be mitogenic for fibroblasts, smooth muscle cells and glial cells (Grotendorst, 1992; Ross *et al*, 1974; Kohler & Lipton, 1974; Westermark & Wasterson, 1976) and stimulates cell replication by acting in tandem with factors such as insulin and somatomedins in the serum. Despite the lack of animal models of chronic wounds PDGF has been shown recently to be more effective than others in reversing states of impaired wound healing in animals (Zederfeld, 1993). PDGF, like TGFβ has the ability to enhance healing and the tensile strength of incisional wounds (Pittlekow, 1991). In full thickness pig skin wounds, PDGF has also been shown to stimulate healing without increasing inflammation, therefore it appears to act as an anti-inflammatory agent by inhibiting the production of complement proteins (Circolo *et al*, 1990)).

Recombinant PDGF has been tested in a randomised phase I/II double-blind study in

patients with type III/IV pressure sores, being topically applied daily. After 28 days, the PDGF-treated sores had reduced in size to 6.4% of their initial volume compared with 21.8% in the placebo group (Robson *et al*, 1992). PDGF has also been incorporated into various biomaterial matrices, such as fibronectin, fibrin, hyaluronic acid, chondroitin sulphate and collagen and its effect on *in vitro* fibroblast behaviour analysed (Roy *et al*, 1993). The results showed that PDGF enhanced replication when incorporated into both fibrin and hyaluronic acid.

1.7.2 Transforming Growth Factor Beta (TGFß)

TGFß has been purified from human platelets, serum, embryonic tissue and bovine kidney, with the human kind being known as $TGFß_1$ (Pittelkow, 1991). This growth factor is a homodimeric, disulphide-bonded molecule with a molecular weight of 25kDa. Additional forms $TGFß_{2-5}$ have subsequently been discovered by DNA library screening and cloning. TGFß was originally discovered to be secreted by human keratinocytes in culture (Shipley *et al*, 1986) and has since been found to be stored in the alpha granules of platelets from which it is released during coagulation and de-granulation (Pittelkow, 1991). TGFß is involved at all stages in the wound healing process. Following tissue injury, TGFß is released from the platelets and after the influx of monocytes to the injury site and their subsequent conversion to macrophages, further amounts of TGFß are released which facilitate in the recruitment of more inflammatory cells, debridement of tissue by macrophages and granulation tissue formation (Wahl *et al*, 1987). During the formation of granulation tissue, TGFß has been demonstrated to increase the expression of fibronectin, the fibronectin receptor, various types of

collagen, proteinase inhibitors, and is involved in the contraction of collagen by fibroblasts which aids wound closure (Dijke & Iwata, 1989; Montesano & Orci, 1988). TGFß is also known to bind to the core proteins of several proteoglycans (Templeton, 1992).

Studies with TGFß have shown it stimulates granulation tissue formation in pig excisional wounds pre-treated with glucocorticoids, and in conjunction, at higher doses, appears to inhibit epithelialisation (Quaglino et al, 1990; Shipley et al. 1986). In incisional wounds, TGFß improves the wound's tensile strength, if only transiently (Pittlekow, 1991). Recombinant forms of TGFß can now be generated and one these, TGFß$_2$, has recently shown promise in the treatment of full-thickness macular holes in the retina where it improved vision by flattening the surrounding neurosensory retinal detachments (Glaser et al, 1992).

1.7.3   Fibroblast Growth Factors (FGF)

The two major members of this family are basic FGF and acidic FGF and are composed of single chain peptides with a molecular weight of 1.5 x 10^4 Da (Pittelkow, 1991). Both types share 55% gene homology but have structural differences which give them different biological activities. Basic FGF is a mitogen for melanocytes, fibroblasts and keratinocytes and acidic FGF also stimulates fibroblast and keratinocyte replication (Shipley et al, 1986). In addition, the FGF family are also potent stimulators of angiogenesis in many tissues (Pittlekow, 1991).

The FGF family are also known to be heparin binding growth factors as discussed in section 1.3.7.1. It is suggested that basic FGF is bound to heparin or heparan sulphate proteoglycans in the basement membrane and is secreted only when the cells are disrupted by an injury to the tissue (Whalen & Zetter, 1992).

Over the last decade, many animal studies have been carried out in order to determine the effects of the addition of exogenous growth factors to experimentally induced wounds. A single application of FGF at the time of injury has been shown to stimulate epithelialisation in pigs (Hebda, 1990). Another study by Broadley *et al*, (1989), demonstrated that antibodies to basic FGF retarded the early stages of the wound response in rats implanted subcutaneously with polyvinyl alcohol sponges. It has also been shown that FGF acts as an anti-inflammatory agent and this may promote the next stages of the wound healing process to develop. Recombinant bFGF has been topically administered to patients suffering from type III/IV pressure sores. The results from this study demonstrated that patients treated with bFGF had a faster rate of wound closure, and a higher number of healed wounds than those treated with a placebo (Robson *et al*, in press). bFGF has also been shown to facilitate early dermal and epidermal wound healing when coated onto a collagen matrix (Marks *et al*, 1991).

1.8   WOUND MANAGEMENT

1.8.1   Introduction

As the aim of this study was to develop a wound dressing with an active

oligosaccharide component, it is of interest to discuss the likely target wounds such a dressing would be used to treat. Although such a dressing will not be likely to affect the underlying cause of the wounds that these dressings would be used to treat, it is hoped that they would reduce the time to healing and prevent hospital time.

1.8.2   Leg Ulcers

Leg ulcers have been reported in the literature as far back as the eighteenth and nineteenth centuries and were described as a "severe affliction" and a "loathsome disorder" (Loudon, 1982). The main causes in those times of this chronic condition were infection and malnutrition, i.e. scurvy. Today, the main cause of ulcers in the Western world is venous insufficiency, although there are many other contributory factors such as, poor arterial blood supply, malignancy and infection (Morison, 1991). Recent studies in the UK and Europe suggest that 1% of the population develop ulcers, and 20% of these people have an open ulcer at any one time. It is therefore a common complaint especially in the older population where it has been found to be particularly prevalent in women over the age of 65 years (Dale *et al*, 1983). Ulcers are notoriously slow to heal. 50% are open for more than a year, but 10% are still open after 5 years. Lack of healing in these cases is caused by several factors such as, not correcting the underlying cause of the ulcer, poor mobility, malnutrition, tissue damage and infection.

1.8.3   Decubitus Ulcers

Pressure sores (decubitus ulcers) also occur frequently in patients subjected to prolonged bedrest like quadriplegics and paraplegics who suffer skin loss due to the

effects of localised pressure.

1.8.4 Diabetic and Arterial Ulcers

Arteriosclerotic or diabetic disorders of blood flow can lead to the development of gangrene due to the deprivation of vital nutrients and this leads to necrosis.

1.8.5 Treatment of Chronic Wounds

A chronic wound is by definition a wound that will not heal or cannot heal. Poorly healing wounds appear to be a failure of the process of cell replication and the underlying blood flow and therefore enhancement of the growth signal has been one way forward in healing these wounds (Hunt & La Van, 1989).

Collagen dressings, described earlier in this introduction, have been employed in an attempt to encourage healing of wounds, but the extent of healing with collagen alone has not satisfied medical standards (Silver *et al*, 1988). Many growth factors of which some are described in Section 1.7, have been added to collagen matrices in an attempt to promote and accelerate the migration of cells into the wound site and encourage the deposition of new collagen. The high cost of these growth factors however, will be likely to result in an expensive product and would limit the number of patients who may benefit from it. Despite the continued development of new dressings, a product which is both effective at promoting wound healing and economical to produce, is still being sought. For this reason, this study was initiated to try and determine potential active agents that could potentially stimulate wound healing and provide Johnson & Johnson with a proprietary product and leading market position regarding wound dressings.

CHAPTER 2 : MATERIALS AND METHODS

2.1   PREPARATION OF OLIGOSACCHARIDES

2.1.1   Preparation of Hyaluronic acid (HA) & Chondroitin Sulphate (CS)

Oligosaccharides by Enzymatic Digestion

HA (MW 750 kDa, Lifecore, USA) and CS (MW 40-80kDa, Type C from shark cartilage, Sigma, England) were dissolved at 10mg/ml in 0.1M sodium acetate buffer pH 5.4 containing 0.15M NaCl. After incubating at 37°C for 30 minutes, Type V sheep testicular hyaluronidase (2400U/mg, Sigma, England) was added at 0.25mg/ml. Aliquots were taken out at various time intervals into glass test-tubes and immediately immersed in a boiling water bath for 10 mins to stop the enzyme activity. After cooling, to room temperature, each sample was filtered through a 0.45μm syringe filter to remove any debris including enzyme, then dialysed against 3 changes of distilled water using dialysis tubing (Spectra-Por 6 MW cut-off 1000 Da) to desalt the preparations. Finally, the samples were freeze-dried. The above method was adapted from West & Kumar, 1989.

2.1.2   Preparation of HA Oligosaccharides by Sonication

A 10mg/ml HA solution, made in dH_2O, was sonicated using a Kontes Micro Ultrasonic Cell Disrupter, KT 50, setting 20, for varying lengths of time to produce degraded HA samples (Rehakova et al, 1994; Personal communication - Paul Watt). The probe was

cooled on ice after each round of sonication to prevent thermal degradation of the sample. No further preparation was required.

2.1.3 Separation and Purification of HA Fragments by Gel Filtration

Separation of hyaluronic acid digests into oligosaccharide fractions of varying sizes was performed using a Biogel P6 column (1000 x 30mm, Pharmacia, Sweden) equilibrated with 50mM ammonium acetate buffer pH 5.0. The void volume was determined using blue dextran (Sigma, Dorset, England) at a concentration of 10mg/ml. The hyaluronic acid samples were applied to the column in 30ml of the eluting buffer (1mg/ml hyaluronic acid) and eluted at 1ml min^{-1} and 3ml fractions collected using an automatic fraction collector. This method was adapted from Cowman *et al*, 1984.

2.1.4 Preparation of ORC Oligosaccharides Fragment 1 (MW Range 5 - 40kDa)

Insoluble ORC fabric (Surgicel®) was obtained from Johnson & Johnson Medical Inc. New Jersey, USA. Fragment 1 was prepared by Peter Doyle at Johnson & Johnson Medical, Stirling as follows : a 20mg/ml suspension of ORC fabric in 6M sodium hydroxide was prepared and incubated at 37°C for 45 minutes. The reaction was stopped by the addition of concentrated hydrochloric acid until no further precipitation of ORC occurred. The suspension was then centrifuged at 15,000 rpm for 10 minutes. The pellet was dialysed against water using a Spectra/Por® membrane with a molecular weight cut-off of 1000 Da. The salt concentration was estimated by comparing the addition of 0.5ml of 0.25M silver nitrate to a 0.1% (w/v) sodium chloride and a

10mg/ml solution of ORC. This was carried out in glass test tubes and the precipitates formed following the addition of silver nitrate were compared visually to assess the level of salt in the ORC solution. After 3 days the salt concentration was estimated to be an acceptable level of less than 0.1% (w/v). The ORC was then freeze-dried and used for *in vitro* studies. Molecular weight was determined as described in section 2.2.7.

2.1.5   Preparation of ORC Oligosaccharide Fragment 2 (MW Range ~40kDa)

0.5g of ORC fabric was placed in 50ml of 0.1M sodium bicarbonate solution and placed in a water bath at 37°C for 15 minutes. The reaction was stopped by the addition of concentrated hydrochloric acid until precipitation of ORC was completed. The suspension was centrifuged at 15,000 rpm for ten minutes and dialysed using Spectra/Por® membrane (MW cut-off 1000Da) for 3 days against water. The salt concentration was estimated as described previously in section 2.1.4. After 3 days the salt concentration was estimated to an acceptable level of less than 0.1% w/v. The ORC was then freeze-dried and used for *in vitro* studies. Molecular weight was determined as described in section 2.2.7.

2.1.6   Preparation of Pectin Oligosaccharides by Alkali Hydrolysis

A 10mg/ml solution of pectin (High Methoxyl pectin, HP Bulmer) in 6M sodium hydroxide was prepared and incubated at 37°C for 45 minutes after which the reaction was stopped by the addition of concentrated hydrochloric acid until precipitation of pectin occurred. The suspension was then centrifuged at 15,000 rpm for 10 minutes.

Dialysis of the pellet against water was then carried out using Spectra/Por® membrane with a molecular weight cut-off of 1000 Da. The salt concentration was estimated by comparing the addition of 0.5ml of 0.25M silver nitrate to a 0.1% (w/v) sodium chloride solution with the precipitate formed on its addition to a 10mg/ml solution of pectin. After 3 days the salt concentration was estimated to an acceptable level of less than 0.1% w/v. The pectin was then freeze-dried and used for *in vitro* studies.

2.2 Molecular Weight Determinations of Polysaccharides

Different methods to determine the molecular weights of various polysaccharides were assessed in this study in order to obtain the most reliable method for each polysaccharide.

2.2.1 Molecular Weight Estimation by Viscometry

The relative viscosity was measured using an Ubbelohde suspended level viscometer (Figure 2.1a), secured in a water bath using a retort stand. The method was developed by Fermentech Ltd., Edinburgh and was calibrated for the molecular weight determination of HA. The method was therefore limited in its use with other polysaccharides and their fragments, but was however, used in this study as a comparison to other available methods. The temperature of the water was kept constant at 25°C ± 0.5°C. Using a syringe and 0.45µm filter, 13ml of PBS was dispensed into tube A of the viscometer. Then, with a dampened finger over tube B, PBS was sucked up tube C using a pipette filler until the meniscus was in the bulb above mark 1. After removing the pipette filler and finger, the time taken for the liquid to travel to mark 2
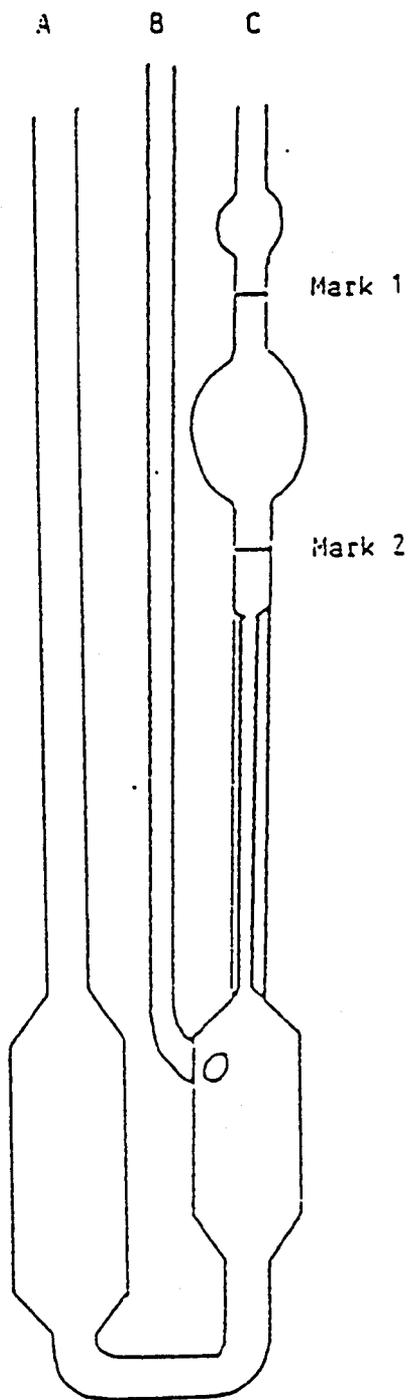
Figure 2.1 :   The Ubbelohde suspended level viscometer

1. $n_{sp} = \dfrac{T_1}{T_0} - 1$

2. $\ln_{rel} = \dfrac{\ln T_1}{T_0}$

3. $[n] = \dfrac{\sqrt{2}(n_{sp} - \ln_{rel})}{c}$

4. Molecular Weight $=$ antilog $\dfrac{\log [n] + 3.244}{0.75}$

Figure 2.1(b) Calculation of HA molecular weight by the Ubbelohde viscometer

was measured and was repeated until 3 readings fell within 0.2 seconds of each other (T_0). Using a new syringe and a new $0.45\mu m$ filter, 4.5ml of a 1mg/ml solution of polysaccharide was loaded through tube A, then with a finger over tube B and the pipette filler on tube C, air was blown through the solution to achieve adequate mixing. Tube B was then covered again and the solution sucked up until the meniscus was above mark 1. After removing the pipette filler and finger, the time taken for the solution to reach mark 2 was measured and repeated until three readings were within 0.2 seconds of each other (T_1). This method has proved to be efficient at measuring large molecular weight, viscous solutions, however it has limitations when the molecular weight falls. Another drawback of this method is that it must be calibrated for each different polysaccharide and this is time consuming.

The calculation for determining molecular weight by this method is shown in Figure 2.1(b)

2.2.2 Molecular Weight Determinations of HA, CS and ORC by HPLC.

A Kontron HPLC system was used (models 450mtz, 422, 425, 430). Two gel permeation columns were run in series : the first was a $10\mu m$ "Aquagel" (Jones Chromatography, Strathaven, Scotland) and the second a TSK gel G300pwxl (Anachem, Luton, England). The size exclusion columns were eluted with 5mM tetrabutylammonium phosphate (Sigma, Dorset) in dH_2O at a flow rate of 1ml/min^{-1}. This method was developed by Peter Doyle at Johnson & Johnson Medical.

Calibration of the column was performed using dextrans with molecular weights of 1200, 11700, 47250, 97000 and 326000 Da (Crawford Scientific, Strathaven, Scotland), with detection of the polysaccharides using a refractive index detector with the range equal to 0.2 x 10^{-3} ΔRI. Molecular weight was determined by running standard curve of the dextrans and comparing the elution time of the oligosaccharides to the standard curve. A example of a standard curve is shown in Figure 3.6. This method has its limitations with polydisperse polysaccharides as sharp defined peaks cannot be obtained therefore the weight estimations are not accurate. This method, however has its strengths at separating out the purer oligosaccharides as shown in Chapter 3.

2.2.3 Molecular Weight Determination of HA Oligosaccharides
by Pulsed Amperometric Detection

Hyaluronic acid fragments were sized using anion exchange chromatography with pulsed amperometric detection. The equipment was a Dionex Bio LC with a Carbopac PA1 column. The flow rate was 1 ml/min with a gradient eluant system as shown in Table 2.1. The gradient system was developed by Cathy Scott, University of Stirling. HA was dissolved at 1mg/ml in 18 mΩ deionised, filtered water and degassed thoroughly before injection.

Pulsed Amperometric Detection (Dionex) had limitations in that there were that no standards available to compare the molecular size to and so only qualitative data was obtained and was compared to those obtained by other methods. Another limitation of this method was that sulphated molecules could not be applied to this particular

Time (min)	% 200mM NaOH	% 200mM NaOH + 750mM NaOAc
0	100	0
1	100	0
2	100	0
90	0	100
90.1	0	100
120	0	100
120.1	100	0

Table 2.1 :   Gradient eluant system for Dionex chromatography of anionic polysaccharides. The flow rate was 1ml/min with a high pressure limit of 1800 psi and a low pressure limit of 300 psi.

column.

2.2.4 Molecular Weight Determination of HA Oligosaccharide by NMR

Hyaluronic acid oligosaccharides were prepared and examined as follows. Samples were dissolved in 0.5ml 99.8% ^2H_2O, buffered to pH7 with 10mM sodium phosphate mixture and referenced with sodium 3-trimethylsilyl [$^2H_4$] propionate as internal standard. After microfiltration through 0.45μm nylon filters, samples were dried using a rotary concentrator and exchanged several times with 0.5ml 99.8% ^2H_2O and then once with 99.6% ^2H_2O. ^{1}H-NMR spectra at 400 MHz were obtained at 23°C on a JEOL GSX-400 spectrometer fitted with a 5mm probe. All chemical shifts are given relative to internal 3-trimethylsilyl [$^2H_4$] propionate at 0.0ppm. NMR was carried out by Dr. Thomas Huckerby, University of Lancaster, England. The number of protons were calculated from this method thereby giving an accurate measurement of molecular weight.

NMR gives an accurate characterisation of the samples, however its limitations are in the amount of time needed to prepare the samples for analysis, the availability of the machine and the skill to interpret the data.

2.2.5 Molecular Weight Determination by Chemical Analysis

Chemical analysis is a rough tool used to determine molecular weight. It is a quick, easy and effective method.

2.2.5.1 Uronic Acid Content

Uronic Acid was determined by using the modified assay of Bitter & Muir (1962). A stock solution (0.025M) was prepared consisting of 9.5g sodium tetraborate dissolved in 1 litre of concentrated sulphuric acid. To a 1ml sample, 5ml of the tetraborate-acid solution was added. After thorough mixing, the solution was heated for 10 minutes in a boiling water bath and cooled to room temperature. 0.2ml of 0.125%(w/v) Carbazole (Sigma, Dorset) was prepared in absolute ethanol was added to the solution. The resulting solution was heated for 15 minutes in a boiling water bath, cooled, then the absorbance read at 530nm. Carbazole solutions are sensitive to light and were stored therefore in dark bottles during use. Analyses were performed on a Cecil 2020 spectrophotometer using glucuronolactone standards from 0 - 0.2 μmol/ml.

2.2.5.2 Estimation of N-Acetylamino Sugars

Reducing sugars were determined by a modified method of Elson & Morgan (1933). A 0.7M solution of boric acid was prepared and the pH raised to 9.2 with potassium hydroxide. A dimethylaminobenzaldehyde (DMAB) solution was also prepared by dissolving 10g of DMAB (Sigma, Dorset) in glacial acetic acid containing 12.5% (v/v) concentrated HCl. This was diluted 10 fold in glacial acetic acid before use. To a 0.5ml sample was added 0.1ml of the Boric acid solution, followed by heating in a boiling water bath for 5 minutes. After cooling on ice, 3ml of diluted DMAB was added and mixed thoroughly and the test tubes incubated at 37°C for 20 minutes. After cooling, the tubes were read immediately at 586nm. N-acetylglucosamine and N-acetylgalactosamine were used as standards at between 0 - 0.2μmol/ml. All analyses

were carried out on a Cecil 2020 spectrophometer.

2.2.5.3   Estimation of Number of Repeating Units in HA and CS

The number of repeating disaccharide units present in any HA and CS chain can be estimated by measuring the amount of uronic acid (method 2.2.5.1) and reducing power (method 2.2.5.2) present in the sample. The ratio of uronic acid to reducing N-acetylglucosamine and N-acetylgalactosamine (μmol/ml) is an estimate of the molecular weight of the sample. 1 disaccharide unit is equal to 400 Da. From this the number of disaccharide units can be determined as follows:

$$\frac{\text{Uronic acid (}\mu\text{mol/ml)}}{\text{Reducing Power}} = \text{Average no. of disaccharide units per chain}$$

2.2.6   Molecular Weight Determination of HA and CS Oligosaccharides
    by PAGE

Gel electrophoresis and densitometry were employed to determine molecular weight. The problems encountered by this method were mainly due to the polydispersity of the polysaccharides (i.e.) long smears obtained on the gel. The method chosen was more accurate at determining the higher molecular weight samples. The small oligosaccharides were too small to run on the gel and were estimated to have much higher weights than determined by another other method. Gels were prepared as described by Cowman *et al*, (1984) to final concentration of 20% (w/v) acrylamide/0.67% NN'-methylene bis-acrylamide (Table 2.2). The concentrated buffer

1.   <u>10 x Reservoir Buffer (pH 8.3)</u>

1M Tris

0.01M di-sodium EDTA

Add 6M HCl until pH rises to 8.3

2.   <u>Stock acrylamide separating gel</u>

20% acrylamide : 0.67% bisacrylamide

3.   <u>Separating gel (10%)</u>

25ml of 20% acrylamide/0.67% bisacrylamide stock solution

25ml of 0.2M Tris/HCl/0.002M EDTA

0.3ml of 10% ammonium persulphate solution

85µl TEMED

4.   <u>Sample Buffer</u>

0.2% Bromophenol Blue in 1 x running buffer

5.   <u>Stain</u>

0.5% Alcian Blue in 2% acetic acid

6.   <u>Destain</u>

2% acetic acid

Table 2.2   :   Solutions for Polyacrylamide Gel Electrophoresis of HA & CS

Fragments

stock contained 0.2M-Tris/HCl/0.002M-disodium EDTA, pH8.3. Equal volumes of these two solutions were mixed at room temperature and deaerated for 15 minutes. For polymerisation, 0.6ml of 10% (w/v) ammonium persulphate in water (freshly prepared) and 0.17ml of NNN'N'-tetramethylethylenediamine were added per 100ml of gel solution. The gels (15cm long and 14cm wide) were cast between glass plates spaced by perspex strips. The gels were overlayed with buffer to prevent drying out and allowed to polymerise overnight at room temperature before use. Samples containing 150 µg of material in less than 50 µl volume were mixed with a one-tenth volume of 0.2% (w/v) Bromophenol Blue (dissolved in 2M-sucrose in 0.1MTris/HCl/EDTA buffer) and applied to the gel. The mobility of Bromophenol Blue matched that of a chondroitin sulphate digest with approximately 13 repeating units (Cowman *et al*, 1984). The reservoir buffer was 0.1M Tris/HCl/EDTA. Gels were electrophoresed at 125V for 20 min and then at 250V for approximately 1.5h. The gels were stained in 0.5% (w/v) Alcian Blue in 2% (v/v) acetic acid for 45 mins, then destained for 15 mins in 2% (v/v) acetic acid. Photographs were then taken of gels. Molecular weight estimations were carried out as described in section 2.2.8.

2.2.7   Molecular Weight Determination of ORC Oligosaccaharides and
   Pectin by PAGE

Solutions used in the preparation of these polyacrylamide gels are shown in Table 2.1. The method was developed for ORC by Peter Doyle, Johnson & Johnson). The gels were cast between glass plates spaced by perspex strips as above and were overlaid with buffer and allowed to polymerise overnight at room temperature. 20µl aliquots of

1.   Reservoir Buffer (pH 8.3)

0.1M Tris

1mM EDTA

Raise to pH 8.3 with 6M HCl

2.   Stock acrylamide separating gel

20% acrylamide : 0.67% bisacrylamide

3.   Separating gel

25ml of 20% acrylamide/0.67% bisacrylamide stock

25ml of water

70μl TEMED

0.4ml of 10% ammonium persulphate solution

4.   Sample buffer

3ml of glycerol

19mg EDTA

0.25ml of running buffer

1mg of Bromophenol Blue

5.   Stain

0.1% Alcian Blue in 7% acetic acid

6.   Destain

7% acetic acid

Table 2.3   :   Solutions for Polyacrylamide Gel Electrophoresis of ORC and
                            Pectin Fragments

approximately 10mg/ml of the standards (Section 3.1.6) and 10mg/ml of each ORC sample were dissolved in the sample buffer then added to the gel. The gels were run at 150V for approximately 4 hours, after which they were stained with 0.5% (w/v) Alcian Blue in 7% (v/v) acetic acid for 45 minutes, then destained with 7% (v/v) acetic acid. Calculation of molecular weight was carried out as described in section 2.2.8.

2.2.8 Densitometry Analysis of Polysaccharides

Polysaccharides invariably produce smears when run on polyacrylamide gels (Cowman *et al*, 1984). In order to help analyse the molecular weight range of these oligosaccharides, the gels were analysed by densitometry. Images of the gels were captured using a Nikon TV camera onto the Apple McIntosh program "Optilab®" using a method developed by Derek Silcock, Johnson & Johnson. The program then calculated the most dense area of each smear and displayed the smear as a graph of pixel intensity. The closer the value to 0, the more dense the area. The captured image was calibrated to ensure that measurements taken represented those on the actual gel. This method gave an measurement of the peak molecular weight of the dispersed polysaccharide smear.

2.3   CELL CULTURE

2.3.1   Cell types

(a)   L929 mouse connective tissue fibroblasts, kindly donated by Dr. S. Srivastava, Bioengineering Department, University of Strathclyde.

(b)   Bovine Aorta Endothelial Cells (BAEC), kindly supplied by Prof. Mark Ferguson, Department of Cell and Structural Biology, University of Manchester.

(c)   Rat Wound fibroblasts were isolated at Johnson & Johnson Medical Biolpolymer Group (see section 2.3.3)

(d)   Human skin fibroblasts, kindly supplied by Dr. Michael Edwards, Department of Dermatology, University of Glasgow.

(e) 3T3 Swiss Mouse fibroblasts, kindly supplied by Prof. Birgitt Lane, University of Dundee.

2.3.2   Cell Culture Reagents

Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) Fetal Calf Serum (FCS) was used to maintain all the cell types. This was supplemented with 1% (v/v) penicillin/streptomycin solution, 1% (v/v) glutamine and 9mM sodium bicarbonate (all Gibco BRL, Paisley, Scotland). Hank's balanced salt solution (HBSS) without calcium and magnesium (Gibco BRL, Paisley, Scotland) was used to wash cell monolayers before trypsinisation. The trypsin solution was prepared by diluting 10x concentrated trypsin-EDTA (Gibco BRL, Paisley, Scotland) with HBSS to give a single strength solution (Ferrao & Mason, 1993).

Methylene Blue powder and 100% Leishman's stain were both obtained from Sigma Chemicals, Dorset.

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2.3.3   Isolation of Rat Wound Fibroblasts

The cells were isolated by the method of Finesmith *et al*, 1990. Adult Wistar male

rats, aged 3 months, were each implanted with 4 polyvinyl alcohol sponge discs beneath the panniculus carnosus, next to the body musculature, as previously described (Davidson *et al*, 1985), while under pentobarbitone anaesthesia (45mg/kg). At 14 days post-implantation, the animals were sacrificed by CO_2 asphyxiation. The sponges were placed in DMEM containing 20% (v/v) FCS, amphotericin (25µg/ml, GIBCO BRL, Paisley, Scotland), penicillin/streptomycin (1000U/ml, GIBCO BRL, Paisley, Scotland) and placed on ice. Under sterile conditions, the encapsulated adventitia was removed and the sponges were cut into small sections (1-2mm^2) using a scalpel and scissors. The minced sponges were placed in DMEM containing 10% (v/v) FCS, 200 units of bacterial collagenase (Type 1A) and penicillin/streptomycin (10x) and incubated at 37°C for 4 hours. The sponges were agitated by pipetting and the supernatants were centrifuged at 1200rpm for 5-10 minutes. The pellets were resuspended in DMEM with 20% (v/v) FCS, 10x penicillin/streptomycin and placed in 75cm^3 flasks. The cells were grown in standard cell culture DMEM/10% (v/v) FCS after the first passage and were maintained as described in sections 2.3.6 & 2.3.7.

2.3.4   Isolation of Human Skin Fibroblasts

Isolation of human skin fibroblasts was carried out by Dr. Mike Edwards, Dept. of Dermatology, University of Glasgow.

Small skin biopsies from the forearm were taken from healthy volunteers. The skin samples were placed in DMEM containing 20% (v/v) FCS, amphotericin (25µg/ml, GIBCO BRL, Paisley, Scotland), penicillin/streptomycin (1000U/ml, GIBCO BRL, Paisley, Scotland) and placed on ice. Under sterile conditions, the skin samples were

minced finely with curved scissors and added to a solution of DMEM containing 10% (v/v) FCS, 200 units of bacterial collagenase (Type 1A, Sigma Chemicals) and penicillin/streptomycin (10x, Gibco Ltd) and incubated at 37°C for 4 hours. Every 30 mins, the mixture was agitated by pipetting and then the supernatants were centrifuged at 1200rpm for 5-10 minutes. The pellets were resuspended in DMEM with 20% (v/v) FCS, 10x penicillin/streptomycin and placed in 75cm^3 flasks. The cells were grown in standard cell culture DMEM/10% FCS after the first passage and maintained as described in sections 2.3.6 & 2.3.7.

2.3.5 Isolation of Bovine Aorta Endothelial Cells

Isolation of BAEC cells was carried out by Prof. Mark Ferguson's group at The University of Manchester. Fresh aortas were transported from the abattoir to the laboratory within one hour. Fat and other loose connective tissue were removed from the aorta, then it was cut length ways and the inside surface was washed with copious amounts of sterile phosphate buffered saline. The aorta was laid on a flat surface and the inside was gently scraped with a sterile scalpel. The scraped material from one aorta was suspended in 4 ml of collagenase dispase solution (1mg/ml in serum free DMEM) and incubated at 37°C for 45 minutes. The cell suspension was transferred to a plastic tube and centrifuged at 600 x g for 10 minutes. The enzyme solution was removed by vacuum aspiration and the cell pellet was resuspended in DMEM supplemented with 10% serum. The cell suspension was transferred to a 75cm^3 cell culture flask and incubated at 37°C and 5% CO_2. The medium was replenished after 24 hours and thereafter at 3 day intervals. The cultures were found to be confluent within 3-5 days.

2.3.6 Maintenance of cells

All cell types were grown in $25cm^3$ flasks and incubated at 37°C in an atmosphere containing $95\%O_2/$ $5\%CO_2$. The cells attached to the plastic surface under these conditions to form a monolayer.

2.3.7 Trypsinisation of Cells

Medium was aspirated from the confluent cell monlayers and 5ml of HBSS added for 5 minutes before being removed. 1ml of trypsin solution (section 2.3.2) was added to the flask and incubated at 37°C until all of the cells had rounded and detached from the flask surface. The cell suspension was then transferred to a sterile centrifuge tube and centrifuged at 1000rpm for 5 minutes. The supernatant was then aspirated and the cells resuspended in an appropriate volume of culture medium, containing 10% (v/v) FCS and 10% (v/v) DMSO. 1ml aliquots were transferred to cryovials, placed in the freezer tray and left overnight in the liquid nitrogen vat. The vials were then transferred to a permanent position in the racking system.

2.3.8 Recovery of Frozen Cells

Frozen cells were removed from the liquid nitrogen vat and placed in water at 37°C. Immediately after the vial had thawed, the cells were transferred to a culture flask and 5ml of DMEM with 10%(v/v) FCS was added drop by drop. The cells were incubated overnight to allow attachment. The medium containing the cryopreservative

(DMS0) was aspirated and replaced with fresh medium containing 10%(v/v) FCS and

the cells allowed to grow to confluence.

2.4 CELL CULTURE ASSAYS

2.4.1 Standard Curves

For each cell type, a standard curve of cell number versus absorbance at 650nm was

constructed. Cells were seeded at 0-9x10^4 cells/200µl per well in Dulbecco's Modified

Eagles Medium (DMEM) containing 10% fetal calf serum (FCS), with six replicates for

each density. The cells were allowed to attach for 4 hours, then the plates were fixed

with methanol (section 2.4.2) and then assayed by the methylene blue method described

in section 2.4.4. The mean and standard deviation of the absorbance measurements at

650nm were plotted against cell number. Standard curves are shown in Chapter 3 for all

cell types used in this study.

2.4.2 Methanol Fixation of Cells

100 µl of methanol was placed into each well containing attached cells. After 5 mins,

the methanol was removed by inverting the plate onto tissue paper. The plates were

air dried before any further assays were carried out.

2.4.3 Proliferation Assay

A modified version of that described by Ferrao & Mason, (1993) was used. Cells were

seeded at 5000/200µl per well in DMEM containing 10% (v/v) FCS and were left to

attach for 4 hours at 37°C/5%CO$_2$. The cell number was obtained by using the method described in section 2.4.5. Following attachment, the medium was discarded and replaced with 200µl of serum-free DMEM and left to incubate overnight. After disposal of the medium, 200µl of oligosaccharide solutions were added at various concentrations to the wells in DMEM containing 2%(v/v) FCS (6 replicates of each), and left to incubate for 1, 2 and 3 days. Cells were fixed with methanol (section 2.4.2) following incubation. The wells were then assayed for proliferation by the methylene blue method described in Section 2.4.4.

2.4.4 Methylene Blue Assay for Measurement of Cell Proliferation

A modified version of the Oliver *et al,* (1989) method was employed. 100µl of 0.1% (w/v) methylene blue in 10mM Borate Buffer pH 8.4 was dispensed into each well containing methanol fixed cells, with a multichannel pipette and left to stain for 30 minutes. Excess dye was discarded by overturning the plate into a large beaker and the plates washed four times with 300µl of 10mM borate buffer. 100µl of 20% Ethanol in 0.1M HCl was then added to each well to elute the dye from the cells. The plate was placed in a plate reading spectrophotometer (Dynatech MR5000) where the absorbances of each well were read at 650nm (A$_{650}$). The percentage of proliferation was calculated by assuming the average absorbance of the control values to be 100% and therefore the average absorbance of the samples would be directly proportional to the 100%.

2.4.5 Cell Counting Using a Haemocytometer

The method used was taken from Sigma Biosciences Catalogue, p201. Cells were

detached from the plastic by firstly washing with Hank's Balanced Salt solution (Gibco BRL, Paisley, Scotland) for 5 mins, followed by the addition of 40μl of trypsin-EDTA solution to each well. The plate was incubated at 37°C/5%CO_2 until the cells detached from the surface. An aliquot was then taken and placed under the coverslip on the Neubaeur improved haemocytometer (Fisons, England). The cells were counted on the grid and expressed as the number of cells per ml. The calculation is shown below :

Cells per ml = average count per square x dilution factor x 10^4

2.4.6 Chemotactic Assay to Measure Cell Migration

A modified version of Postlethwaite *et al*, (1978) was employed to measure cell migration.

A suspension of cells was prepared at 400,000 per ml in serum-free DMEM following trypsinisation as described in section 2.3.7. Oligosaccharides were dissolved at different concentrations in serum-free DMEM and 25μl of each concentration placed in the bottom wells of a Neuroprobe microwell chemotaxis chamber, in triplicate. A polycarbonate filter with the relevant pore size for the type of cells being used (8μm for fibroblasts), was placed carefully on top of the bottom wells and the gasket seal placed between the filter and the top section of the chamber. This was pre-incubated for 10 mins at 37°C after which 50μl of the cell suspension was added to each of the top wells and incubated for the required period for each cell type (fibroblasts - 3.5h). DMEM alone was used as a negative control, while the positive control was 10pg/ml Transforming Growth Factor β (TGFβ), (Sigma). The filters were then removed

carefully with forceps, fixed with methanol (section 2.4.2) and then dipped into 100% Leishman's stain for 10 mins. Cells were then counted in 20 fields of view for each sample at x40 magnification on the light microscope.

2.5 ASSAYS TO DETERMINE THE EFFECTS OF OLIGOSACCHARIDES & POLYSACCHARIDES ON WOUND HEALING IN ANIMAL MODELS

2.5.1 Introduction

Polyvinyl Alcohol Sponges containing potential active factors were implanted subcutaneously in rats to establish the ability of the polysaccharides & oligosaccharides to influence wound healing and allow a comparison to the *in vitro* data obtained for the same factors. The subcutaneous model was chosen as it allowed multiple sample analysis on each animal and an animal licence was easily obtained for this type of minor surgery. The following methods were employed to carry out and analyse this study.

2.5.2 Polyvinyl Alcohol Sponge Implantation

Adult Wistar male rats, aged 3 months, were each implanted with 4 polyvinyl alcohol (PVA) sponge discs beneath the panniculus carnosus, next to the body musculature, as previously described (Davidson *et al*, 1985), while under pentobarbitone anaesthesia (45mg/kg). PVA sponges were manufactured by Unipoint Industries Inc., North Carolina, USA, as an implant material used to fill cavities in reconstructive and other

types of surgery. Discs of 10 x 3mm were cut and sterilised by autoclaving, and implanted in five rats using the subcutaneous implant model. 100μl of polysaccharide solutions were injected directly into the implanted sponge through the skin and was carried out 3 days post-implantation. The rats were sacrificed by CO_2 asphyxiation followed by cervical dislocation at 7 and 10 days and the sponges sectioned by wax histology to visualise the invasion of the sponge by cells (see section 2.7).

2.6   Processing of PVA Sponges

Embedding, cutting and staining of PVA sections was carried out by staff at the Department of Pathology, Stirling Royal Infirmary, Stirling, Scotland.

Firstly the sponges were cut in half and embedded in paraffin. Sections were then cut at 3um on a Reichert-Jung 2040 microtome. The sections were placed on gelatin coated slides and placed in an oven at 56°C to melt the wax. They were then dipped in a series of alcohol washes to remove the wax. The sections were air dried until staining was carried out.

2.7   STAINING OF PVA WAX SECTIONS

2.7.1   Histological Staining of PVA Wax Sections with Haemotoxylin & Eosin

(H & E)

Staining of PVA sections was carried out at the Stirling Royal Infirmary as follows. Each slide was dipped in Mayer's haematoxylin (Sigma, England) for 5 mins and rinsed with tap water until the water ran clear. The slides were then dipped in a 1%

solution of eosin (Sigma, England) for 8 -10 secs and again washed with Tris Buffered Saline pH 7.4, (TBS) until the water ran clear. They were air-dried before observing under the light microscope (Nikon Optiphot, Japan).

2.7.2 Visual Analysis of H & E Stained Histological Sections

Sections were examined by light microscopy at both low and high power magnifications (Nikon Optiphot, Japan). The degree of granulation tissue formation, inflammation, blood vessel formation and fibrin content was examined for each sponge in triplicate. Granulation tissue formation was assessed by the presence of fibroblasts and new connective tissue laid down and these were identified by the presence of pink areas on the slide (nuclei were blue/black), visualised under the microscope at x40 magnification.

The sponges containing potential actives were compared against controls containing PBS only and were graded overall with a negative, zero or positive score and this grading was carried out by 2 or 3 independent assessors. There were up to a maximum of five animals used per sample with replicates of each sample on each animal. A total of 10 scores were therefore obtained at each time point. From this data, it was hoped that if any polysaccharide had stimulated granulation tissue formation, the majority of the scores would be positive and that the 2 or 3 independent assessors would all achieve the same overall result. Statistical analyses were carried out on the results obtained as described in section 2.8, however the method was not validated.

2.7.3   Anti-Sera Used for Immunocytochemical Analysis

Antibody staining of the H & E wax sections was also carried out to observe the presence of laminin, monocytes & macrophages and endothelial cells. This analysis would give an indication if inflammation was present, if blood vessels had formed and if laminin had been deposited.

To observe laminin, a polyclonal rabbit anti-laminin (Serotec) was used as the primary antibody followed by sheep anti-rabbit IgG-FITC conjugate (Serotec). Monocytes and macrophages were visualised using a mouse monoclonal mouse anti-rat monocyte/macrophage antibody, MCA 341 (Serotec), followed by a rabbit anti-mouse IgG FITC conjugate (see section 2.7.3). The presence of endothelial cells in the sections was detected using a mouse monoclonal antibody MCA 276 (Serotec) followed by a rabbit anti-mouse-FITC conjugate.

2.7.4   Immunocytochemistry Using Fluorescently Labelled
Antibodies

Wax embedded sections were prepared by staff at Stirling Royal Infirmary as described in section 2.6 and retained for immunocytochemistry. Firstly, the wax was melted in an oven at 56°C. The slides were then dipped in xylene for five minutes to remove the wax. This was followed by rehydration of the sections in decreasing concentrations of isopropanol (100%, 95% & 70%) with the slides bathing for 3 min

in each concentration. Following a final rinse in distilled water, the slides were placed in a bath containing 0.05M Tris buffered saline, pH 7.4 (TBS) for 5 mins. The sections were then labelled with $20\mu l$ of 1/25 dilution in TBS of MCA 341 or MCA 276 antiserum for one hour or 1/200 and 1/500 of rabbit anti-laminin for one hour in a humid chamber. The antiserum was decanted and followed with three one minute TBS washes. The sections were then incubated with $20\mu l$ of rabbit anti-mouse IgG-FITC conjugate, diluted to 1/50 in TBS or a 1/40 dilution of sheep anti-rabbit IgG-FITC for one hour in a humid chamber. The conjugate was decanted and sections washed in three changes of TBS for one minute each. Sections were mounted using permanent aqueous mounting medium. Sections were visualised immediately by fluorescence microscopy using a Nikon fluorescent lamp and Nikon Optiphot Microscope (Japan). Photographs were taken using Kodak 400 ASA film. This method was developed by Johnson & Johnson.

2.8   STATISTICAL ANALYSES

2.8.1   Methylene Blue Proliferation Assay

Statistical analysis was carried out using the Apple MacIntosh™ program, Multistat™. The sample data was entered into the program and an unpaired Student's t-test performed. This is a parametric test that tests the null hypothesis that two sets of data come from the same population, taking into account the standard deviations and numbers of items of the two groups.

t = mean difference / SEM of differences x (n-1)

Where : SEM = standard error mean ; n = number of samples

A confidence level of 95% was selected, therefore if the calculated probability was $p < 0.05$, the Null hypothesis was rejected and the sample mean was therefore significantly different from the control mean.

2.8.2 Chemotaxis Assay

The Apple MacIntosh™ program , Multistat™, was also used for analysing data from this assay. Means and standard error of the means were calculated for each sample in triplicate. An unpaired student's t-test was then applied to the data and if $p < 0.05$, the Null hypothesis was rejected and the sample mean was therefore significantly different from the control mean.

2.8.3 Histological Data

Statistical analysis was carried out using the Apple MacIntosh™ program Excel™. The sample data was entered into the program and an unpaired Student's t-test performed. This is a parametric test that tests the null hypothesis that two sets of data come from the same population, taking into account the standard deviations and numbers of items of the two groups.

A confidence level of 95% was selected, therefore if the calculated probability was $p < 0.05$, the Null hypothesis was rejected and the two samples were considered to be significantly different.

CHAPTER 3 : PREPARATION OF POLYSACCHARIDE FRAGMENTS

3.1 Introduction

It has been suggested from previous studies that HA oligosaccharides between 3 and 16 disaccharides in length are angiogenic *in vivo* and stimulate the proliferation of endothelial cells *in vitro* (West & Kumar, 1989). These results indicated a possible role for the breakdown products of HA in wound healing as the proliferation of endothelial cells in new blood vessel formation is an important step in the healing process. This work initiated the idea of investigating the effect of other HA-like polysaccharides, such as other GAGs and plant polysaccharides, on the proliferation and migration of cells involved in the wound healing response. This would be carried out by investigating both *in vitro* and *in vivo* models, and characterising the specific size of fragment or fragments which are bioactive. Johnson & Johnson Medical Biopolymer Group were interested in developing new wound dressings containing bioactive molecules to stimulate the healing of chronic wounds, particularly leg ulcers. Amendments to an existing filed patent including data generated in this study has now resulted in a granted UK patent (Harvey, Light & Watt), No.9206492. Polysaccharides, including those derived from plants, were cited in the patent for their use in the treatment of chronic wounds. This chapter details the methods by which oligosaccharide fragments of selected polysaccharides were generated and characterised.

3.2 Production and Analysis of Hyaluronic Acid and Its Oligosaccharides

Initially it was thought prudent to produce HA oligosaccharides and test whether these were in fact bioactive as described by other observers (Gorham *et al*, 1974; Cowman *et al*, 1984). It was hoped that the HA products produced in this study would serve as positive controls for subsequent experiments with other polysaccharides.

3.2.1 Molecular Weight Determination of Intact HA

Molecular weight estimation of the starting material was determined both by polyacrylamide gel electrophoresis (PAGE) (Maniatis *et al*, 1975; method 2.2.6) and viscometry (section 2.2.1; Fermentech Ltd., Edinburgh). Molecular size estimation was important, firstly, to compare PAGE and viscometry methods and secondly, to compare values of molecular weight calculated with those detailed by the manufacturer. The manufacturer of the HA used in this study, Lifecore Inc., claim their HA to have an average MW of 750,000Da, as estimated by viscometric analysis.

3.2.2 PAGE of Undigested HA

PAGE is an established method used for the separation of polymeric GAGs (McDuffie, 1981; Nader *et al*, 1981). The use of PAGE for the separation of these polymers, however, yields rather diffuse bands, resulting from charge density and molecular size heterogeneity (Cowman *et al*, 1984). For the purpose of this study, PAGE was used to give an approximate value of molecular size estimated by running dextran sulphates of known molecular weights simultaneously on the gel. Dextran sulphates with average

molecular weights of 5, 40 and 500kDa were run alongside undigested HA as molecular weight indicators. Fig 3.1 demonstrates the profiles obtained for the dextran sulphate standards. Molecular weights were estimated by measuring the most densely stained area of the band using a densitometer (method 2.2.8). Fig 3.2 demonstrates that undigested HA travelled from 0 cm to 0.22cm on the gel which suggests that its molecular weight is greater than that of the 500kDa dextran standard which travelled 0.29cm. This result shows therefore that by using PAGE, HA has a molecular weight of greater than 500kDa. PAGE analysis of such large molecular weight polysaccharides is clearly not accurate, however, this method is useful when trying to obtain information about the fragmentation of HA polymers.

3.2.3   Molecular Weight Estimation of HA by Viscometry

Fermentech Ltd., also a supplier of HA, supplied a validated method to size HA samples by viscometry (method 2.2.1). Using this method, Lifecore HA was determined to have an average molecular weight of 828kDa using the formula in Figure 2.1(b). PAGE could only give a value of >500kDa for the weight of the undigested polymer, whereas viscometry gave a more precise measure. Subsequently, in this study, the molecular weight of undigested HA was considered therefore to be 828kDa. The manufacturers value and the value obtained in this study may be different due to a number of reasons. Firstly, the method used to calculate the molecular weight may have been different and also the type of viscometer used may have varied. It is also possible that different batches of HA produced have variations in molecular size. The limitations of using viscosity as an estimation of molecular size will be discussed later

Lane   a   b   c
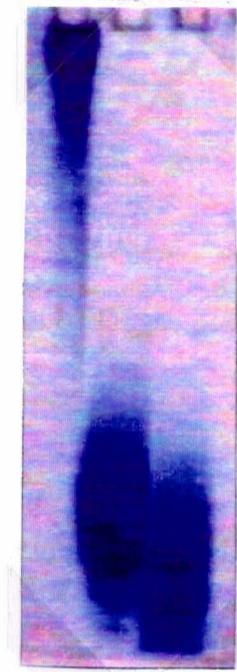
Lane (a)   Lane (b)   Lane (c)
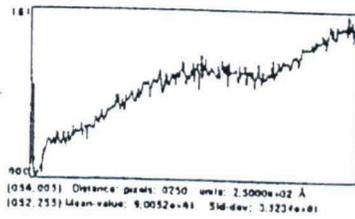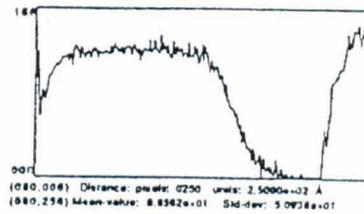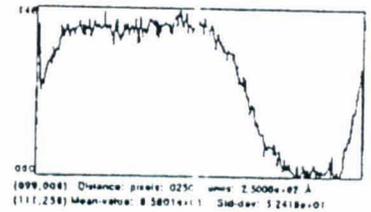
Distance to most dense area   Distance to most dense area   Distance to most dense area
= 0.29cm   = 10.44cm   = 13.34cm

Figure 3.1   :   Polyacrylamide gel electrophoresis and densitometry scans of
                   dextran sulphate standards. Lane a - 10mg/ml 500kDa
                   dextran sulphate (20µl loaded), Lane b - 10mg/ml 40 kDa dextran
                   sulphate (20µl loaded), Lane c - 10mg/ml 5 kDa dextran sulphate
                   (20µl loaded). The scans run from left to right on the graph and the
                   most dense areas are represented by the lowest numbers on the
                   scale.

Lane a b c

Lane (a)	Lane (b)	Lane (c)
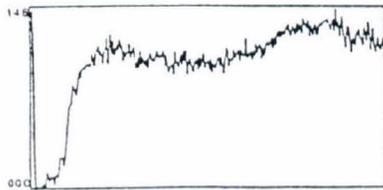
(136,004) Distance: pixels: 0250 units: 2.5000e+02 Å
(148,254) Mean-value: 1.0377e+02 Std-dev: 3.3592e+01

(158,005) Distance: pixels: 0250 units: 2.5000e+02 Å
(162,255) Mean-value: 6.6992e+01 Std-dev: 5.2759e+01

(158,005) Distance: pixels: 0250 units: 2.5000e+02 Å
(191,255) Mean-value: 9.6426e+01 Std-dev: 2.1332e+01

Distance to most dense area
= 0 - 0.22 cm

Distance to most dense area
= 4.5 - 7.8cm

Distance to most dense area
= 9.57cm

Figure 3.2 : Polyacrylamide gel electrophoresis and densitometry scans of HA digests. Lane a - undigested HA, Lane b - 5 min HA digest, Lane c - 24hr digested HA.The scans run from left to right on the graph and the most dense areas are represented by the lowest numbers on the scale.

in the chapter.

3.3 Depolymerisation of HA

Depolymerisation of HA can be carried out by several methods including, enzymatic digestion (Gorham *et al*, 1974), chemical hydrolysis (Rowley & Halliwell, 1982: Swann, 1969) and sonication (Rehakova *et al*, 1994). One of the most controllable method however, has been shown to be the addition of testicular hyaluronidase which breaks the HA into repeating units of glucuronic acid and N-acetylglucosamine (Gorham *et al*, 1974). Testicular hyaluronidase is specific for the ß1-4 bond between the N-acetylglucosamine and glucuronic acid residues in the HA chain. Molecular sizing of these repeating structures can be determined by a colorometric test which measures average number of disaccharide units per chain, and this can be easily converted into the equivalent molecular weight (see methods 2.2.5.1- 2.2.5.3).

3.4 Hyaluronidase Digestion of HA

In order to generate potentially bioactive fragments, it is necessary to depolymerise the intact HA molecule. Sections 3.4.1 to 3.6 describe a number of these methods and the types of oligosaccharides produced.

Hyaluronic acid was digested by hyaluronidase over a time course of 24 hours in order to establish the rate and degree of HA breakdown. Table 3.1 shows the digestion of 10mg/ml HA with 0.25mg/ml testicular hyaluronidase (2400U/mg) at 25°C as measured by determining the ratio of uronic acid to reducing N-acetylglucosamine

Incubation Time	Uronic acid/reducing N-acetyl glucosamine	Ratio UA : reducing NAG	Average Molecular Weight (Da)
0 mins	-	-	828,000*
5 mins	19.8/1.94	10 : 1	4000
10 mins	32.3/1.93	17 : 1	6800
15 mins	23.1/2.43	10 : 1	4000
30 mins	22.0/2.67	8 : 1	3200
45 mins	20.2/2.83	7 : 1	2800
60 mins	24.9/3.14	8 : 1	3200
90 mins	33.7/3.27	10 : 1	4000
120 mins	21.8/3.9	6 : 1	2400
3 hrs	24.6/4.36	6 : 1	2400
4 hrs	28.4/5.27	5 : 1	2000
5 hrs	27.9/5.45	5 : 1	2000
6 hrs	30.8/6.14	5 : 1	2000
24 hrs	22.3/10.06	2 : 1	800

* Molecular weight measured by viscometry (Method 2.2.1)

Table 3.1 :   Digestion of 10mg/ml HA (MW 828 kDa) with 0.25mg/ml testicular hyaluronidase (2400U/mg) over a 24hr time period at 25°C. The table shows the average molecular weights of each aliquot as determined by uronic acid and reducing N-acetylglucosamine content (Method 2.2.5).

present in the samples. Digestion of HA was extremely rapid under these conditions, falling from an average molecular weight of 828,000 Da to an average of 4000 Da (equal to 10 disaccharide units), after only 5 minutes. After the 24hr digestion period, the average molecular weight had dropped to 800 Da, equivalent to 2 disaccharides. This result supports that reported in the literature (Gorham *et al*, 1974). It must be stressed, however, that the values obtained for the molecular weights of the HA digests were an average and other sizes of HA oligosaccharides were present in the samples also as shown by the large smears obtained on gels and densitometric analysis (Fig 3.2).

3.4.1 Depolymerisation of HA by Sonication

Previous in-house studies carried out by Johnson & Johnson Medical Biopolymer Group suggested a role for sonication in the depolymerisation of HA, however it was apparent from these studies that the lowest molecular size obtained by this method was approximately 50,000Da using dextran sulphate standards (P.Watt, personal communication;). Due to time constraints however, this work was not taken any further, but it is unlikely that small oligosaccharides would be generated by this method. Ross *et al*, (1993) and Rehakova *et al*, (1994) have also reported that sonication is effective at producing HA oligosaccharides in the range of 80,000 -1.38 x10^6 Da as determined by gel filtration.

In this study two samples of HA were sonicated (method 2.1.2) for (a) 4 x 8 secs and (b) 8 x 8 secs and their molecular weights estimated by viscometry (method 2.2.1). The HA sample which was sonicated for 4 x 8 secs had an apparent molecular weight of 739 kDa, therefore sonication for this length of time resulted in only a small decrease in

the average molecular weight of the HA. Sonication for 8 x 8 secs, however, resulted in a reduction of the average molecular weight to 500kDa. Sonication for longer periods of time would degrade the HA further however, time constraints made it impossible to develop this work further. Sonication therefore appears to produce HA solutions with lower molecular weights than the starting material, however, as this project requires very small oligosaccharide fragments, sonication was deemed an unsuitable method for the desired purpose.

3.5   Isolation of Selected Oligosaccharides

West & Kumar (1989) suggested that oligosaccharides between 3 and 16 disaccharides were bioactive both *in vitro* and *in vivo*. For this reason, the aim of this study was to produce discrete sizes of HA for use as both molecular weight markers and bioactive agents. The 24h HA digest was chosen for separation of HA oligosaccharides because it contained a range of sizes from 2 disaccharide upwards (see section 3.4).

3.5.1   Separation of HA Oligosaccharides by Gel Chromatography

Separation of oligosaccharides from the 24hr HA digest was carried out by gel chromatography, using a Biogel P6 column (method 2.1.3), which separates molecules with molecular weights of between 1000 and 6000 Da (Bio-Rad, Watford, UK).

The oligosaccharides produced were susbsequently analysed by chemical analysis (method 2.2.5) to determine their molecular weight . It was discovered however, that the eluant used during the separation (ammonium acetate, pH 5.0) interfered with the measurement of reducing N-acetylglucosmine. A study was then carried out to

determine the effect of the buffer and this is shown in Fig 3.3. In order to resolve this interference problem, it was therefore necessary to remove the ammonia and acetic acid by freeze-drying the samples and then resuspend them in water. The results of the study demonstrate that when samples were dissolved in ammonium acetate the detection of N-acetyl glucosamine at A_{586} was much lower than those samples dissolved in water or freeze-dried and resuspended in water.

Chromatographic separation of the 24hr HA digest on a Biogel P6 column, resulted in the detection of 4 discrete oligosaccharides by measuring the amount of uronic acid detected in each tube (Figure 3.4). Recovery of HA fragments compared to total HA (30mg) loaded was not quantitated but visual inspection suggested the presence of only a few milligrams per peak thereby indicating some high molecular weight HA remaining. The Vo suggests that no high MW HA was present, however it may have eluted before the blue dextran standard and therefore has not been recorded.

3.5.2   Molecular Weight Determination of Individual Peaks by
Chemical Analysis

The molecular weight of each oligosaccharide peak was determined by the ratio of uronic acid to reducing N-acetylglucosamine. The ratios (Table 3.2) indicated that peaks 1 and 2 were both hexasaccharides, peak 3 was a tetrasaccharide and peak 4 was a disaccharide. Peaks 1 and 2 eluted very close to each other and so it is possible that they were in fact a single broad peak and not two separate ones.
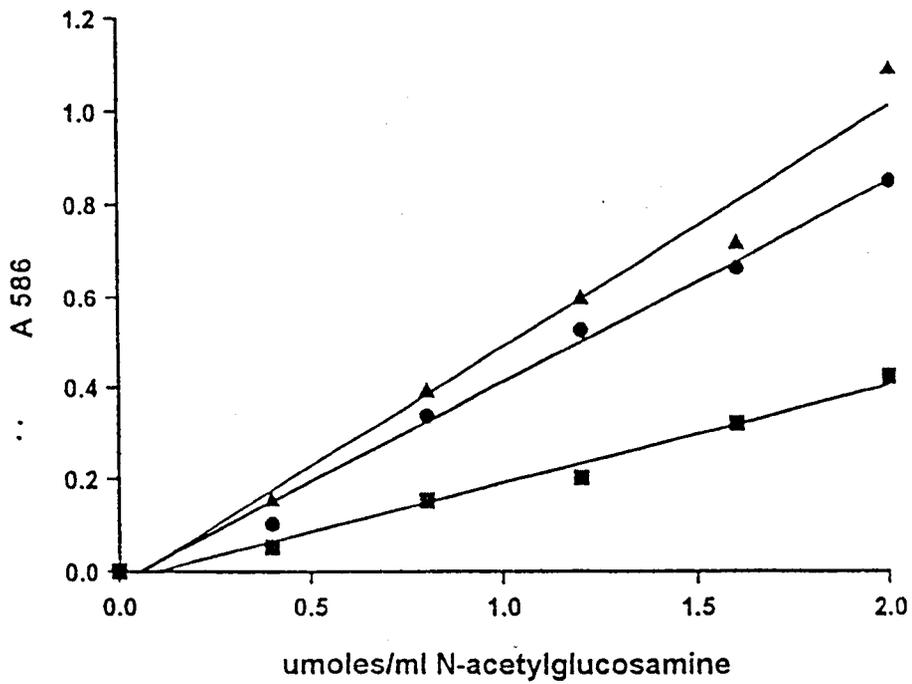
Figure 3.3 :   The effect of ammonium acetate on the detection of N-
acetylglucosamine (NAG) as determined by absorbance at 586nm.
The graph shows the effect of dissolving NAG in water (●),
ammonium acetate (■) or ammonium acetate followed by freeze-
drying and resuspending in water (▲).

Peak Number	Ratio of Uronic acid : reducing N-acetyl glucosamine (μmol/ml)	Ratio	No. of disaccharides
1	1.54/0.53	2.9 : 1	3
2	2.28/0.88	2.6 : 1	3
3	2.75/1.55	1.8 : 1	2
4	0.32/0.3	1 : 1	1

Table 3.2 : Molecular weight estimations of eluted peaks from Biogel P6 gel permeation chromatography (Fig 3.4). Molecular weights were determined by the ratio of uronic acid to reducing N-acetyl glucosamine (Method 2.2.5.2).
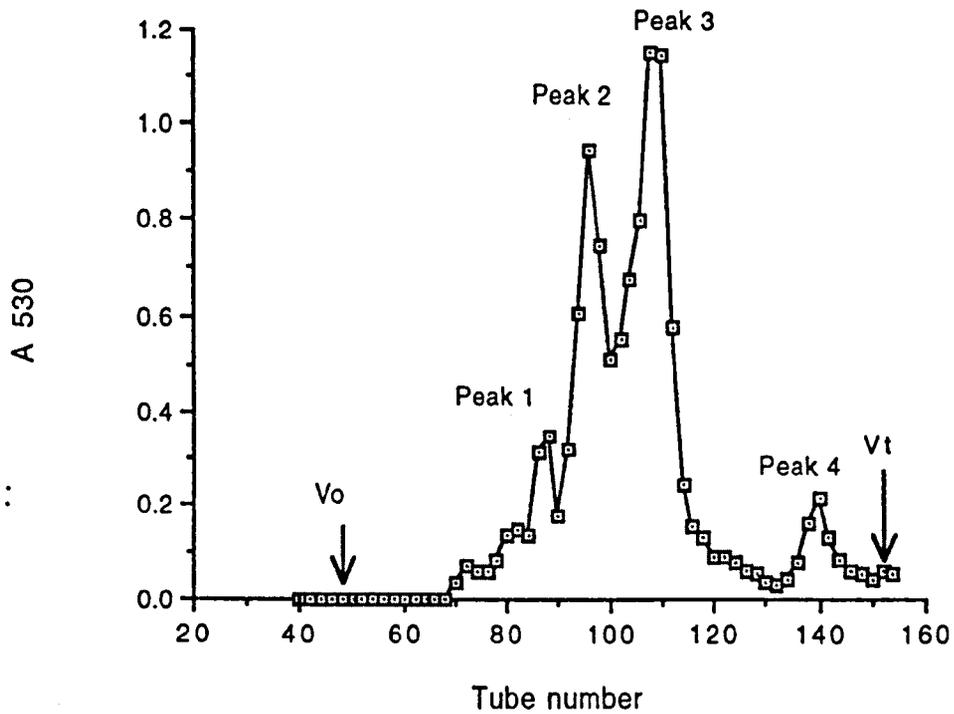
Figure 3.4 : Separation of oligosaccharides produced by digestion of HA (MW 828kDa) by hyaluronidase for 24hr. Approximately 30mg of sample was applied to a column (1000 x 30mm) of Biogel P6, and eluted with 50mM ammonium acetate, pH 5.0. 3ml fractions were collected, analysed for uronic acid content (method 2.2.5.1) and absorbance read at 530nm.

3.5.3 Molecular Weight Determination by Dionex Chromatography

Standards for the Dionex were not readily available as it was in fact the aim of this experiment to produce them. The information obtained from the Dionex therefore served only to confirm that the samples had different molecular weights but no size estimation could be undertaken (Fig 3.5)

3.5.4 Further Characterisation of Peak 3 by HPLC

Further molecular weight characterisation was carried out on peak 3, which was the biggest peak collected and provided enough freeze-dried material for analysis. Peak 3 was analysed by HPLC(method 2.2.2). Dextran standards with molecular weights of 1200, 11700, 47250, 97000 and 326,000 Da were used to calibrate the column (Fig 3.6). The results of this analysis suggested that peak 3, with a retention time of 16.16 mins, had a molecular weight of 1200 Da, which represented a hexasaccharide (Fig 3.7). This result was contradictory to the chemical analysis which indicated that peak 3 was a tetrasaccharide (Table 3.2). As HA standards were not available commercially, dextrans were used as the best alternative. It is possible however that they may have given inaccurate estimations of molecular weight due to the presence of sulphate groups which HA molecules do not possess.

3.5.5 Molecular Weight Determination of Peak 3 by NMR

In an attempt to obtain a more accurate measure of molecular weight of Peak 3, analysis by Nuclear Magnetic Resonance (NMR) was performed (method 2.2.4). This type of
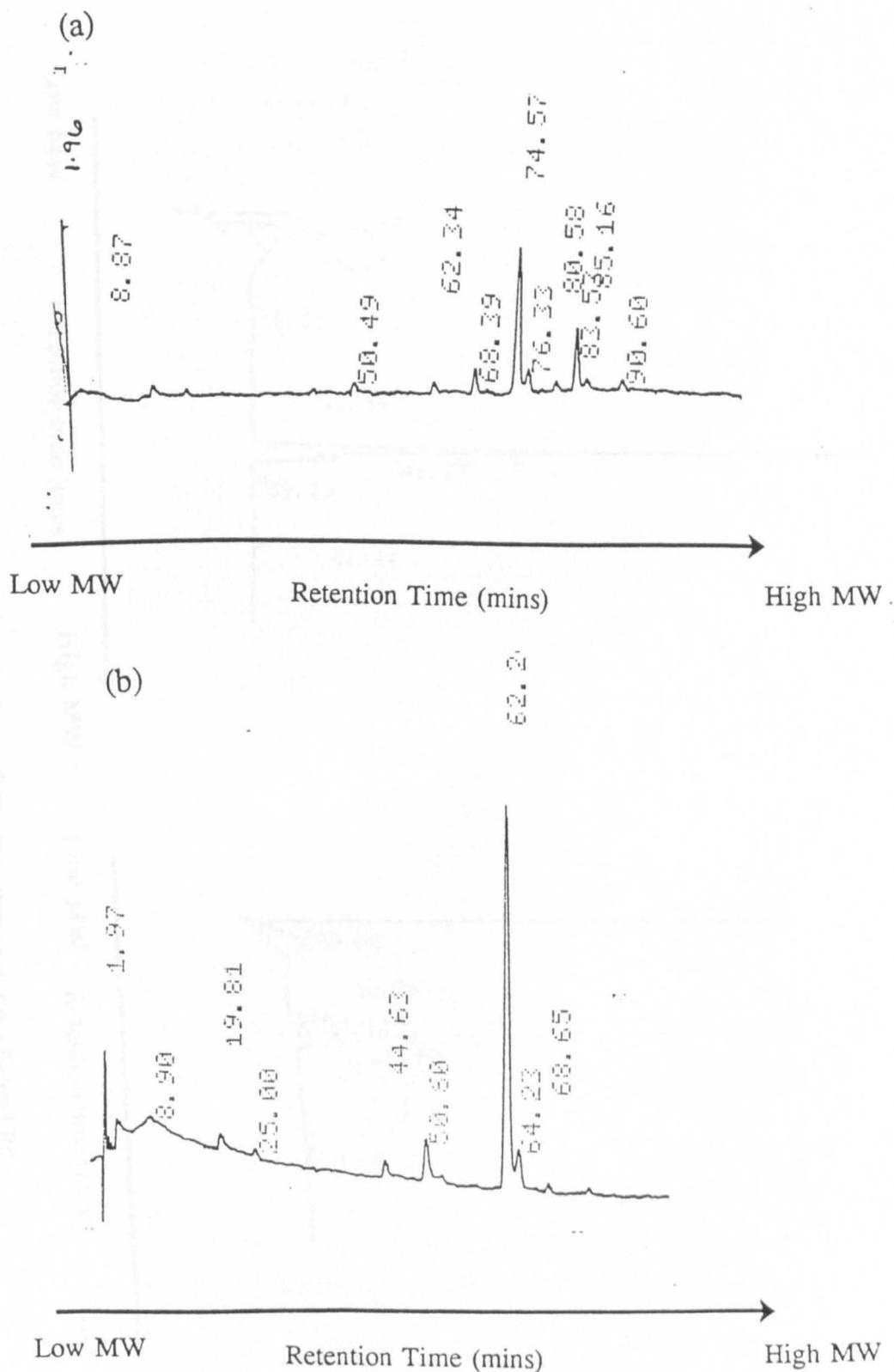
(a)

1.96

8.87

50.49

62.34

68.39

74.57

76.33

80.58

82.585.16

90.60

Low MW　　　　Retention Time (mins)　　　　High MW

(b)

1.97

8.90

19.81

25.00

44.63

50.80

62.2

64.23

68.65

Low MW　　　　Retention Time (mins)　　　　High MW

Figure 3.5 : Dionex traces of peaks 1-4 isolated by separation on a Biogel P6
column. The method used for the detection of these peaks is described
in method 2.2.5.1. (a) Peak 1 (b) Peak 2
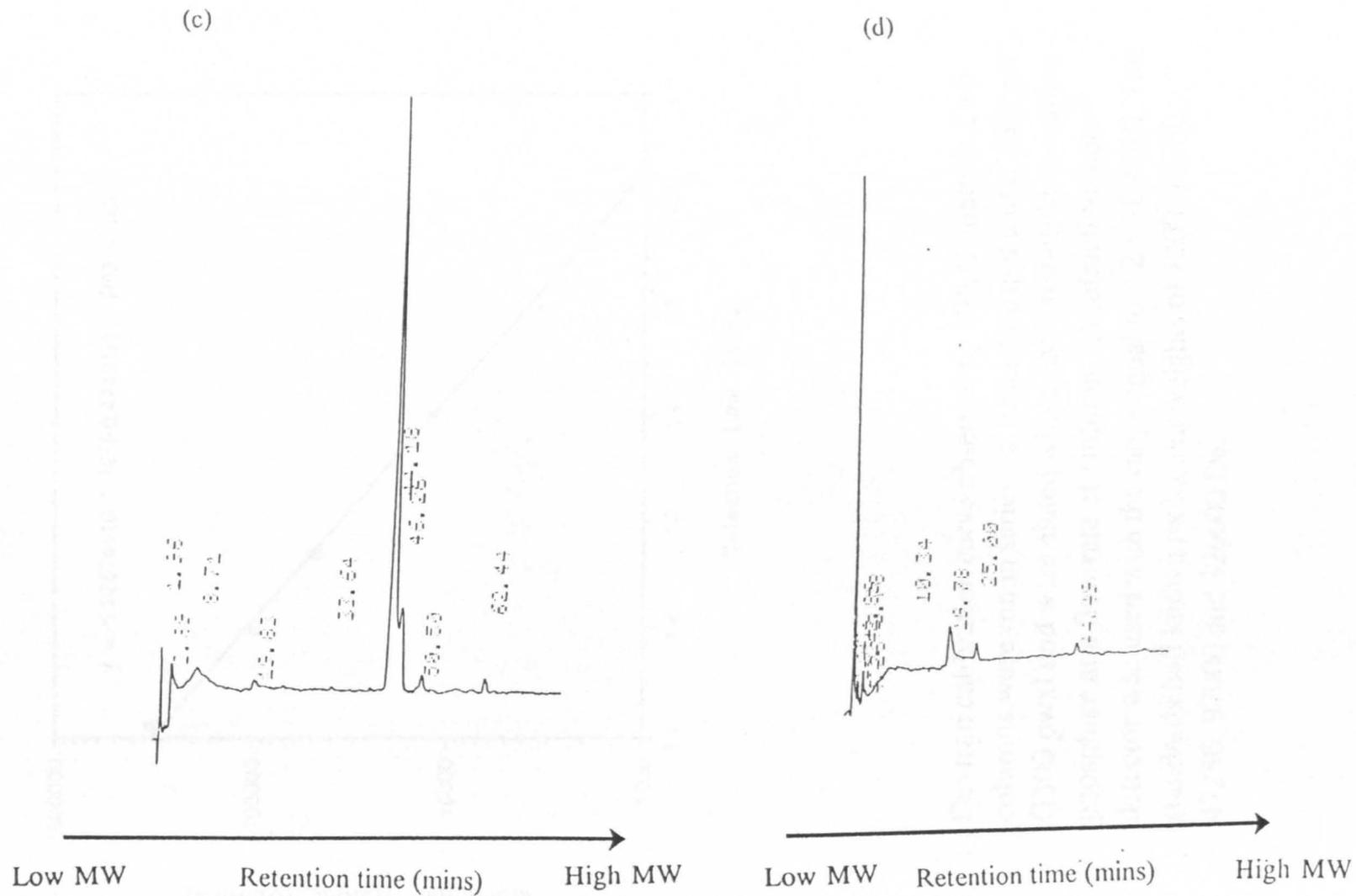
Figure 3.5 : Dionex traces of peaks 1-4 isolated by separation on a Biogel P6 column. The method used for the detection of these peaks is described in method 2.2.5.1. (c) Peak 3 (d) Peak 4

y = 5.2261e+10 * 10^(-0.47431x)   R^2 = 0.998
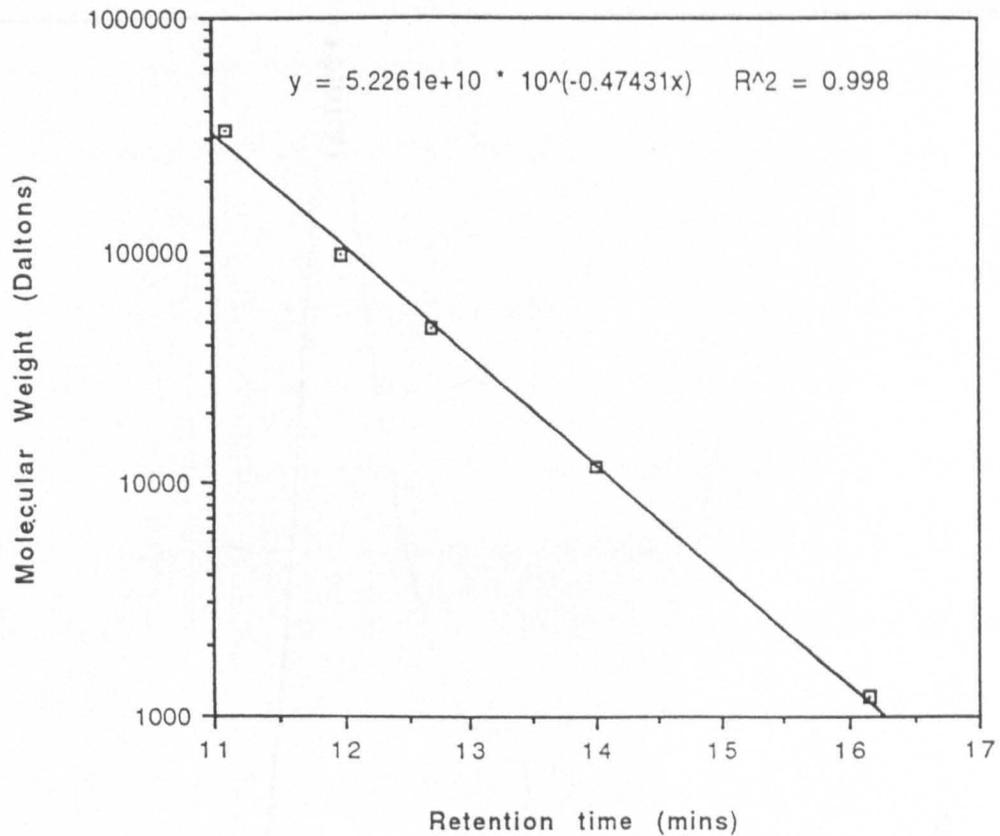
Figure 3.6   :   Dextran calibration curve observed by HPLC analysis. Two columns were run in series - a 10μm Aquagel and a TSK gel G300 pwxl and were eluted with 5mM tetrabutylammonium phosphate at a flow rate of 1ml/min . A refractive index detector was used with the range equal to 0.2×10^{-3} ΔRI. The standards had known molecular weights of 1200, 11700, 47250, 97000 and 326000 Da.
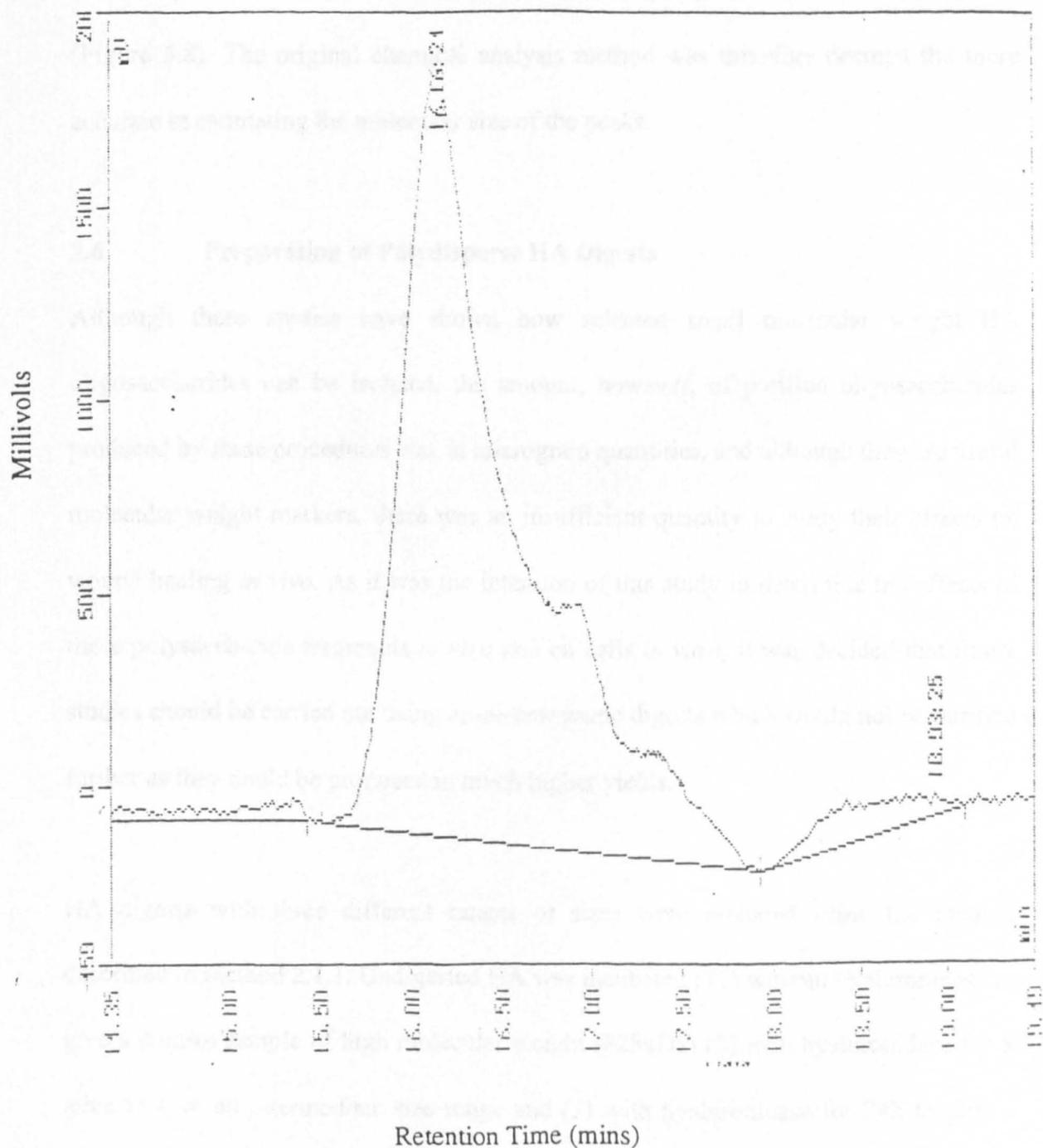
Figure 3.7 :   HPLC analysis of peak 3, obtained by separation of a 24h HA digest on
              a Biogel P6 column. Two columns were run in series - a TSK gel +
              G300pwxl and were eluted with 5mM tetrabutylammonium phosphate
              at a flow rate of 1ml/min . A refractive index detector was used with
              the range equal to 0.2 x 10⁻³ ΔRI. The molecular weight of the peak
              was estimated from the dextran calibration curve (Fig 3.6)

analysis has been used previously to determine the molecular weight of polysaccharides (Huckerby *et al*, 1990). NMR analysis confirmed that peak 3 was a tetrasaccharide (Figure 3.8). The original chemical analysis method was therefore deemed the more accurate in estimating the molecular size of the peaks.

3.6   Preparation of Polydisperse HA Digests

Although these studies have shown how selected small molecular weight HA oligosaccharides can be isolated, the amount, however, of purified oligosaccharides produced by these procedures was in microgram quantities, and although they are useful molecular weight markers, there was an insufficient quantity to study their effects on wound healing *in vivo*. As it was the intention of this study to determine the effects of these polysaccharide fragments *in vivo* and on cells *in vitro*, it was decided that future studies should be carried out using crude enzymatic digests which would not be purified further as they could be produced in much higher yields.

HA digests with three different ranges of sizes were prepared using the method described in method 2.1.1. Undigested HA was incubated : (1) without hyaluronidase to give a control sample of high molecular weight (828kDa) (2) with hyaluronidase for 5 mins to give an intermediate size range and (3) with hyaluronidase for 24h to give a digest containing small oligosaccharides (see section 3.4).

3.6.1   Molecular Weight Determination of HA by Viscometry

The molecular weight of the undigested HA sample was determined to be 828kDa by
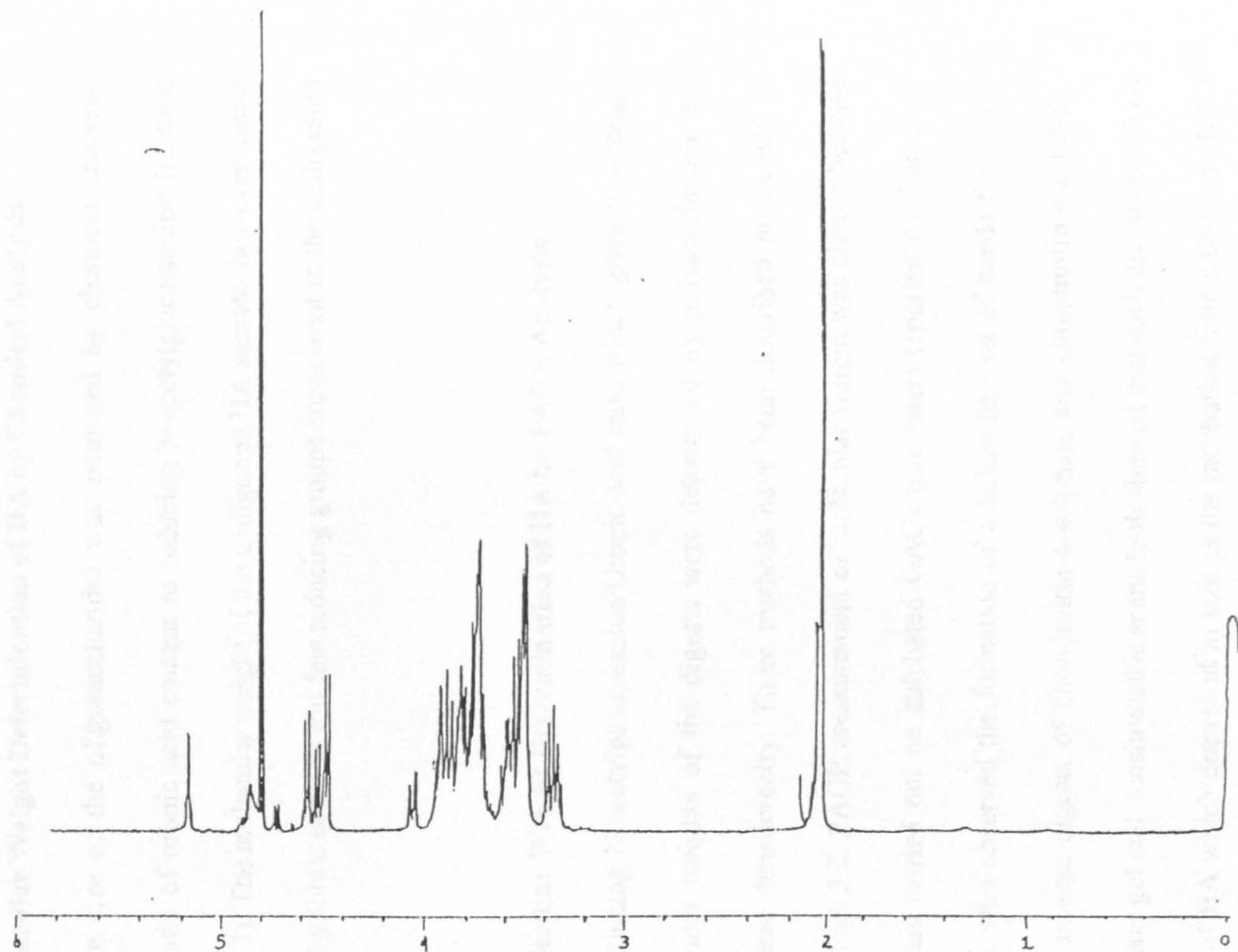
Figure 3.8 : H^1 - NMR spectra of HA oligosaccharide (peak 3) eluted by gel permeation chromatography using using Biogel P6. Spectra were obtained at 23°C on a JEOL GSX-400 spectrophometer. The internal standard was sodium 3-trimethylsilyl [^{2}H$_4$] propionate.

viscometry as described previously in section 2.2.1. Viscosity measurements on the 5

and 24h digests were not be carried out as such low molecular weight species have

negligible viscosity.

3.6.2   Molecular Weight Determination of HA by Chemical Analysis

The approximate size of the oligosaccharides was estimated by chemical analysis,

measuring the ratio of uronic acid content to reducing N-acetylglucosamine (method

2.2.5.3)(Table 3.3). The molecular weight of the undigested HA sample, however, could

not be analysed by this method as too few reducing groups are present in the undigested

sample.

3.6.3   Molecular Weight Determination of HA by PAGE Analysis

In addition to reducing N-acetylglucoasmine/Uronic acid ratio which gives an average

MW, the molecular weights of the digests were determined by polyacrylamide gel

electrophoresis and densitometry. These methods have been described previously in

section 3.2.2 & Figs 3.2. PAGE measurement of molecular weight was chosen because

of similar analysis carried out on sulphated GAGs (see later) reported by Turner &

Cowman (1984), who observed the formation of a ladder-like set of bands for partial

testicular hyaluronidase digests of chondroitin-4-sulphate and chondroitin-6-sulphate.

The electrophoretic gel and densitometric scans both showed that each digest contained

a range of sizes of HA which decreased in size as the incubation time increased (Table

3.4; Fig 3.2). Dextran sulphate standards with defined molecular weights of 5, 40 and

500 kDa were included in the electrophoretic gel to give an indication of the molecular

Sample	Distance to most dense area (cm)	Approximate MW (kDa)
Dextran sulphate 500kDa	0.29	500
Dextran sulphate 40kDa	10.44	40
Dextran sulphate 5kDa	13.34	5
Undigested HA	0 - 0.22	>500
HA (5 min digest)	4.5 -7.8	>40, <500
HA (24h digest)	9.57	~ 40

Table 3.4 :   Molecular weight estimations of HA digests by comparison to the
distance travelled by dextran sulphates on a 10% polyacrylamide gel.
Distances to the most intense blue staining were measured by
densitometry (method 2.2.8).

Incubation Time of HA	Uronic acid/N-acetyl glucosamine (μmol/ml)	Average no. of disaccharide units	Approx. Molecular weight (Da)
0 mins	-	-	828,000 *
5 mins	30/6.2	5	2000
24h	21/11.77	2	800

* Molecular weight analysis by viscometry

Table 3.3 :   Digestion of 10mg/ml HA (MW 828kDa) with 0.25 mg/ml testicular hyaluronidase (2400U/mg) at 5 mins and 24h incubation periods at 25°C. The table shows the average molecular weights of each aliquot as determined by uronic acid and N-acetyl glucosamine content. (Method 2.2.5).

weights of the digests (Table 3.4; Fig 3.1). Charge to mass ratios however, also affect mobility of polysaccharides and it has been shown that sulphated GAGs migrate quicker than non-sulphated GAGs (Turner & Cowman, 1984). This effect must be remembered when comparing the dextran sulphate and HA gel scans. Ladders were not obtained during this study, but instead polydisperse smears were observed. Due to the presence of smears, the distance from the top of the gel to the most dense area of the of the HA bands were measured by densitometry to give a molecular size range compared to those obtained for dextran sulphate (Table 3.4; Fig 3.2).

The results from PAGE suggest much greater molecular weights for each digest than using chemical analysis as might be expected due to the smaller oligosaccharides running off the bottom of the gel, however PAGE followed by densitometric analysis demonstrates the presence of a range of molecular weights and therefore an average has been calculated from the range. Inaccuracies in the PAGE method for MW estimation may also have occurred due to the difference in charge to mass ratios of the dextran sulphate standards and HA.

3.6.4 Conclusion

Chemical analysis of molecular size by measuring the ratio of uronic acid to reducing N-acetylglucosamine has previously been reported to give accurate molecular weight determinations so long as the HA has first been partially depolymerised (Gorham *et al*, 1974). In conclusion, as PAGE loses the smaller oligosaccharides off the bottom of the gel, chemical analysis, which measures all molecules present, is a more accurate method of analysis. Further evidence for the accuracy of chemical analysis of purified fragments

is shown in section 3.4, where NMR analysis of a suspected tetrasaccharide (value obtained by chemical analysis) gave the same value.

A summary of the methods used to determine molecular weight and the results obtained for the different oligosaccharide preparations are shown in Table 3.7a.

In conclusion, oligosaccharides of HA could be produced by several different methods, giving various molecular weights ranges. The weight of the oligosaccharides could also be determined by various methods, some more accurate than others. For cruder fractions, a simple hyaluronidase digestion was fine, however, purification was required to give fragments of a specific size.

The amount of pure HA fragments isolated was not sufficient for animal studies, therefore the crude oligosaccharide fragments produced were retained to examine their effect on cell proliferation & chemotactic ability *in vitro*. Similar work was reported by West & Kumar (1989) when they examined the effect of a range of HA oligosaccharides on endothelial cell proliferation *in vitro*. It was hoped that their effect on cells in culture would give an indication as to their ability to effect cells *in vivo*.

3.7   **Production and Analysis of Chondroitin Sulphate and its**
        Fragments

3.7.1   **Introduction**

Chondroitin sulphate (CS) has a very similar structure to that of HA (Chapter 1 , Fig 1.1) and its role in wound healing has been previously documented (LeBoeuf *et al*,

1987; Westergren-Thorsson *et al*, 1991). As reported in section 1.4.2, CS has been used

in medical devices such as burn dressings, and so its potential activity in a wound

healing situation has already been recognised but not proven. The involvement of CS in

the wound healing response and its structural similarities to HA led to the decision to

depolymerise the polysaccharide to investigate the effects of small CS oligosaccharide

on wound healing.

Like HA, CS can be depolymerised by testicular hyaluronidase (Knudson *et al*, 1984) as

it also contains ß1-4 bonds between the N-acetylgalactosamine and uronic acid residues

comparable to HA.

3.7.2   Molecular Weight Determination of Intact CS by Viscometry

Molecular determination of the starting material was important in order to establish the

degree of depolymerisation over a time course. Viscosity measurements as used for the

determination of undigested HA molecular weight, could not be used, as the starting

viscosity of the CS was too low to be measured accurately by this method.

3.7.3   Molecular Weight Determination of Intact CS by HPLC

Analysis of intact CS by HPLC (method 2.2.2) was employed, using a dextran sulphate

standard with the molecular weight range of 40-80 kDa. The manufacturer, Sigma,

estimated the molecular weight of the CS by HPLC analysis to be between 40 and 80

kDa. The HPLC trace (Fig 3.9) demonstrates the presence of 3 peaks for the dextran

sulphate which correspond to the range of molecular size from 40 to 80 kDa within the

sample tested. Dextran sulphate peak 1 contained the high molecular weight fragments
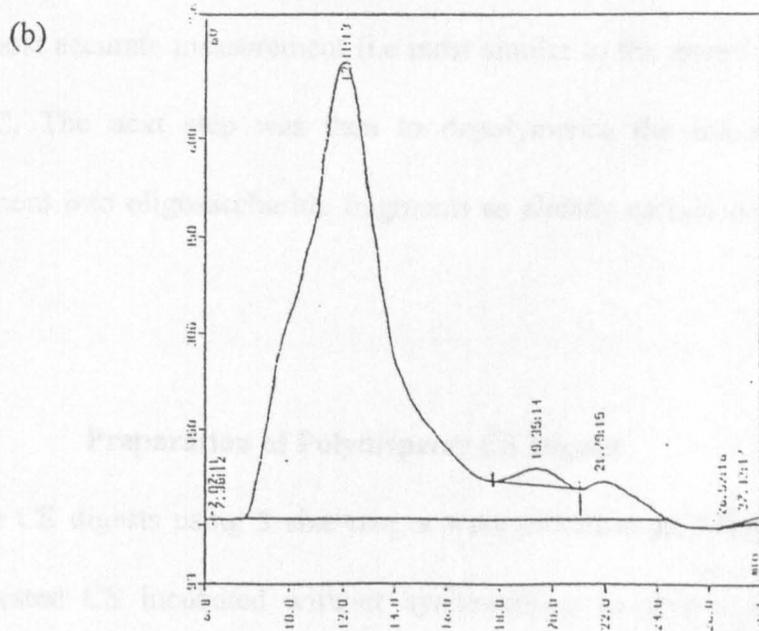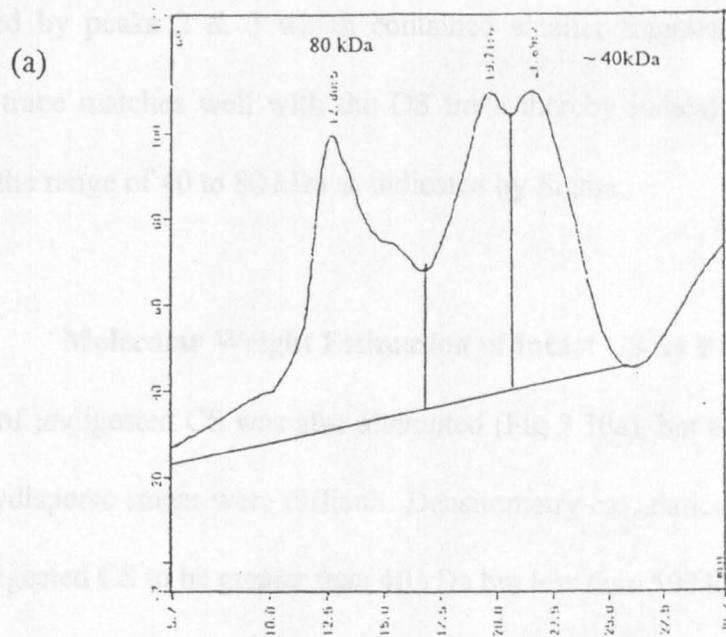
Figure 3.9 : HPLC analysis of (a) 10mg/ml dextran sulphate with a molecular weight of between 40 and 80 kDa and (b) 10mg/ml chondroitin sulphate C. Two columns were run in series - a 10 μm "Aquagel" and a TSK G300pwxl and were eluted with 5mM tetrabutylammonium phosphate at a flow rate of 1ml/min'. UV absorbances were measured at 210nm.

followed by peaks 2 & 3 which contained smaller fragments respectively. The CS HPLC trace matches well with the DS trace thereby indicating the CS to indeed be within the range of 40 to 80 kDa as indicated by Sigma.

3.7.4   Molecular Weight Estimation of Intact CS by PAGE

PAGE of undigested CS was also attempted (Fig 3.10a), but accurate estimations from the polydisperse smear were difficult. Densitometry calculations of the smear indicated the undigested CS to be greater than 40 kDa but less than 500 kDa.

Following molecular weight analysis by several different methods, the results indicated the most accurate measurement (i.e most similar to the manufacturer's value) to be by HPLC. The next step was then to depolymerise the intact CS by hyaluronidase treatment into oligosaccharide fragments as already carried out on intact HA (section 3.6).

3.8.   Preparation of Polydisperse CS Digests

Crude CS digests using 3 size ranges were produced as follows (method 2.1.1) : (1) undigested CS incubated without hyaluronidase to give a control sample of high molecular weight (40-80kDa), (2) incubation with hyaluronidase for 5 mins to give an intermediate size range and (3) incubation with hyaluronidase for 24h to give a digest containing small oligosaccharides.

Lane a   b   c

Lane (a)	Lane (b)	Lane (c)
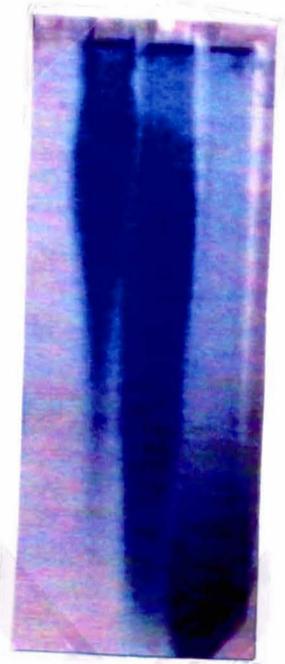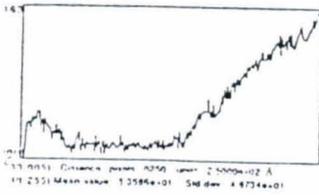
Distance to most dense area	Distance to most dense area	Distance to most dense area
= 2.47 - 7.83cm	= 5.22 - 10.44cm	= 10.44cm
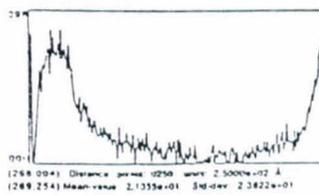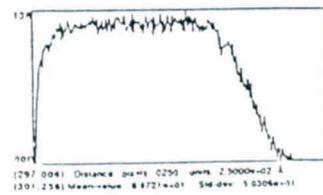
Figure 3.10 :   Polyacrylamide gel electrophoresis and densitometry scans of CS
digests. Lane (a) undigested CS (b) 5 min CS digest (c) 24hr CS digest
The scans run from left to right on the graph and the most dense
areas are represented by the lowest numbers on the scale. Dextran
standards are shown in Figure 3.1

3.8.1 Molecular Weight Estimations of CS Oligosaccharides by Chemical Analysis

Chemical analysis shows the decrease in molecular size of CS as the incubation time increases (Table 3.5). After 5 minutes, the oligosaccharide has a molecular weight in the range of 4 kDa and after 24hrs this has decreased to 2 kDa.

3.8.2 Molecular Weight Estimations of CS Oligosaccharides by PAGE

Comparison of the smears obtained for CS and the dextran sulphate standards on PAGE, suggest the 5min and 24h digests to have molecular weights of between 40-500 kDa and ~40 kDa respectively (Table 3.6 & Fig 3.10). PAGE results have previously shown that small oligosaccharides run off the bottom of the gel (section 3.6.3), however, it still provides a useful visual assessment of the degree of polydispersion and comparison of the molecular weight changes between samples. A summary of the methods used to measure molecular weight are shown in Table 3.7b.

As in the case of the HA fragments, it was the intention of this study to investigate the effects of these fragments in animal models, however, there was an insufficient amount of material to carry out the *in vivo* studies, so like HA, *in vitro* studies were carried out only.

Incubation time of CS	Uronic acid/N-acetlygalactosamine (μmol/ml)	Average no. of disaccharide units	Approx. Molecular Weight (kDa)
0 mins	-	-	40-80 kDa [*]
5 mins	20/1.96	10:1	4 kDa
24h	16/3.04	5:1	2 kDa

[*] Molecular weight estimation by HPLC

Table 3.5 : Molecular weight estimations of CS digests by chemical analysis of uronic acid concentration and N-acetyl glucosamine content (method 2.2.5).

Sample	Distance to most dense area (cm)	Approximate MW (kDa)
Dextran sulphate 500kDa	0.29	500
Dextran sulphate 40kDa	10.44	40
Dextran sulphate 5kDa	13.34	5
Undigested CS	2.47-7.83	>40, <500
CS (5 min digest)	5.22-10.44	>40, <500
CS (24h digest)	10.44	40

Table 3.6 : Molecular weight estimations of CS digests by comparison to the distance travelled by dextran sulphates on a 10% polyacrylamide gel. Distances to the most intense blue staining were measured by densitometry (method 2.2.8).

(a)

Digest	Viscometry	PAGE	Chemical Analysis	HPLC
Undigested HA	828kDa	>500 kDa	N/A	N/A
HA 5 min digest	N/A	40-500 kDa	2 kDa	N/A
HA 24h digest	N/A	~40 kDa	0.8 kDa	0.8-1.2 kDa

(b)

Digest	Viscometry	PAGE	Chemical Analysis	HPLC
Undigested CS	N/A	40-500 kDa	N/A	40-80 kDa
CS min digest	N/A	40-500 kDa	4kDa	N/A
CS24h digest	N/A	40 kDa	2 kDa	N/A

Table 3.7 :   Summary of (a) HA and (b) CS molecular weight determinations by different methods.

3.9 Preparation and Analysis of Plant Polysaccharides and Their Oligosaccharides

3.9.1 Introduction

Certain plant polysaccharides have structures similar in many ways to animal polysaccharides (see chapter 1, section 1.5). Several of these plant polysaccharides were chosen to determine if their breakdown products had any wound healing bioactivity *in vitro* and/or *in vivo*.

The polysaccharides tested were Oxidised Regenerated Cellulose (ORC) and pectin. ORC was chosen due to its structural similarity to HA (see Figure 1.3) whereas pectin shares similarities with chondroitin sulphate HA (Fig 1.3). Both these polysaccharides have also been clinically used in the treatment of wounds (see chapter 1, sections 1.5.1 & 1.5.2), although ORC's main role is help in haemostasis and to prevent adhesion.

3.9.2 Oxidised Regenerated Cellulose

As stated in the introduction (chapter 1, section 1.5.2), ORC is a derivatised biopolymer produced from cellulose by oxidation with nitrogen tetraoxide in a chlorofluorocarbon solvent, and consists of 83% glucuronic acid residues (Saferstein & Boardman, 1992) (section 1.5.2). This material possesses a structural resemblance to GAGs, in that it contains uronic acid residues. ORC is both patented and manufactured by Johnson & Johnson Inc. as a haemostat and a barrier to adhesion. Recently a patent has been filed (Doyle, 1996) on ORC fragments and collagen-ORC composites

covering the potential bioactivity of ORC and its fragments. ORC and its fragments are therefore of great significance to Johnson & Johnson in that they may have a potential role in wound healing that previously has not been identified.

3.9.3   Molecular Weight Estimation of ORC

ORC is produced as an insoluble knitted fabric and its molecular weight in this form cannot be determined. ORC can be solubilised in alkali as shown previously by Saferstein& Boardman (1992). Due to the lability of ketone groups, however, the polymer begins to depolymerise even before it dissolves which leads to the formation of fragments - some with terminal aldehyde groups and others containing ketone groups. Resorption of ORC in the body occurs by the beta elimination process and the resulting fragments can then be removed from the blood by the kidneys or metabolised into CO_2 and H_2O. The formation of small oligosaccharide fragments of ORC by solubilisation in alkali was attempted in this study by using a strong alkali - 6M NaOH, and a weak alkali - 0.1M $NaHCO_3$ (method 2.1.4 & 2.1.5). It was hoped solubilisation of ORC using 2 different strengths of alkali would produce fragments of ORC with different polydisperse molecular weight ranges. Depolymerisation by this method involves the addition of ORC to alkali followed by the addition of HCl to stop the reaction and bring the pH back to 7.0. Precipitation of the ORC follows at pH 7.0. The potential problem with this method is that addition of HCl may not stop the depolymerisation reaction. For the purpose of this study however, the degree of polymerisation of the ORC is not important. The aim was to demonstrate if either of the depolymerised solutions of ORC were bioactive. Further studies however could be devised to ensure that

depolymerisation is stopped by HCl by taking aliquots of ORC at varying time points after the addition of HCl and separating on PAGE to detect changes in molecular size.

Following the solubilisation of ORC in NaOH and NaHCO$_3$, the molecular weight of the 2 fragment ranges was determined by PAGE (method 2.2.7. Figure 3.11 shows that solubilisation with 6M NaOH yields fragments with a smaller range of molecular weights than that obtained using 0.1M NaHCO$_3$. The smears of polydisperse ORC are visible on the electrophoretic gel, however, the difference in molecular size between the two treatments is clearly apparent. Average molecular weight estimations of depolymerised ORC by comparison to those of dextran sulphate standards (Fig 3.11) gave average molecular weights of between 5 and 40kDa for the NaOH treated sample and about 40kDa for the NaHCO$_3$ treated sample, however, the inaccuracies of PAGE at estimating the molecular size of small polysaccharides has already been discussed and additional methods of estimation were sought. Measurement by viscosity was impossible due to the low initial size of the sample. For this study, the two ORC preparations were given the names ORC 1 (solubilised in 6M NaOH) and ORC 2 (solubilised in 0.1M NaHCO$_3$) and further investigation into the sizing of the these molecules was noted as potential future work. For this study, no further purification was attempted and all future studies were conducted with the 2 crude fragment ranges.

3.9.4   Pectin and its Oligosaccharides

Pectin (Figure 1.3c) is an abundant plant polysaccharide and was investigated in this project as Johnson & Johnson have patent protection for anionic plant polysaccharides

Lane a b c d

Lanes (a) and (c)   Lanes (b) and (d)

(204.012) Distance: pixels: 0250 units: 2.0000e+02 Å
(204.242) Mean-value: 9.1706e+01 Std-dev: 5.3219e+01

(227.012) Distance: pixels: 0250 units: 2.0000e+02 Å
(227.242) Mean-value: 8.7307e+01 Std-dev: 5.6060e+01
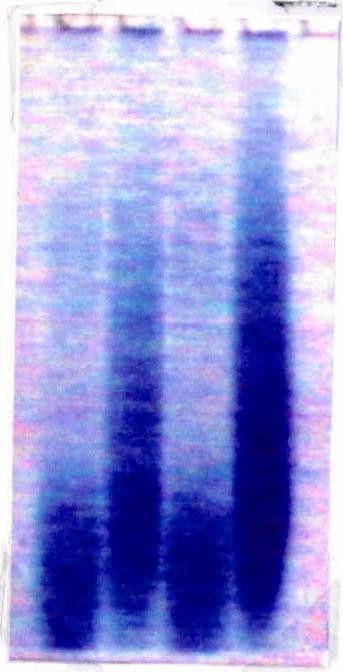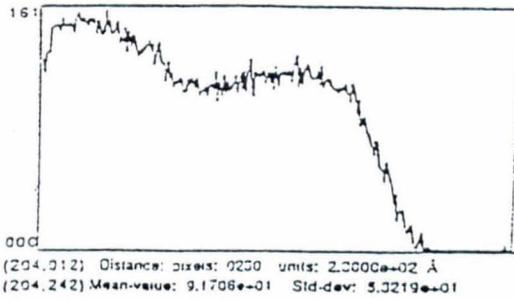
Distance to most dense area   Distance to most dense area
= 11.46cm   = 10cm

Figure 3.11   :   Polyacrylamide gel electrophoresis and densitometry scans
                    of ORC(1) digested with 6M NaOH and ORC(2) digested with
                    0.1M sodium bicarbonate. Lanes a and c - ORC(1)(20μl
                    loaded). Lanes b and d - ORC(2)(20μl loaded). The scans run
                    from left to right on the graph and the most dense areas are
                    represented by the lowest numbers on the scale.

which may be used in collagen based wound healing devices (UK Patent: Harvey, Light & Watt, No.9206492), and so as pectin is readily available and inexpensive, any bioactivity shown *in vitro* or *in vivo* would be beneficial to the company in the search for new, innovative products.

3.9.5   Molecular Weight Estimation of Pectin

HP Bulmer manufacture the high methoxyl pectin used in this study, and give a typical molecular weight value of between 500 and 750 kDa for this product as estimated by HPLC analysis. The average molecular weight of pectin in this study was estimated by viscometry and PAGE and gave average values of 645 and 500 kDa respectively (Fig 3.12a). Both these values fall within the manufacturer's limits, and although PAGE has been shown to be inaccurate at estimating low molecular weight polysaccharides, it seems a good method for estimating the size of large polysaccharides.

Depolymerisation of pectin was attempted using 6M NaOH as shown previously by ORC. This work, however, was not completed and only preliminary data is reported here. Degradation by 6M NaOH of pectin can be detected on PAGE as shown in Figure 3.12b. Further studies would be needed to optimise this reaction and investigate the conditions to produce full degradation of the polysaccharide.

A molecular weight estimation was calculated by PAGE for the alkali treated sample to give a molecular range (Fig 3.12b). The results indicate the molecular size range of the alkali treated pectin to be between 40 and 500kDa. The degraded pectin was retained for future bioactive studies.

Lane a   b

:
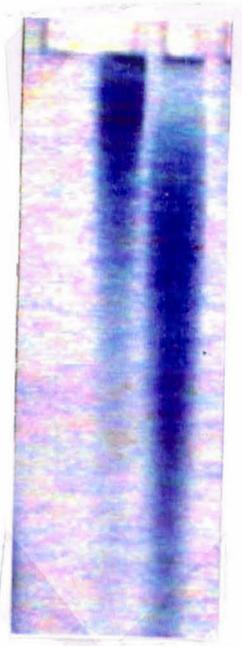
Lane (a)   Lane (b)
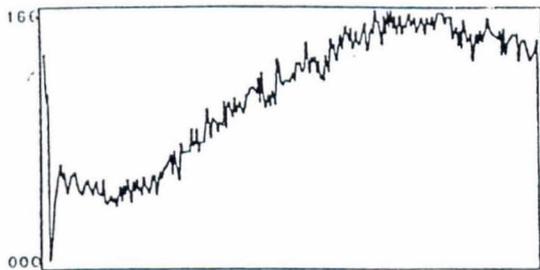
(219,004) Distance: pixels: 0253 units: 1.4442e+01 cm   (244,004) Distance: pixels: 0250 units: 1.4271e+01 cm
(223,257) Mean-value: 1.1026e+02 Std-dev: 4.3288e+01   (252,254) Mean-value: 6.4904e+01 Std-dev: 3.3443e+01

Distance to most dense area   Distance to most dense area
= 0.29cm   = 8.99cm

Figure 3.12 :   Polyacrylamide gel electrophoresis and densitometry scans of pectin
                and a digest of pectin. Lane (a) undigested pectin (b) pectin treated
                with 6M NaOH. The scans run from left to right on the graph and the
                most dense areas are represented by the lowest numbers on the scale.
                Dextran standards are shown in Figure 3.1

3.10 Preparation and Storage of Polysaccharides & Their Fragments for *In Vitro* Testing

All the polysaccharides and fragments described in the previous sections were dialysed against water to remove any salts which may interfere with cell culture, using Spectra-Por 6 tubing with a MW cut off of 1000. They were then filtered through a 0.45 um filter to remove precipitated enzyme (when necessary), freeze-dried and stored at 4°C prior to *in vitro/in vivo* testing.

CHAPTER 4

THE EFFECT OF POLYSACCHARIDES ON THE PROLIFERATION CULTURED CELLS

4.1   Introduction

The *in vitro* effect of polysaccharides and their fragments on the proliferation and chemotaxis of cells involved in the wound healing process was carried out in an attempt to ascertain their potential effect *in vivo*. Proliferation of cells such as fibroblasts (Postlethwaite *et al*, 1978), endothelial cells (Marks *et al*, 1991) and epithelial cells (Marks *et al*, 1991) during wound healing, is of the utmost importance in order to restore cell loss due to injury i.e. damaged skin, blood vessels and connective tissue. Cells such as epithelial cells need to proliferate to restore the epidermis and protect the healing wound underneath. Chemotaxis of cells such as fibroblasts towards the site of injury is therefore also necessary to ensure that the damage is repaired by laying down new collagen to give the healing wound strength and also are involved in the contraction of the wound itself (Postlethwaite *et al*, 1978). This response is mediated by signals in the wound attracting the specific cells required.

It was hoped that any stimulation above control values of proliferation or chemotaxis of various cell types in the presence of polysaccharides *in vitro*, might indicate a potential role for these materials in the healing of chronic wounds *in vivo* and speed up the healing process.

79
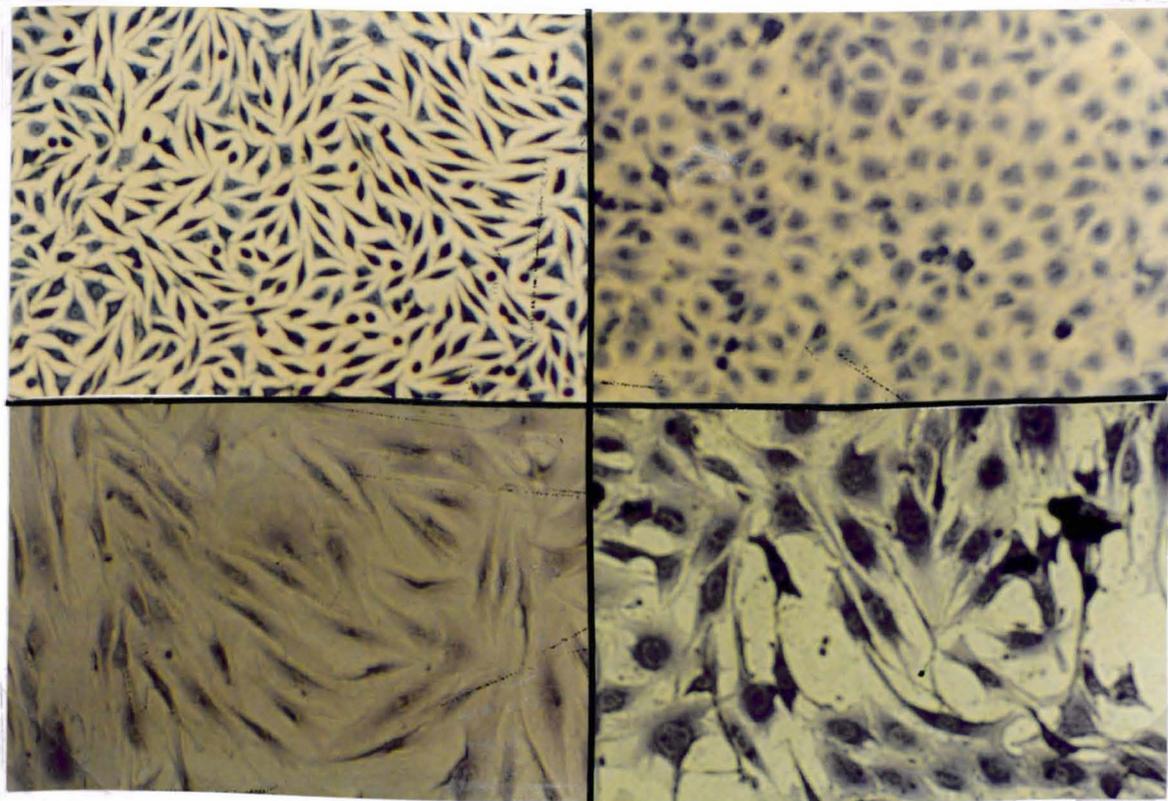
Figure 4.1   :   Photographs of the various cell types used in this study.
All cells were stained with 0.1% methylene blue in
10mM borate buffer pH 8.4 as described in Methods.
(a) L929 fibroblasts, (b) Bovine aortic endothelial cells,
(c) Rat wound fibroblasts and (d) human dermal
fibroblasts. Magnification x 40.

The polysaccharides prepared as described in Chapter 3 were examined for their *in vitro* effect on the proliferation and chemotaxis of several different types of cells. Fibroblasts were the first cell type tested as they are essential to the repair of damaged tissue at the wound site where they lay down new collagen, produce growth factors & proteoglycans and draw the edges of the wound together (Lynch *et al*, 1989; Morgan & Pledger, 1992). The second cell type employed in this study were endothelial cells, which line the blood vessels. The formation of blood vessels (angiogenesis) by endothelial cells at the wound site is an important stage in the wound healing process as new vessels bud from intact vessels and grow into the wound giving it a new blood supply (Fowler, 1989; Morgan & Pledger, 1992), thereby providing the area with oxygen. Epithelial cells were not investigated in this study as it was felt that in chronic wounds, fibroblasts and endothelial cells were the cell types which initiated the wound healing process and so they should be studied first. Photographs of the cell types used in these experiments are shown in Figure 4.1 (except 3T3 cells which were not photographed) attached to tissue culture plastic and stained with methylene blue dye to visualise the cells (method 2.4.4). The sources of the cells were as follows : (a) L929 mouse fibroblasts, (b) Rat wound fibroblasts, (c) Human skin fibroblasts, (d) Bovine aortic endothelial cells (BAEC) and (e) 3T3 Swiss mouse fibroblasts Mixed sources of cells were used in order to assess differences between species. Mouse L929 and 3T3 cells and BAEC cells were readily available and were used for this reason. Rat skin fibroblasts were isolated during this project and were thought to be a good cell type to study as the results could be compared to *in vivo* rat data later in this study. The only source of human cells available was skin fibroblasts. It was hoped that these cells would give an indication to what may

be happening to these cells *in vivo*, however it is recognised that different human cell types would be needed to give results which could be compared to human wound healing.

4.2   Cell Proliferation

4.2.1   Assessment of Cell Counting Methods

The measurement of cellular proliferation can be determined by several different methods. The most widely used assay depends on the incorporation of [^{3}H] Thymidine into dividing cells where the total amount of [^{3}H] Thymidine incorporated at the end of the proliferation experiment is measured, or, the percentage of labelled cells counted by autoradiography and microscopy (West & Kumar, 1989). Other cell counting methods include colorometric techniques using dyes such as MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), (Carmichael *et al*, 1987; Vistica *et al*, 1991)) and Neutral Red (Triglia *et al*, 1991; Fautz *et al*, 1991) which are most commonly used as indicators of cell death, as the dyes are only taken up by living cells. The methods mentioned above, although accurate cell counting methods, are time consuming due to the incubation times needed to take up the dyes, which renders them unacceptable for the large numbers of samples to be screened in this study. As a result of this, alternative methods were sought for the measurement of cellular proliferation.

Cell counting using a counting chamber such as a haemocytometer, was the first method chosen to be assessed (method 2.4.5). Cell counting using a haemocytometer requires the cells to be detached from the surface of the well in a 96 well plate, followed by

counting on a grid marked on the surface of the counting chamber. The number of cells present are expressed as the number per ml. This method, however, is time consuming and only small numbers of samples can be analysed at the one time.

The second method to be assessed was that of Oliver *et al*, (1989) using the Methylene Blue dye binding assay (method 2.4.4). The methylene blue assay was described by Oliver as a rapid and convenient method for counting cultured cells in microwell plates and was used in Oliver's paper to assess the effect of growth factors on cell proliferation. The methylene blue assay therefore had the potential in this study to allow the rapid screening of many samples. The Methylene Blue dye binding assay (method 2.4.4) operates on the principal that the basic dye will bind to the negatively charged molecules within the cell. Addition of HCl in Ethanol, with a pH less than 2, allows the acid groups to be protonated, liberating the Methylene Blue dye into the elution solvent and a single absorption peak observed at A_{650} has been reported to be an accurate and reproducible reflection of the cell number stained with the dye (Oliver *et al*, 1989; Horobin, 1982), however this method has not been validated.

4.2.2   Comparison of Methylene Blue Dye Binding to Haemocytometer Counting

L929 fibroblasts were the initial cell type used for these studies as they were readily available, grew quickly and passaged well. L929 cells were used to compare the linearity of the methylene blue and cell counting methods over a range of cell densities. Cells were seeded between 0 and 6×10^4 per well in 96 well plates and the data obtained

used to construct standard curves. Cells seeded at higher densities (above 6 x 10^4) were found not to fall on the linear scale and their absorbance readings after staining with methylene blue were greater than the values detectable on the plate reader, therefore a maximum density of 6 x 10^4 was used.

The methylene blue assay demonstrated excellent linearity (R=0.999) over the whole range of cell densities tested (Fig 4.2a) and at the same time showed good reproducibility with a standard deviation of less than 5% between replicates. This result indicates that a linear correlation exists between cell number and dye binding.

The cell counting chamber assay however, displayed a lower degree of linearity with a correlation coefficient of only 0.878 (Fig 4.2b). The graph also demonstrates that the number of cells counted by this method was much lower than the initial number seeded, however, this was probably caused by incomplete detachment of the cells from the surface of the plate during the trypsinisation procedure. The standard deviation between replicates was found to be ±15%, which is much greater than that shown by the methylene blue assay. Direct cell counting on the haemocytometer may also be less accurate due to the small sample size (40μl) taken for the measurements, as any small error in counting using the grid would therefore be multiplied in the calculation and result in an even greater degree of error.

In conclusion, comparison of the two methods revealed the methylene blue dye binding assay to be the most rapid and reproducible at measuring cell numbers and was therefore employed in this study as the standard method for the screening of the effect of polysaccharides on cellular proliferation.
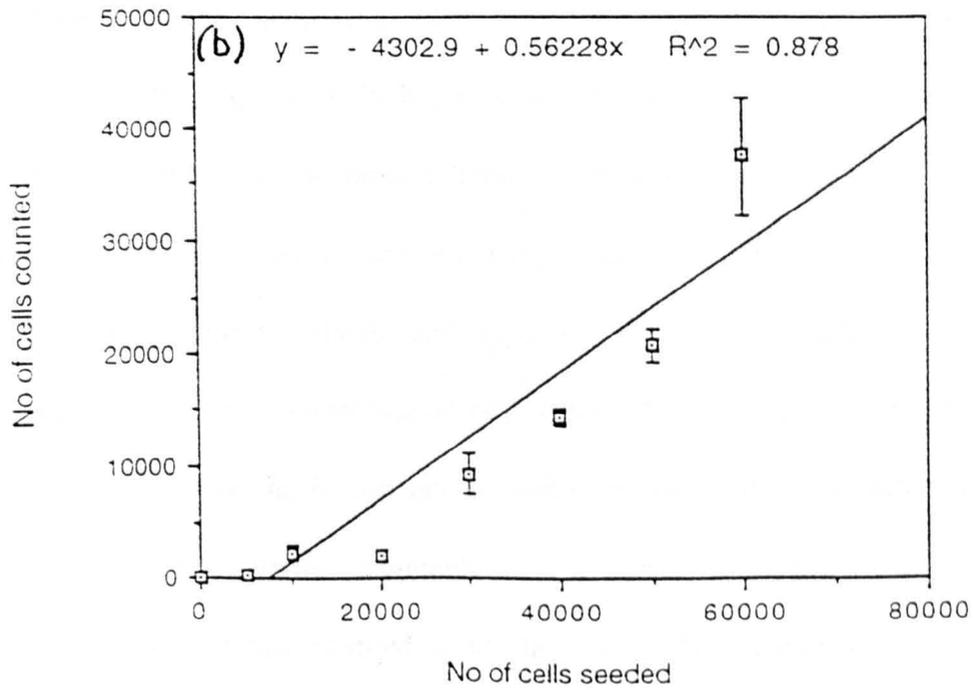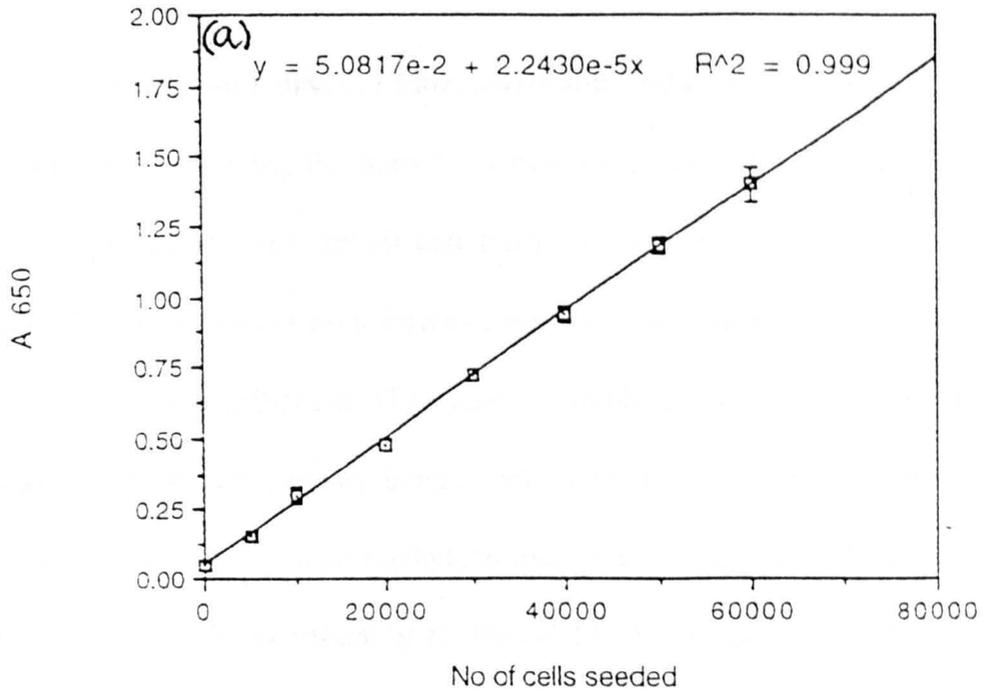
Figure 4.2 : (a) The linear relationship between cell number and methylene blue dye binding. The absorbance of the dye was measured at A_{650}. The standard deviation was $< 5\%$.(b) Graph of the number of cells seeded in a 96 well plate against the number counted after trypsinisation in a haemocytometer. The standard deviation was $< 15\%$.

4.3   Standard Curves of Fibroblasts and Endothelial Cells

Standard curves showing the linearity between methylene blue dye binding and cell number were constructed for all cell types available for the study. The cell types employed were fibroblasts from various sources (see section 4.1) and endothelial cells. Fibroblast cell lines (L929 and 3T3), primary fibroblasts (rat wound fibroblasts, human dermal fibroblasts and primary bovine aortic endothelial cells) were obtained and all were tested for linearity with methylene blue over a range of cell densities. All cell types tested, with the exception of rat wound fibroblasts, showed a linear relationship between cell number and dye binding over the ranges tested, with correlation coefficients of > 0.99 in all cases (Figs 4.3; 4.4; 4.5; 4.6b). Rat wound fibroblasts showed a linear relationship up to 1.9×10^4 cells with r = 0.959 (Fig 4.6a), however, above this concentration, the cells began to detach from the surface of the plate. For *in vitro* studies, therefore, a low initial cell number of rat wound fibroblasts would have to be used in order to prevent detachment at high concentrations. Rat Wound Fibroblasts were the most closely related cell type on which to measure the effect of polysaccharides in an *in vivo* rat wound healing model, however, these cells proliferated very slowly in culture and in fact not enough cells were able to be generated for the screening of polysaccharide fragments in the time period available. Rat wound fibroblasts were therefore omitted from this study. The human dermal fibroblasts however were a good cell type to investigate as the *in vitro* results obtained in this study could correlate with any clinical wound healing studies undertaken in the future.

$y = 0.14922 + 1.6570e\text{-}5x \qquad R^2 = 0.992$

Number of fibroblasts/well
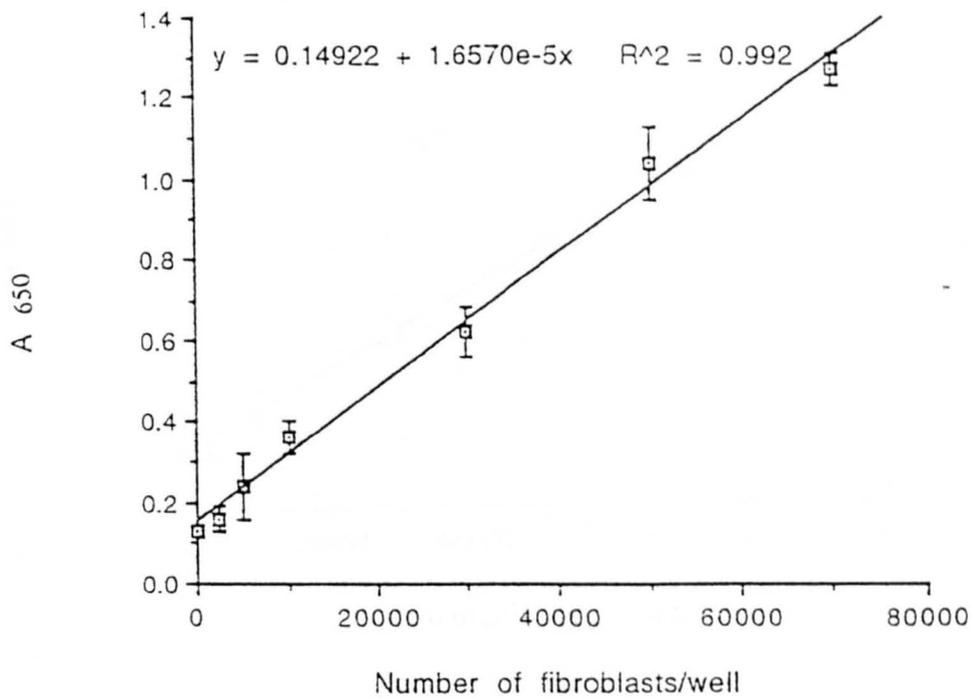
Figure 4.3   :   Linear relationship between A_{650} and the number of L929 fibroblasts seeded. The cultures were harvested after 4h, before significant replication could have taken place. The results are expressed as the mean of six replicates $\pm$ standard deviation.

$$y = 0.18448 + 1.4424e\text{-}5x \qquad R^2 = 0.997$$

No of cells seeded/well
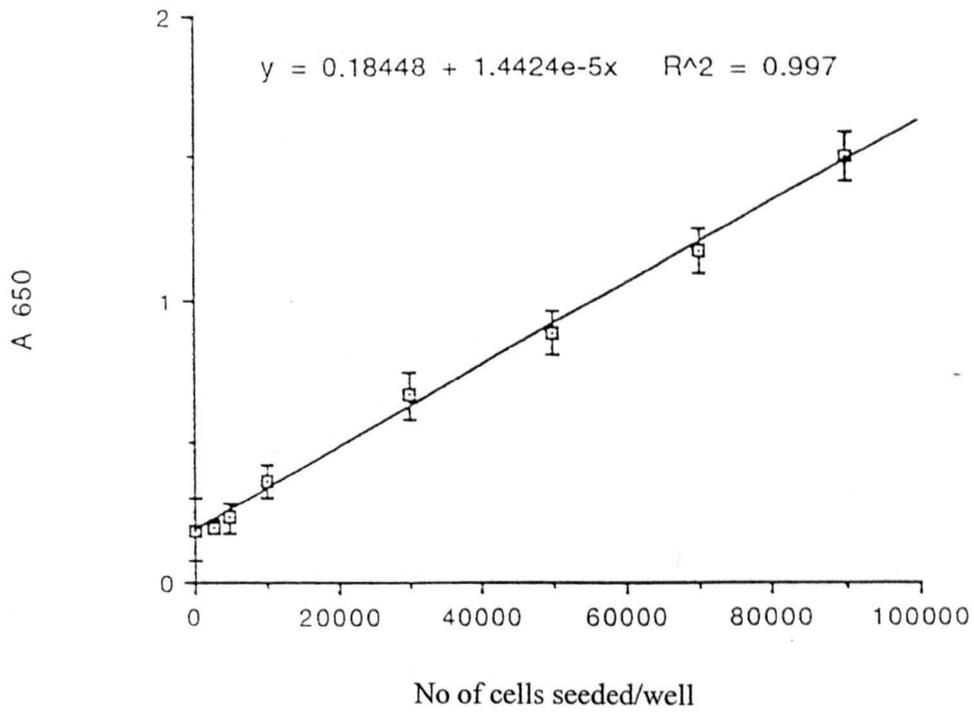
Figure 4.4 :   Linear relationship between methylene blue dye binding (A_{650}) and 3T3 fibroblast cell number. The results are expressed as the mean of six replicates ± standard deviation.
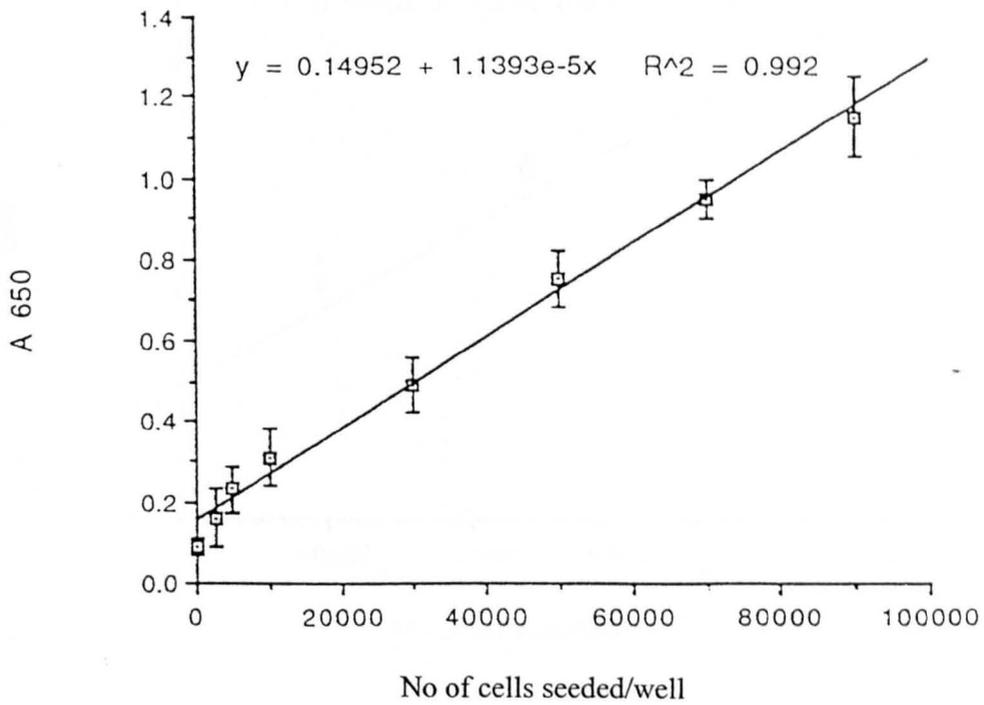
Figure 4.5 :   Linear relationship between methylene blue dye binding (A_{650}) and
BAEC endothelial cell number. The results are expressed as the
mean of six replicates ± standard deviation.
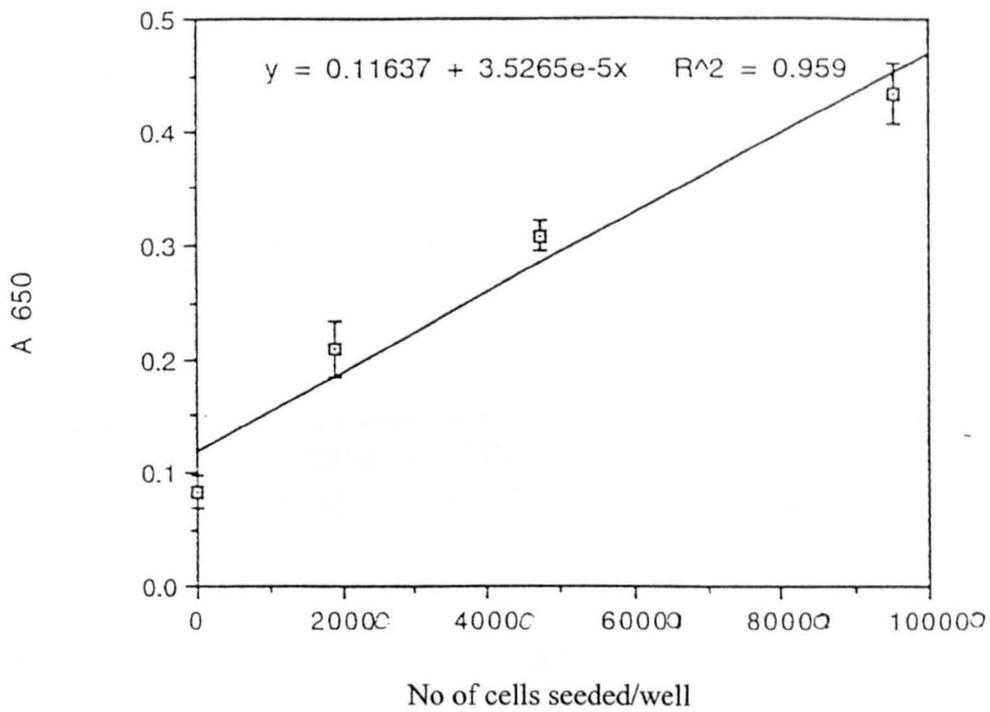
Figure 4.6(a) : Linear relationship between methylene blue dye binding (A650) and
rat wound fibroblast cell number. The results are expressed as the
mean of six replicates ± standard deviation.

$$y = 0.23568 + 1.1634e{-}2x$$

$$R = 0.993$$

No of cells seeded/well
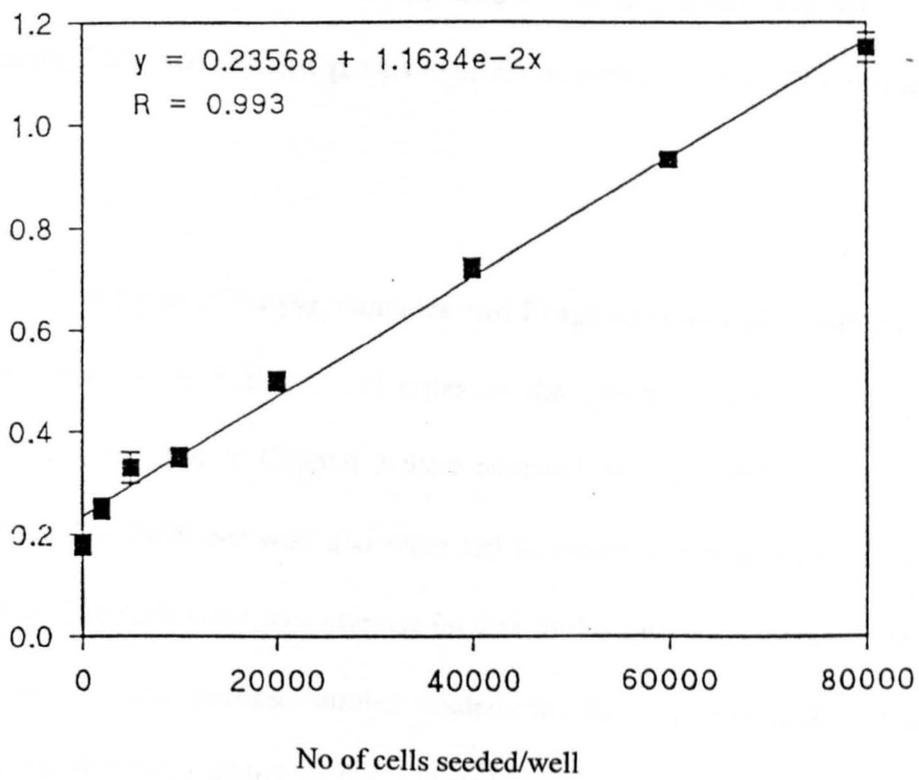
Figure 4.6(b) : Linear relationship between methylene blue dye binding (A650) and human dermal fibroblast cell number. The results are expressed as the mean of six replicates ± standard deviation.

The standard curves for the various cell types indicated the variation in absorbance values between different cell types, for example, at a cell concentration of 5×10^4 cells per well, the absorbance value at A_{650} for L929 fibroblasts was 1.04, whereas 3T3 fibroblasts and BAEC endothelial cells had absorbances of 0.88 and 0.75 respectively. The difference in absorbance between different cell types is therefore due to variations in the amount of dye binding to the cells, and it can be concluded from this observation that different cell types have varying amounts of negatively charged groups such as phosphate, DNA and charged groups in proteins, within the cell (Oliver et al, 1989).

4.4   Effects of Polysaccharides and Fragments on Cell Proliferation

The proliferation of different cell types in the presence of various polysaccharides prepared as described in Chapter 3 were assessed by the methylene blue assay. Cells were seeded at 5000 per well and were left to attach overnight in DMEM containing 10% FCS. The cells were then starved for 24h in the absence of serum to increase their sensitivity to the polysaccharides added in the experimental procedure. The polysaccharides were added to the cultured cells at various concentrations and were diluted using 2% FCS/DMEM (see section 2.4.3).

4.5   Heparin

Heparin is an anionic polysaccharide predominantly containing the repeating unit L-iduronic acid, although D-glucuronic acid is present as a minor constituent, and it is linked to the N-sulphated hexosamine by a ß(1,4) linkage. Heparin has previously been

shown to inhibit the proliferation of several cell types in culture, including human lung and dermal fibroblasts (Ferrao & Mason, 1993; Westergren-Thorsson et al, 1991), vascular smooth muscle cells (Hoover et al, 1980), cervical epithelial cells (Wright et al, 1985) and glomerular mesangial cells (Groggel et al, 1990), and has also been shown to stimulate granulation tissue formation in vivo (McPherson et al, 1988). Heparin was, therefore, used in this study as a control to which the other polysaccharides could be compared, as any similarity to the effect of heparin in the in vitro situation may give an insight to the potential of the polysaccharide digests prepared in chapter 3 to effect wound healing in vivo.

4.5.1   The Effect of Heparin on Cell Proliferation

Six concentrations of heparin between 0.01 - 500μg/ml were examined for their effect on proliferation over a 5 day incubation period, and were tested on L929 fibroblasts, bovine aortic endothelial cells (BAEC) and 3T3 fibroblasts. Previous evidence on the effect on heparin on the proliferation of human lung and dermal fibroblasts demonstrated a significant inhibition (Westergren-Thorsson et al, 1991; Ferrao & Mason, 1993). In this study, both L929 and 3T3 fibroblast proliferation were also significantly inhibited by the addition of increasing concentrations of heparin (Figs 4.7a & 4.7b). The inhibitory response seen in this study by the addition of heparin was maximal at the highest concentration of heparin (500μg/ml) in both cell lines, however, significant inhibition was also seen to a lesser extent at 10 and 100μg/ml. Proliferation was inhibited by 45% and 57% respectively for L929 and 3T3 fibroblasts, therefore, heparin had varying effects on different cell lines. The difference in the degree of
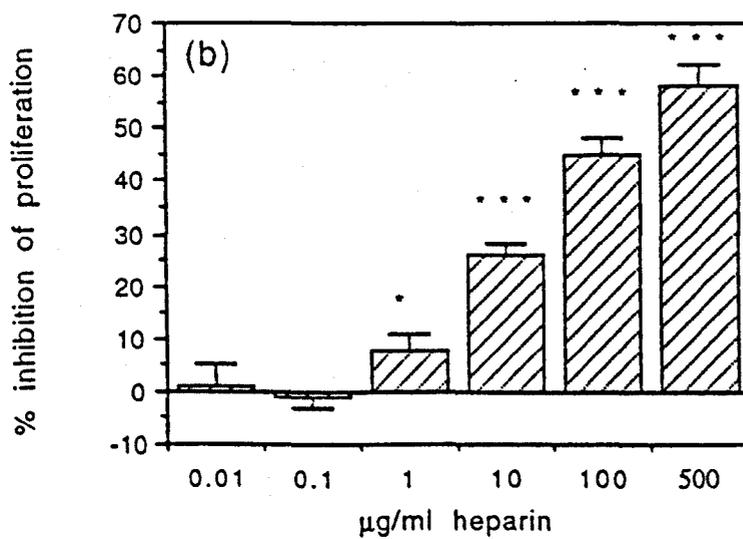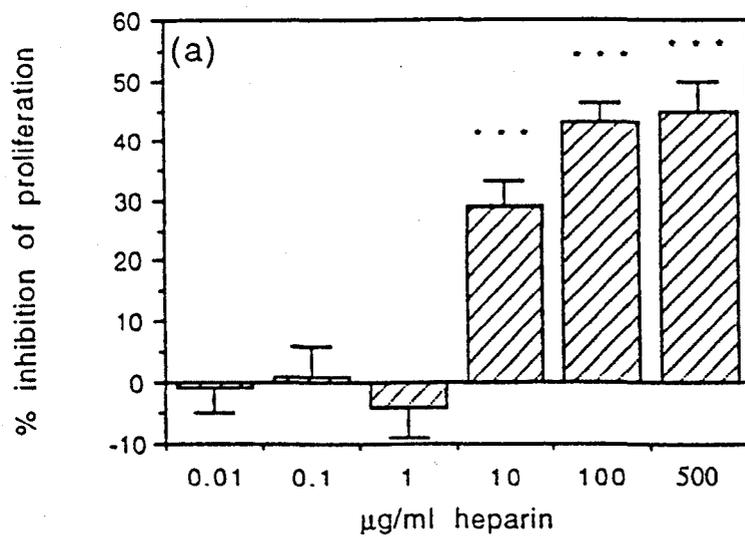
Figure 4.7 : The effect of heparin on cell proliferation. Data are the mean of six replicates at each concentration ± SEM.(a) Inhibition of proliferation of L929 fibroblasts (b) Inhibition of proliferation of 3T3 fibroblasts. Significant results are indicated by $^*P < 0.05$ and $^{***}P < 0.001$.

inhibition may be due to the presence of more heparin receptors on 3T3 cells. BAEC cells however, were unaffected by the addition of increasing concentrations of heparin (Personal communication - E. Lorimer) and suggests two possibilities (i) that heparin can be taken up by the cell and processed without causing an effect on the growth characteristics of the cell or, (ii) that no internalisation of heparin is taking place at all.

4.6　　　Effect of HA And Its Oligosaccharides on Cell Proliferation

The next polysaccharide to be investigated was HA and its fragments. Previous evidence claimed that certain sized fragments of HA stimulated the proliferation of BAEC cells and induced angiogenesis in the chick chorioallantoic membrane (West & Kumar, 1989 ; West *et al*, 1985). This study focused on the effect of crude HA digests on the proliferation of various cell types in an attempt to correlate the results with previous findings and provide new evidence for the effect of HA fragments on other cell sources.

HA digests (undigested, 5mins and 24h) were prepared and characterised as described in Chapter 3 and were tested for their effects on the proliferation of various cell types. Undigested HA isolated from umbilical cord, has been shown previously to have no effect on the proliferation of human dermal fibroblasts (Ferrao & Mason, 1993). Similar effects were seen in this study with HA (MW 828kDa) from Lifecore Inc., for dermal and also L929 fibroblasts (Figs 4.8a & 4.9). BAEC cells however demonstrated stimulation of proliferation ($p < 0.05$) after 2 and 3 days incubation at 100 µg/ml undigested HA (Fig 4.8b). In order to confirm this result, a further experiment was
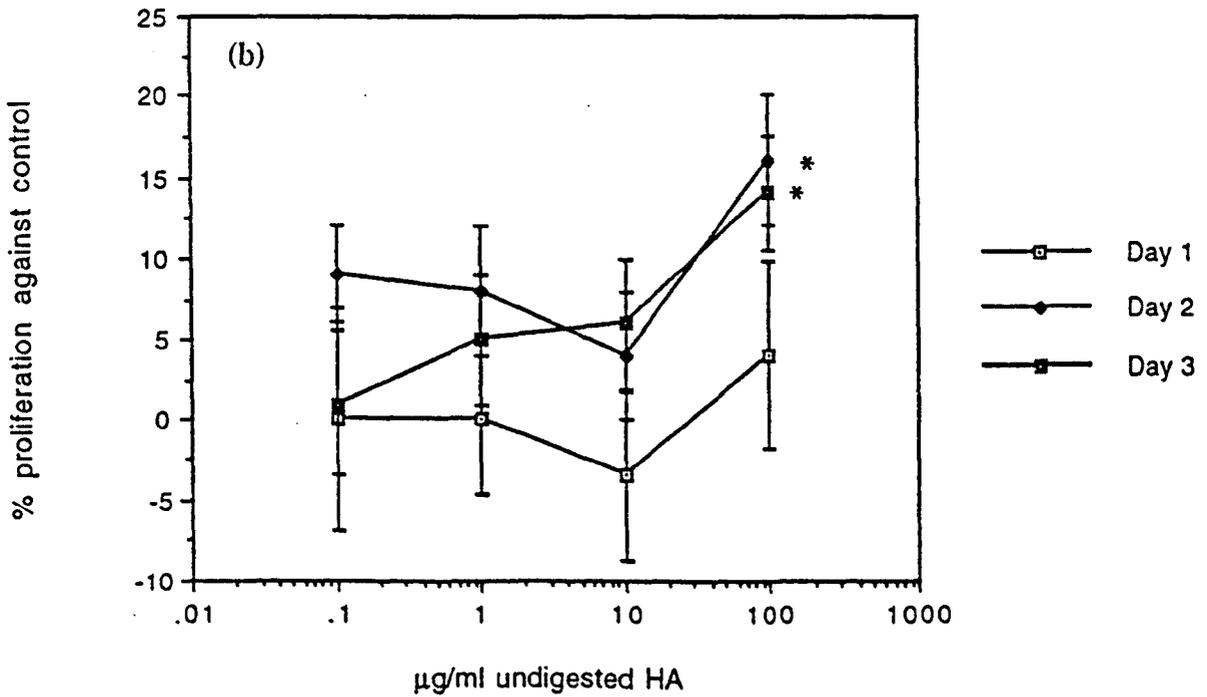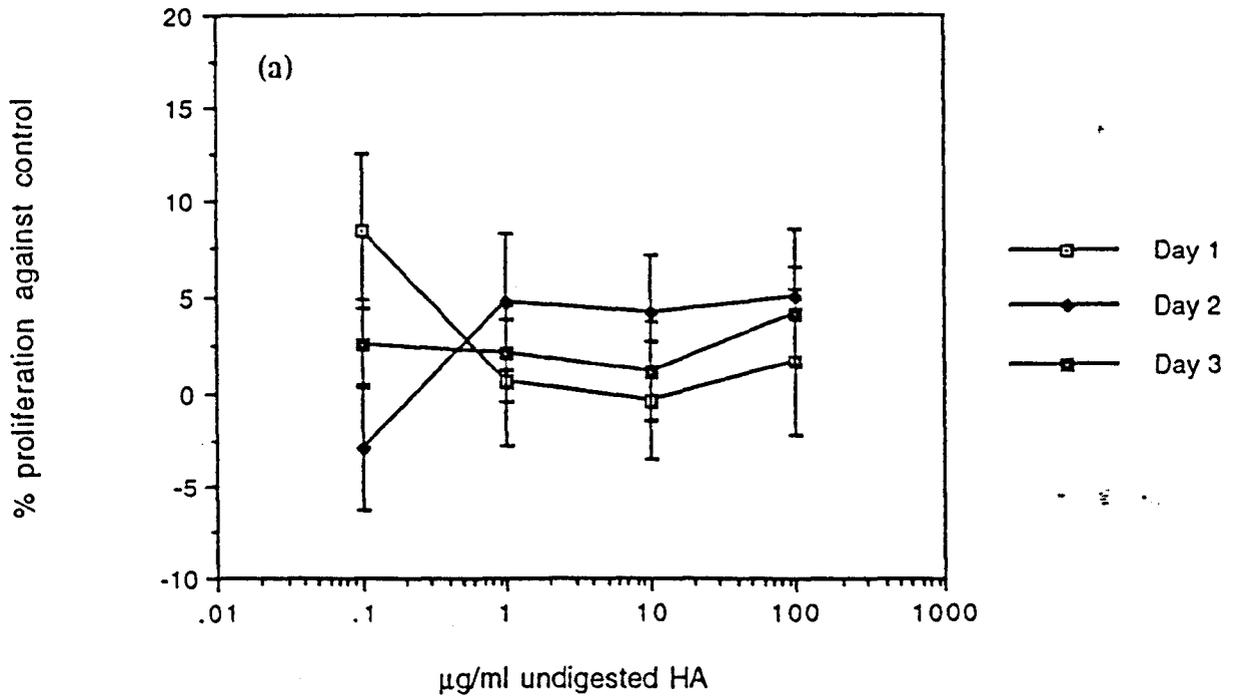
Figure 4.8 : The effect of increasing concentrations of undigested HA on cell proliferation. The data were the mean of 12 analyses at each concentration ± SEM. (a) L929 fibroblasts (b) Bovine aortic endothelial cells. Significant differences are indicated by *P < 0.05
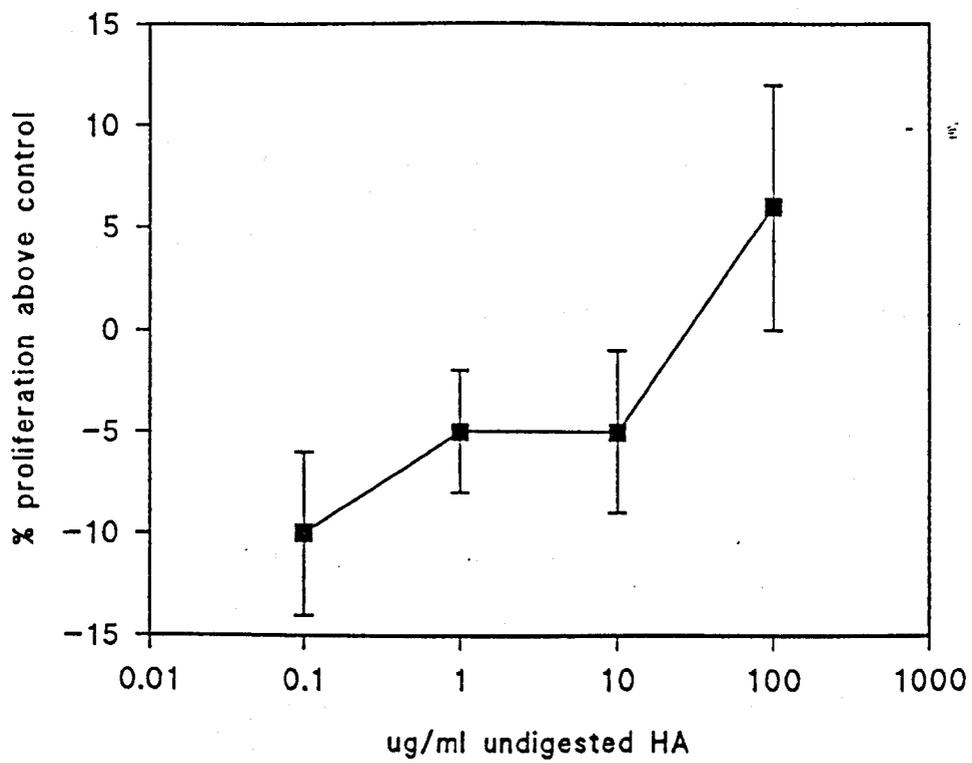
Figure 4.9   The effect of increasing concentrations of undigested HA on human dermal fibroblast proliferation. The data were the mean of 6 analyses at each concentration ± SEM.

undertaken using a reduced level of serum (0.4%) to eliminate firstly the possibility of

stimulation of BAEC cells being due to the presence of serum and secondly to increase

the sensitivity of the cells to the added polysaccharide. The results indicated (Fig 4.10)

that at day 1 & 2, significant stimulation was apparent at 100 μg/ml as seen in the

original higher serum experiment and, therefore, supports the previous finding.

Stimulation appeared later in the high serum experiment and this may be due to factors

present in the serum causing a lag in the proliferation which is overcome by the second

day. The lower concentrations of HA in both experiments, however, had no significant

effect on proliferation. Future studies would be worthwhile to investigate the effect of

concentrations higher than 100 μg/ml to see if the response seen in this experiment was

the optimum to be obtained'. Higher concentrations of HA, however, have been found

to cause high background readings for the methylene blue assay due to non-specific

binding of the polysaccharide to tissue culture plate (personal communication,

E.Lorimer). The problem of background staining when using concentrations of HA over

1mg/ml however, could be overcome by carrying out a different cell counting method

such as one of those described in Section 4.2.

Two other HA digests (5 mins and 24h) were also prepared and characterised, as

described in Chapter 3, and were tested for their effect on cell proliferation. These

digests were chosen as they were within the same molecular weight range as those

investigated by West & Kumar (1989). Both HA (5 mins) and HA (24h) samples had no

significant effect on the proliferation of L929 fibroblasts, but the HA (5 min) digest did

however stimulate the proliferation of both human dermal fibroblasts and BAEC cells

(Figs 4.11b & 4.13a). HA (5min digest) had the greatest effect on the proliferation of

human dermal fibroblasts at day 3 of incubation, where stimulation was seen at 1, 10 and 100µg/ml (Fig 4.13a). BAEC also showed a stimulation of proliferation at day 1 but only at a concentration of 100µg/ml (Fig 4.11b). The 24h digest, however, had no effect on human dermal fibroblasts or BAEC cells (Figs 4.12b & 4.13b). Future work could involve investigation of higher concentrations of HA digests to determine if increasing the dose of HA causes an increase in the proliferation of the cells. Interestingly, there are apparent differences in the ability of HA (5 mins) to stimulate the proliferation of fibroblasts from different sources, whereas undigested HA exhibited no effect on either L929 or human dermal fibroblasts. Differences in stimulatory activity on cells isolated from different sources may be due to the number of specific receptors on the surface of the cells (Culty *et al*, 1992). Differences in receptor number has been demonstrated by actively proliferating 3T3 cells in culture which have been shown to contain HA receptors, however, other cells such as corneal and stomach epithelial cells notably lack HA receptors (Alho & Underhill, 1989). HA is known to have a six sugar sequence which binds to the HA receptor on the surface of the cell (Underhill & Toole, 1979; Alho & Underhill, 1989), therefore, this suggests that L929 cells may lack HA receptors, or that those present may be of low affinity. A lack of HA receptors on the cell surface would stop the entrance of HA into the cell and, therefore, prevent any intracellular effect, however on the contrary, the receptors may actually be present on the surface but the endocytosis and subsequent processing of HA may have no effect on the proliferation of the cell.

In summary, the results obtained in this study demonstrate that HA digested for 5 mins has the ability to stimulate the proliferation of both BAEC and human dermal
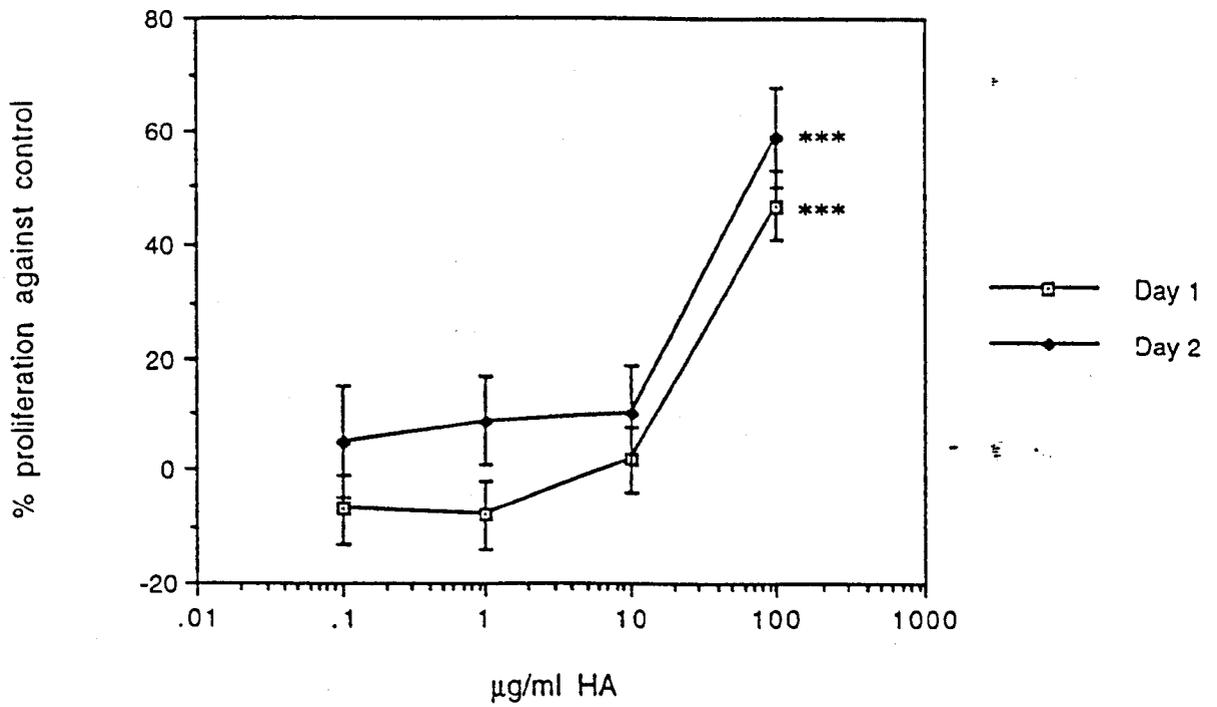
Figure 4.10   The effect of undigested HA on the proliferation of bovine aortic endothelial cells incubated in low serum conditions (0.4%). Significant differences are shown by ***P < 0.001
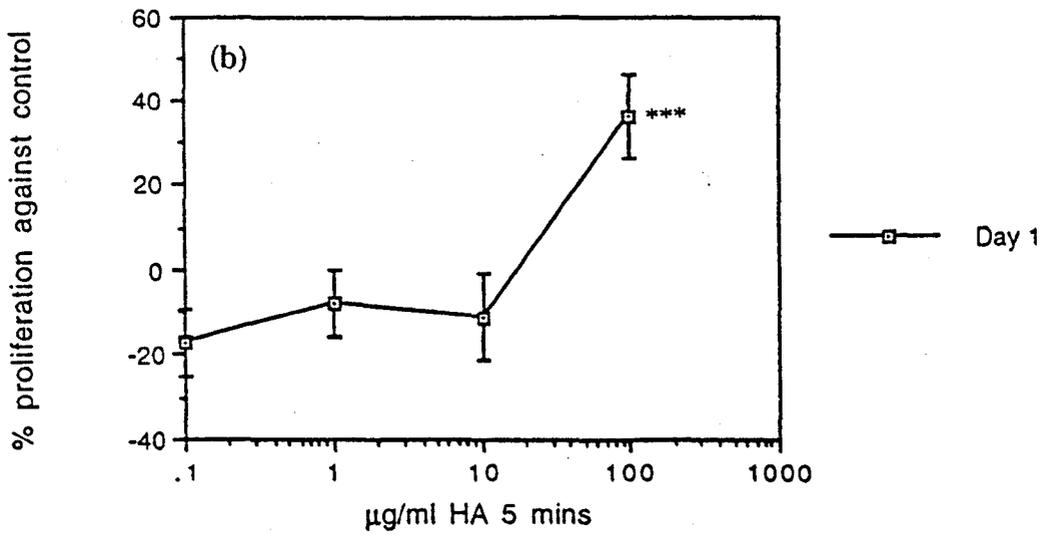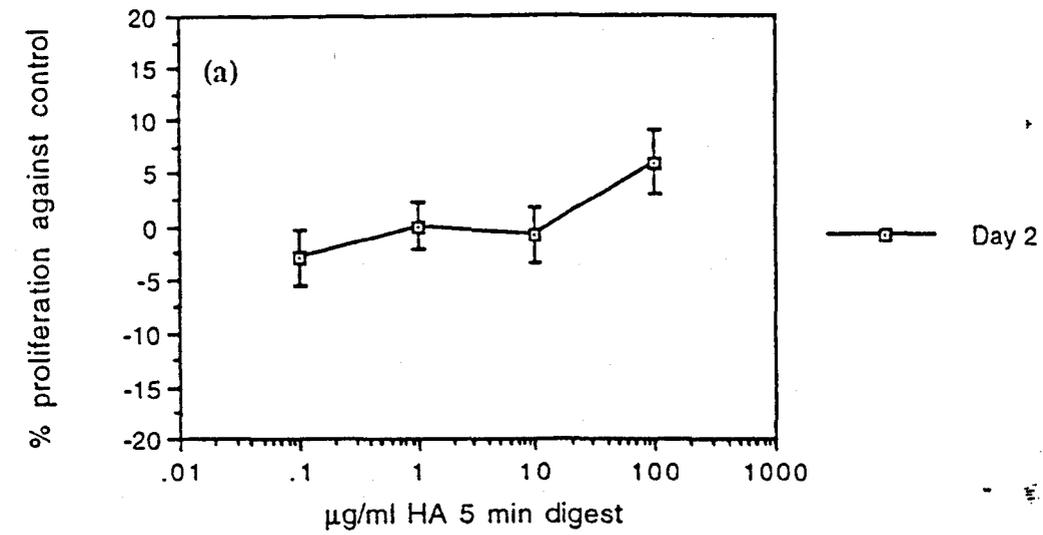
Figure 4.11   The effect of increasing concentrations of HA (5 min) digest on cell proliferation. The data were the mean of 12 analyses at each concentration. (a) L929 fibroblasts incubated for 2 days (b) Bovine aortic endothelial cells incubated for 1 day.
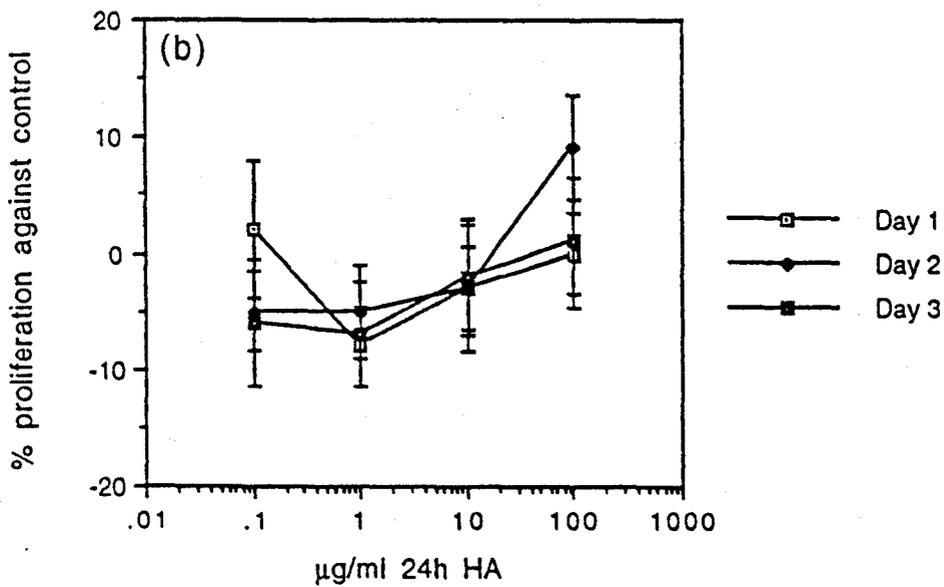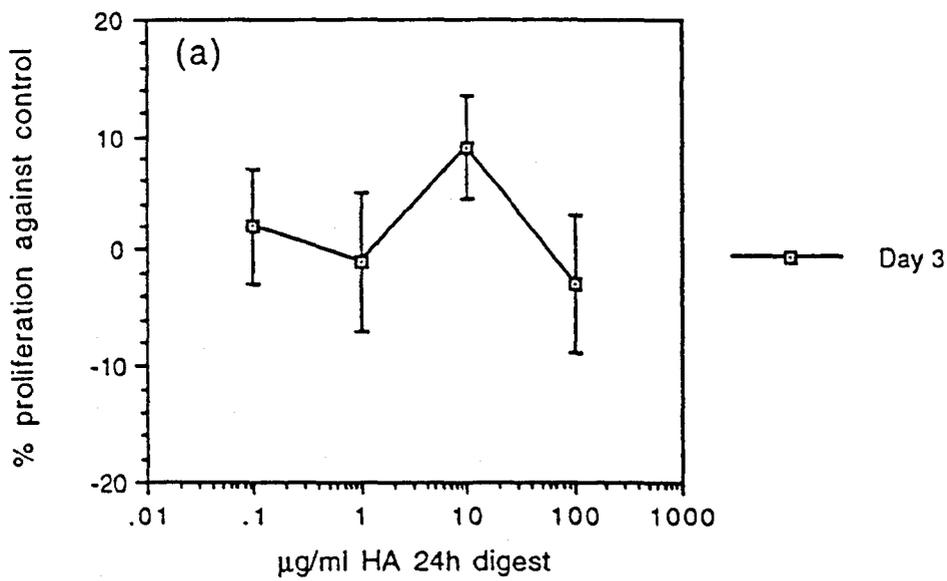
Figure 4.12   The effect of increasing concentrations of HA (24h digest) on the proliferation of (a) L929 fibroblasts incubated for 3 days and (b) BAEC endothelial cells incubated for 1,2 & 3 days. Results are the means ± SEM of 12 analyses.
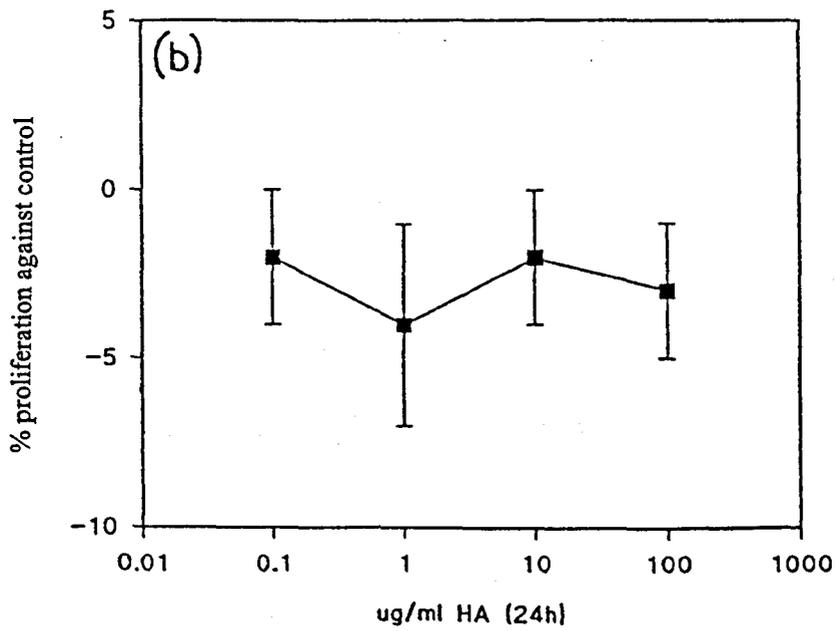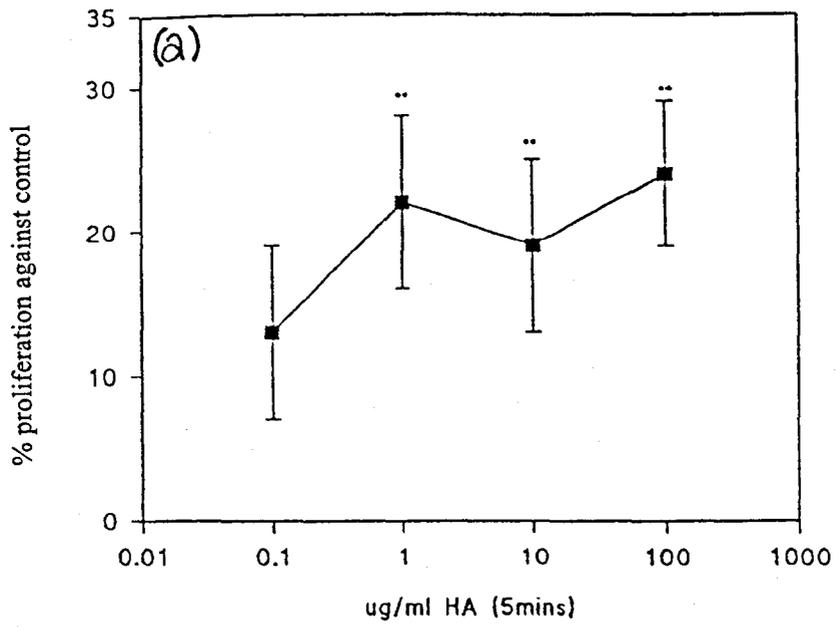
Figure 4.13 : The effect of increasing concentrations of HA digests on human dermal fibroblast proliferation. (a) HA (5 min) fragment and (b) HA (24h) fragment. The data were the mean of 6 analyses at each concentration ± SEM. Significant differences are indicated by **P< 0.01

fibroblasts whereas undigested HA was shown to stimulate BAEC proliferation only.

West & Kumar (1989) demonstrated that specific size ranges of HA oligosaccharides had an *in vitro* effect also. Their study however used three fractions of HA containing >16 disaccharides (F1), 10-16 disaccharides (F2) and 3-10 disaccharides (F3). The results obtained in their study can be compared to the results mentioned above. BAEC cells were stimulated in this study by the 5 min digest of HA and in West & Kumar's study, the F2 & F3 both had the same effect. HA oligosaccharides between 4 and 25 disaccharides in length, have also been shown *in vivo* to stimulate angiogenesis in the chick chorioallantoic membrane (West *et al*, 1985). The size range of F3 and HA (5 min) overlap, therefore, the two results are comparable. HA (5 mins) also stimulated human dermal fibroblasts to proliferate, however F3 in the West & Kumar paper did not have any stimulatory effects on any other cells tested, including fibroblasts. They also found undigested HA to have no effect on fibroblast proliferation as shown also in this study, however, at concentrations greater than 200 ug/ml, they found undigested HA to inhibit BAEC proliferation whereas BAEC were stimulated at 100 ug/ml in this study. Further studies using higher concentrations of undigested HA would need to be carried out in order to determine if BAEC cells would be inhibited at that level as described by West & Kumar.

4.7   The Effect of Chondroitin Sulphate & Its Oligosaccharides on Cell Proliferation

Chondroitin sulphate (CS) has a very similar structure to that of HA (Chapter 1 , Fig

1.1) and its role in wound healing has been previously documented (LeBoeuf et al, 1987; Westergren-Thorsson et al, 1991. The involvement of CS in the wound healing response and its structural similarities to HA led to the decision to depolymerise the polysaccharide to investigate the effects of small CS oligosaccharides on wound healing.

Three different size ranges of CS were prepared and characterised as described in Chapter 3. The fractions were: undigested CS and CS digests (hyaluronidase digested for 5 mins and 24h). This produced oligosaccharides in the range of 40-80 kDa, 4kDa and 2 kDa.

Undigested CS had no effect on L929 fibroblasts or BAEC cells at 0.1-100 µg/ml (Fig 4.14), but demonstrated a significant stimulation of proliferation of human dermal fibroblasts ($p<0.05$) at concentrations of 10 and 100µg/ml after incubation for 48h (Fig 4.15). The stimulation of human dermal fibroblast proliferation by undigested CS, compares with that found with HA (5mins), and indicates that a specific size range of oligosaccharides may be present in both undigested CS and HA (5 min) digest which promotes the stimulation of dermal fibroblasts. Further analysis of the specific size ranges was not attempted as material was in short supply and time was limited.

Hyaluronidase digested 100 ug/ml CS (5mins and 24h) also stimulated the proliferation of L929 cells at Day 2 to 56% and 30% above control values respectively (Figs 4.16 & 4.18), however, higher concentrations of each digest should be investigated in future work in order to determine if a dose response effect is apparent. Human dermal fibroblasts were stimulated to proliferate by CS (5min digest) at all concentrations
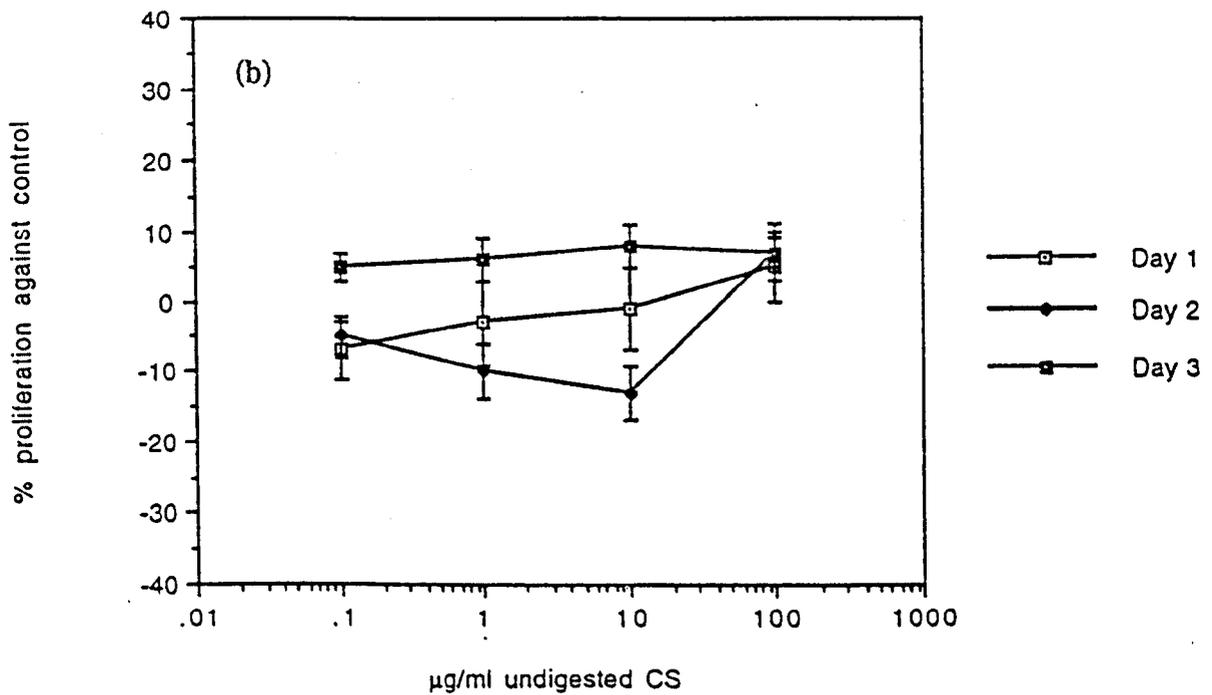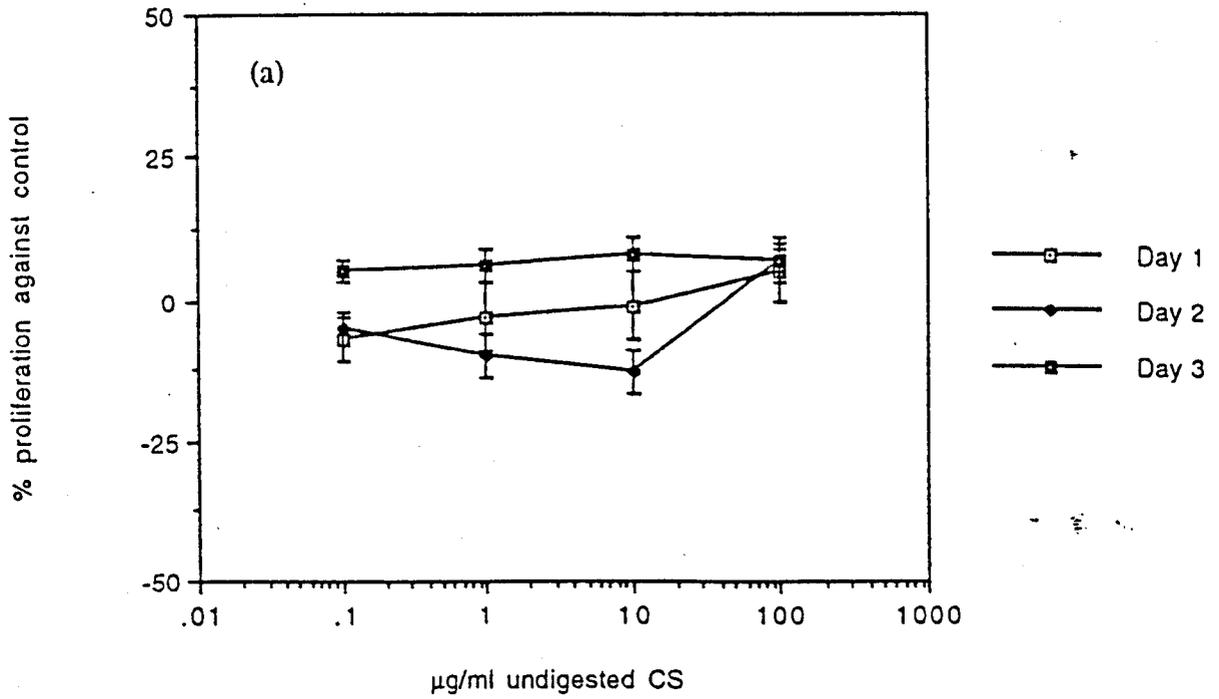
Figure 4.14   The effect of increasing concentrations of undigested
chondroitin sulphate on cell proliferation over a 72h incubation
period. The data are the mean of 6 replicates for each
concentration. (a) L929 fibroblasts (b) BAEC endothelial cells.
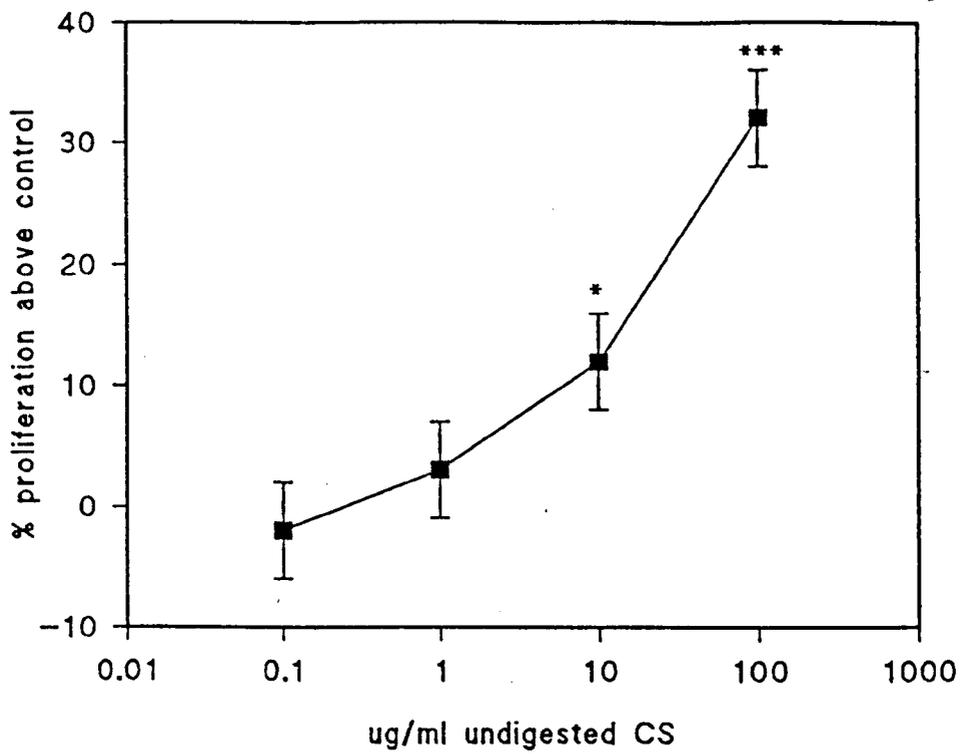
Figure 4.15   The effect of increasing concentrations of undigested CS on human
                     dermal fibroblast proliferation. The data were the mean of 6 analyses
                     at each concentration ± SEM. Significance levels are indicated by
                     *p<0.05 and ***p<0.001
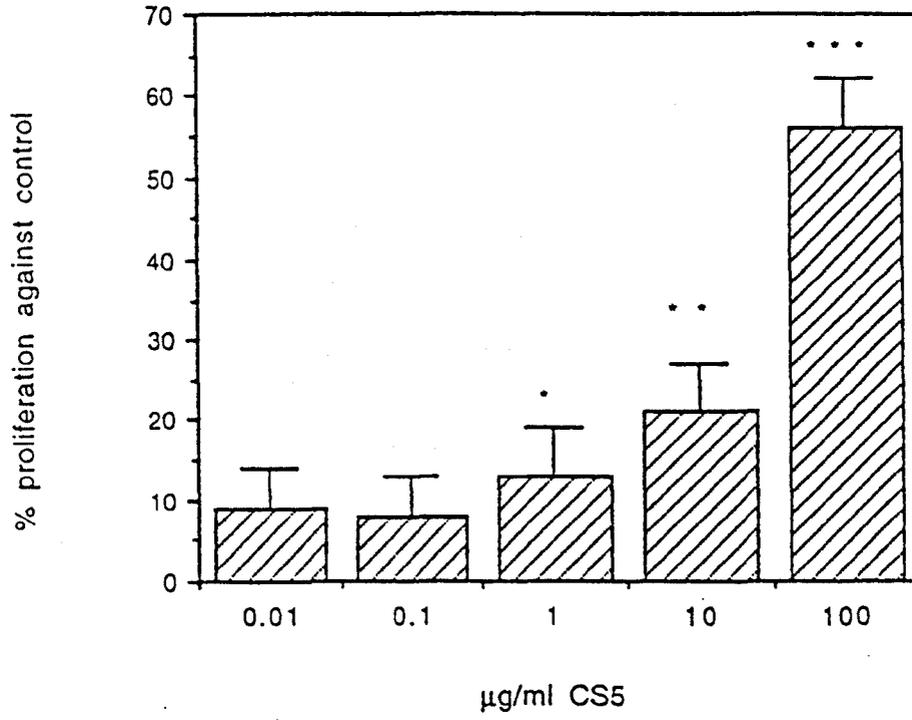
Figure 4.16   The effect of increasing concentrations of CS (5min) digest on the proliferation of L929 fibroblasts. The data are the mean of 12 analyses at each concentration. Significant differences are indicated by *P < 0.05, **P < 0.01 and ***P < 0.001.
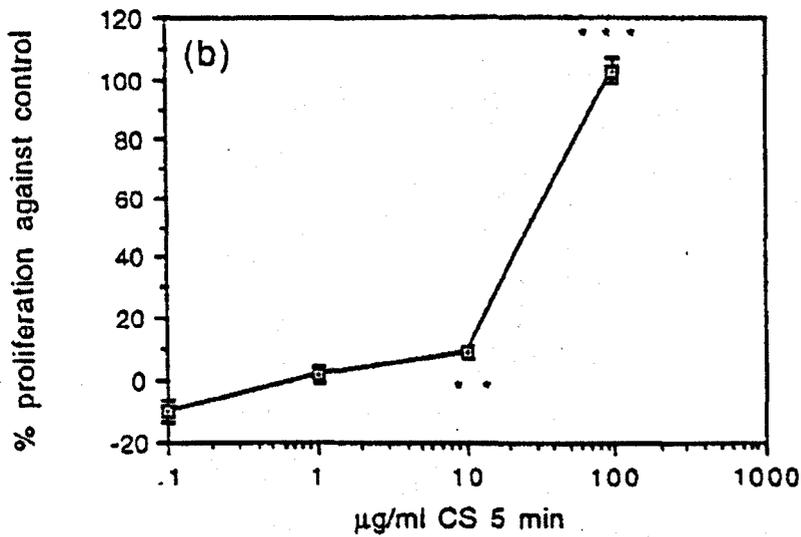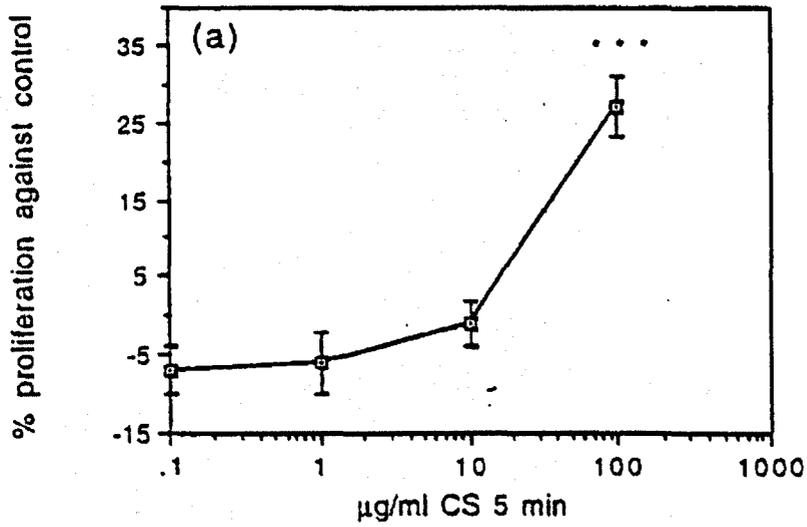
Figure 4.17   The effect of increasing concentrations of CS (5min) digest on the proliferation of BAEC cells. (a) CS (5min) digest added to 2% serum (b) CS (5min) digest added to 0.4% serum. Results are the mean of six analyses at each concentration ± SEM. Significant differences are indicated by **P < 0.01 and ***P < 0.001.
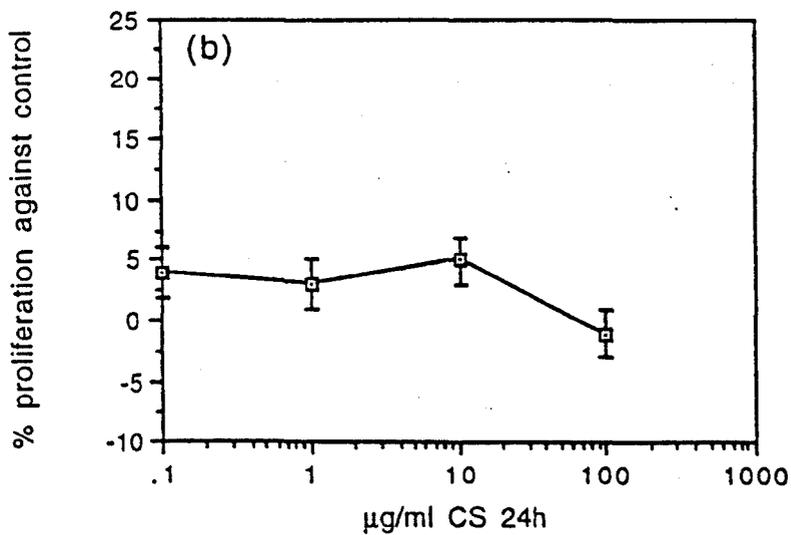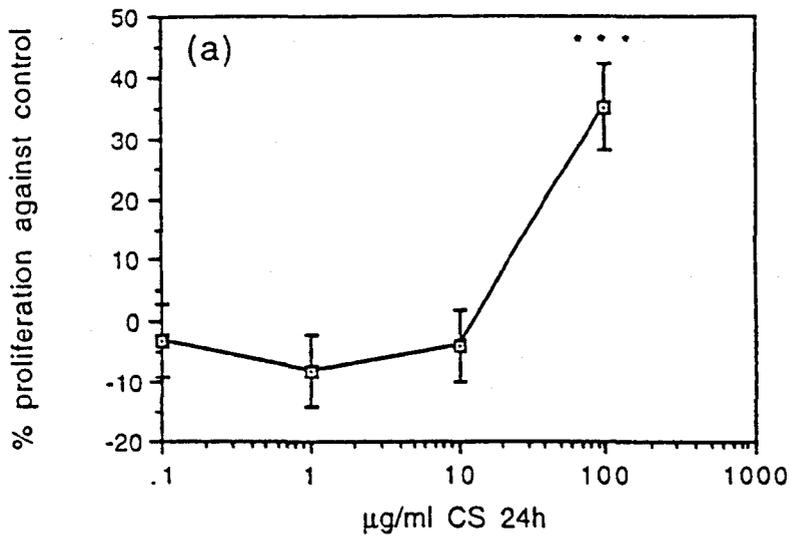
Figure 4.18   The effect of increasing concentrations of chondroitin sulphate
(24h) digest on cell proliferation. (a) L929 cells cultured in 2%
serum and incubated for 2 days. (b) BAEC cells cultured in
2% serum and cultured for 3 days. Results are the mean of 6
analyses at each concentration ± SEM. Significant differences
are indicated by ***P < 0.001.

tested (0.1-100 µg/ml) and showed an increase in stimulation as the concentration increased (Fig 4.19). BAEC cells were less sensitive to the addition of CS (5mins digest), but did however show stimulation of proliferation at 100 µg/ml (Fig 4.17a). A further experiment was carried out with BAEC and CS (5mins) using a lower concentration of serum (0.4%) in the medium in an attempt to reduce the chance of the serum being the stimulating factor. Reducing the serum concentration in this way actually improved the response of BAEC cells to CS (5 mins) as both the 10 and 100 µg/ml concentrations of CS (5mins) stimulated the cells to proliferate to 9% and 103% above control levels (Fig 4.17b). CS (24h) had no effect on the proliferation of human dermal fibroblasts or BAEC cells (Figs 4.18b & 4.20).

The CS digests results clearly demonstrate a difference in the ability of undigested CS and digested CS samples to affect the proliferation of a range of different cell types. Overall, the CS (5min) digest stimulated the proliferation of all the cells tested and therefore would appear to contain the necessary size range of oligosaccharides for this purpose. Comparing the results of the CS digests with those obtained for HA, it appears that both 5 min digests have best capacity to stimulate the proliferation of cells, however, the HA (5 min) digest had an average MW range of 2 kDa, and the CS (5 min) digest had an average MW range of 4 kDa. Further work would be required to isolate individual oligosaccharides to determine which one was causing the stimulation of cells. Future experiments will be discussed later in the thesis.
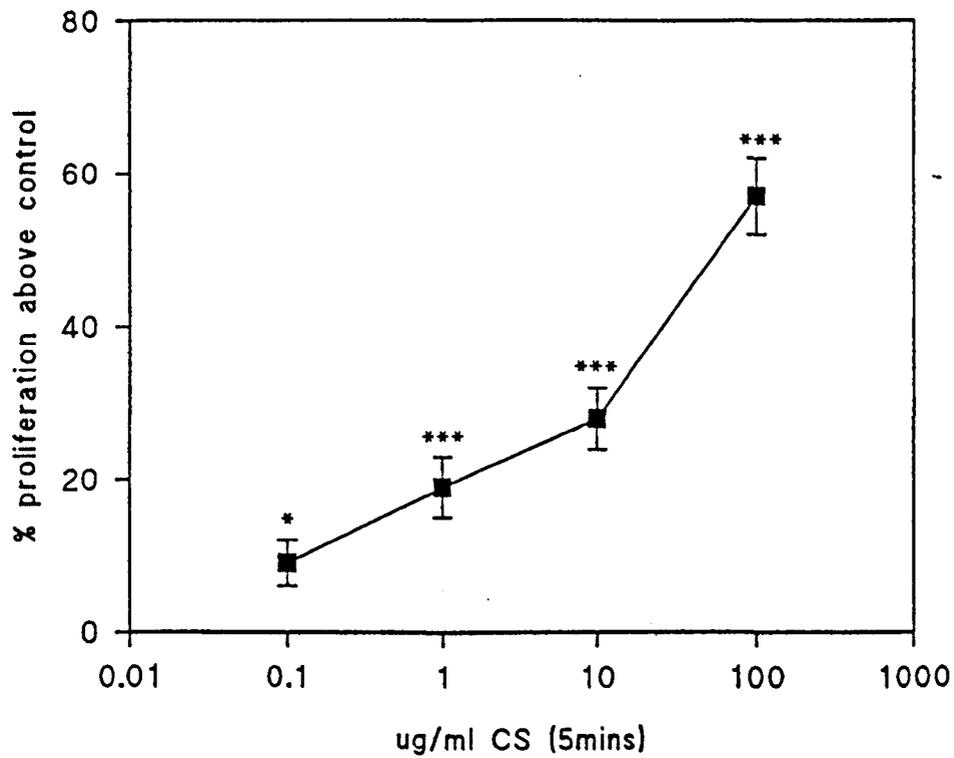
Figure 4.19   The effect of increasing concentrations of CS (5min digest) on human dermal fibroblast proliferation. The data were the mean of 6 analyses at each concentration $\pm$ SEM. Significance levels are indicated by *$p<0.05$ and ***$p<0.001$
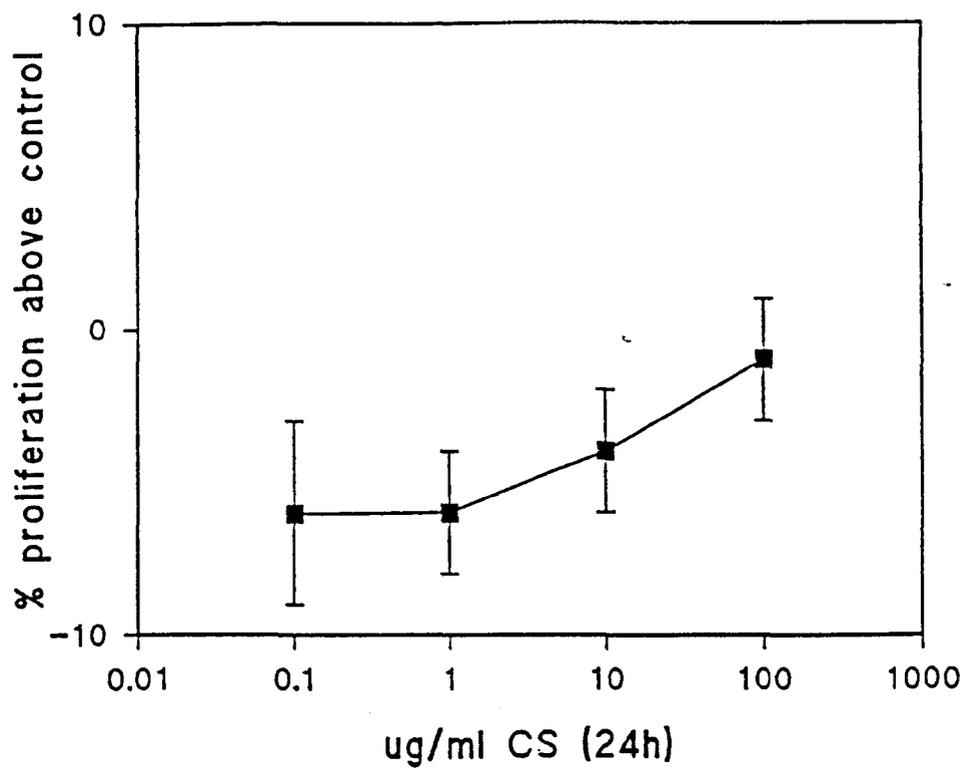
Figure 4.20   The effect of increasing concentrations of CS (24h digest) on human dermal fibroblast proliferation. The data were the mean of 6 analyses at each concentration ± SEM.

4.8   The Effect of Oxidised Regenerated Cellulose Fragments on Cell

Proliferation

Polydisperse ORC fragments (ORC 1 - low molecular weight, (range between 5 and

40kDa) ; ORC 2 - higher molecular weight (average of range 40-500kDa) were

prepared as described in Chapter 3. The effect of six concentrations of each sample

(0.01 - 1000 µg/ml) on the proliferation of a variety of cell types was then measured.

4.8.1   ORC (1) - Low Molecular Weight Fragment Range

As can be seen from Figure 4.21, ORC (1) inhibited the proliferation of both L929

fibroblasts and BAEC cells. The proliferation of L929 fibroblasts was inhibited to a

maximum of 35% at an ORC (1) concentration of 1mg/ml after 24h incubation,

however significant inhibition (25%) was also seen at this time with 100 µg/ml of ORC

(1). Endothelial cell proliferation was also inhibited by the addition of ORC (1) at

concentrations above 10µg/ml. When, however, 3T3 fibroblasts and human dermal

fibroblasts were assessed for their response to the addition of ORC (1), a stimulation

effect was apparent in both cell types. Stimulation was not detected until Day 5 of

culture in the 3T3 cell assay, however, at this time point, both the 10 µg/ml and 100

µg/ml concentrations significantly increased the rate of proliferation (Fig 4.22). All

concentrations of ORC (1) had stimulatory effects on human dermal fibroblasts at Day

1 (Fig 4.23). The results demonstrate that different cell types have different responses to

ORC (1) and furthermore, there are distinct differences in the response of fibroblasts

from different sources.
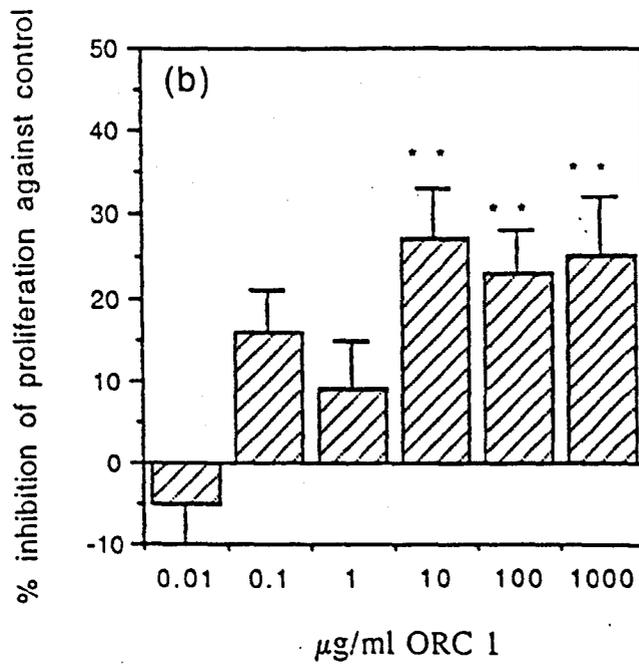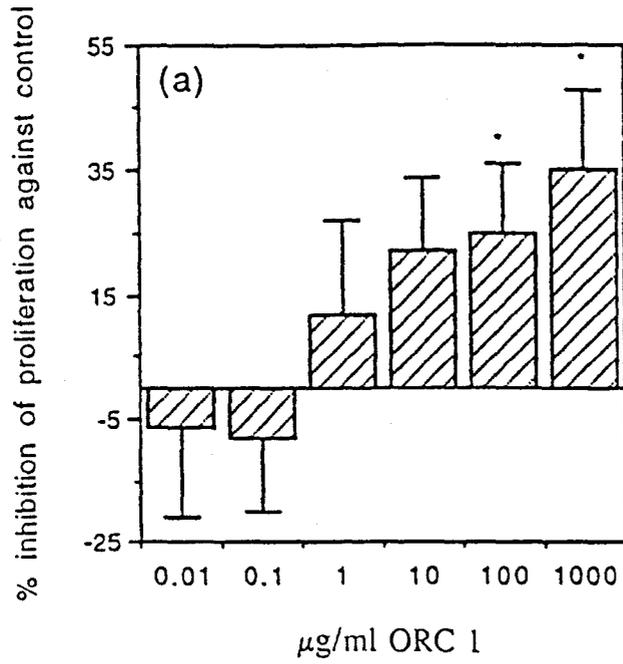
Figure 4.21   The effect of ORC 1 on the proliferation of (a) L929 fibroblasts and (b) BAEC endothelial cells. Results are means ± SEM of six replicates. Significant differences are indicated by *P < 0.05 and **P < 0.01 (analysis by student's unpaired t-test).
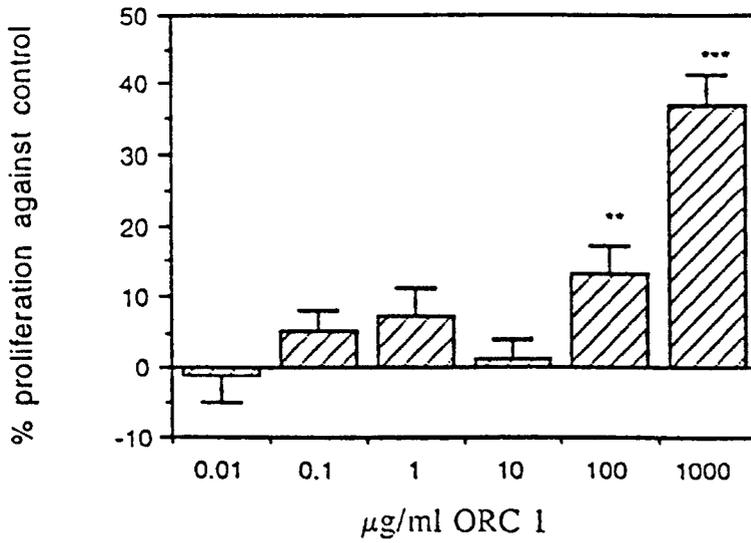
Figure 4.22   The effect of increasing concentrations of ORC 1 on the
proliferation of 3T3 fibroblasts. The data are the mean of six
replicates at each concentration ± SEM. Significant differences
from control are indicated by **P < 0.01 and ***P < 0.001
(analysis by student's unpaired t-test).
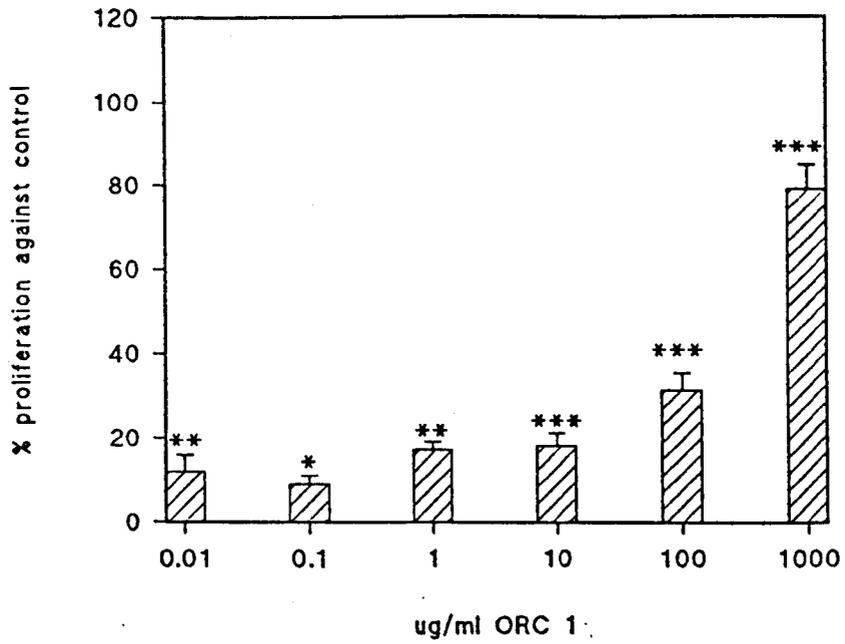
Figure 4.23   The effect of ORC 1 on the proliferation of Human dermal fibroblasts. Results are expressed as means ± SEM of six replicates. Significant differences are indicated by *P < 0.05, **P < 0.01 and ***P < 0.001

4.8.2 ORC (2) - Higher Molecular Weight Fragment Range (40kDa-500kDa)

ORC (2) demonstrated stimulation of proliferation with each cell type tested. At day 5 of incubation, both L929 fibroblasts and BAEC cells were stimulated to proliferate up to 72% above control levels at 1mg/ml ORC (2). A dose response effect was evident for both cell types as indicated by increased stimulation of proliferation as the concentration of ORC 2 increased. The results are shown in Figure 4.24. 3T3 fibroblasts and human dermal fibroblasts both showed stimulation of proliferation (Figs 4.25 & 4.26), however, this was apparent at day 1 which was much earlier than the effect shown by L929 and BAEC cells.

The results from both ORC fragments (Table 4.1) indicate that they have different effects on the proliferation of L929 and BAEC cells, however, both fragments stimulate the proliferation of 3T3 cells and human fibroblasts. It may not be surprising as the two samples contain different fragment sizes, thereby giving rise to differing effects on the proliferation of cells.

ORC 1 and heparin inhibited L929 fibroblast cell proliferation to varying degrees. ORC 1 also inhibited the proliferation of BAEC unlike heparin at similar concentrations.

The two ORC fragments were then used in *in vivo* studies (see Chapter 6). They were chosen specifically because at the time of carrying out the animal work, they were the only samples which had been fully assessed *in vitro* and the results observed *in vitro* had similarities to those shown by heparin, which was also being investigated in the
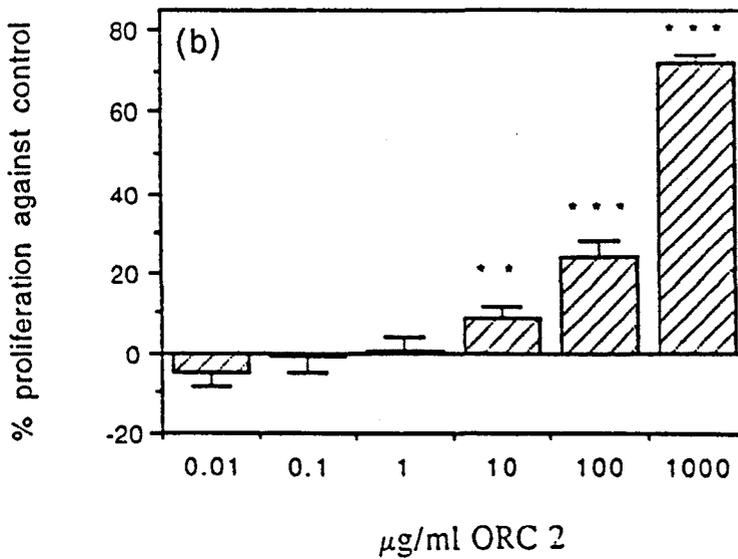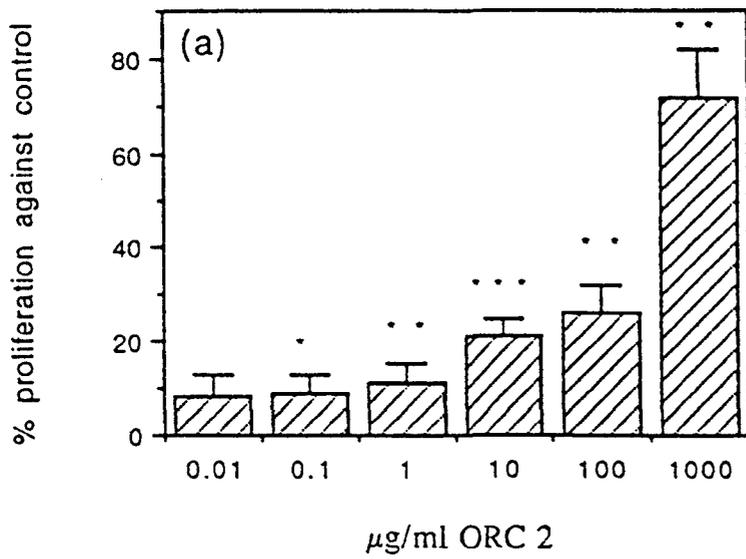
Figure 4.24   The effect of increasing concentrations of ORC 2 on the proliferation of)a) L929 fibroblasts and (b) BAEC endothelial cells. Results are the mean of six replicates ± SEM at each concentration. Significant differences are indicated by *P < 0.05, **P < 0.01 and ***P < 0.001.
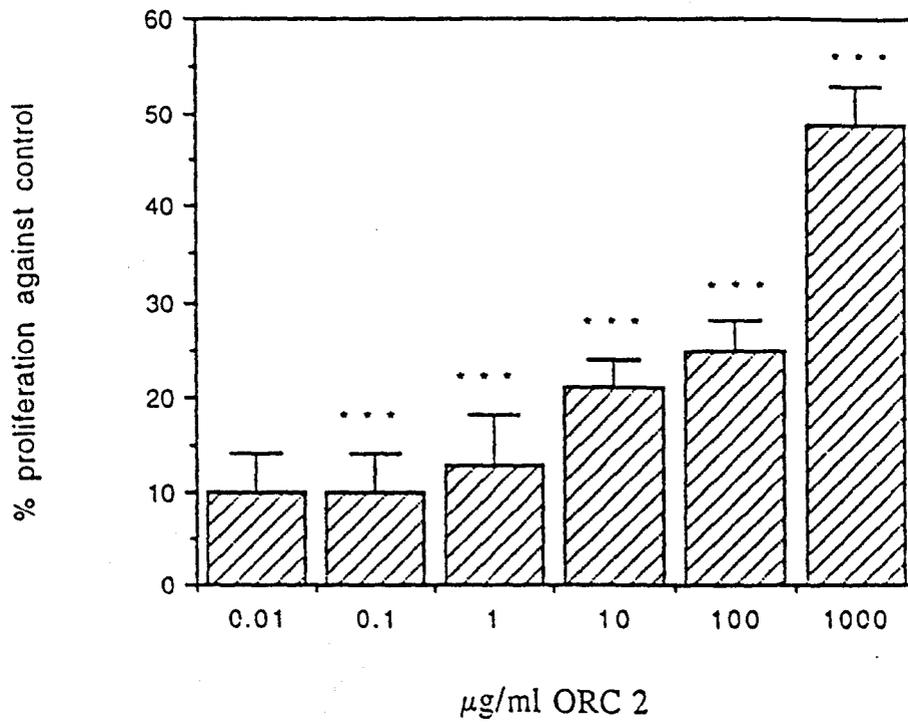
Figure 4.25   The effect of increasing concentrations of ORC 2 on the proliferation of 3T3 fibroblasts. The data are the mean of six replicates at each concentration ± SEM. Significant differences from control are indicated by ***P < 0.001.
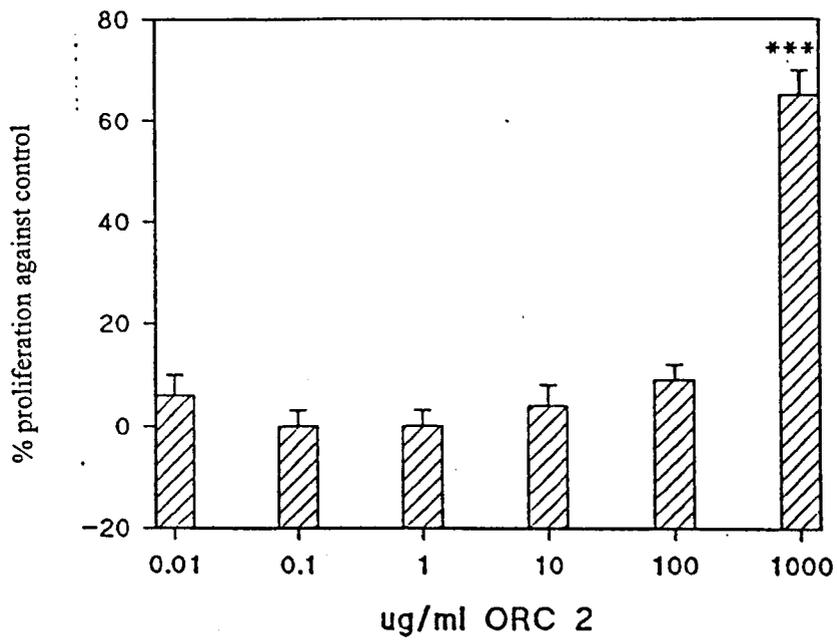
Figure 4.26   The effect of ORC 2 on the proliferation of human dermal fibroblasts. Results are expressed as means ± SEM of six replicates. Significant differences are indicated by ***P < 0.001

	ORC (1)	ORC (2)
L929 Fibroblasts	- 35%	+ 72%
BAEC Endothelial Cells	-26%	+72%
3T3 Fibroblasts	+ 35%	+46%
Human Dermal Fibroblasts	+80%	+65%

Table 4.1 : The effect of ORC (1) and ORC (2) on the proliferation of L929 cells, BAEC cells, 3T3 cells and human dermal fibroblasts. Stimulation of proliferation is indicated by (+), and inhibition by (-).

animal model.

4.9   The Effect of Pectin on Cell Proliferation

The plant polysaccharide, pectin, was also examined for its effect on cellular proliferation. Pectin was selected for testing due to the structural similarities it possesses with other polysaccharides such as the GAGs and ORC (see Chapter 1, Figure 1.3c; section 3.9.4) as well as it being cheap, which is commercially attractive. In this study due to time constraints and availability of cells, only L929 fibroblasts and BAEC cells were tested.

Concentrations of pectin between 1-1000 µg/ml were tested for their effect on L929 fibroblast proliferation. The data shows (Fig 4.27a) that only 1000 µg/ml pectin stimulated the proliferation of L929 fibroblasts (10% above control level). Higher concentrations of pectin, however, were found to gel when the fixative was added at the end of the incubation time. To overcome this problem, an experiment was carried out using other fixatives to observe if the gelation was due to the methanol alone. Table 4.2 illustrates the different fixatives which were employed and the results that followed. The conclusions from this experiment were that 1% Glutaraldehyde in HBSS should be used in future studies to fix pectin samples as addition of it caused no gelation of the polysaccharide. When BAEC cells were assayed, stimulation of proliferation was evident at day 1, however, significant stimulation was only apparent at the 1000 µg/ml concentration at 17% above control levels. The data is shown in Figure 4.27b.
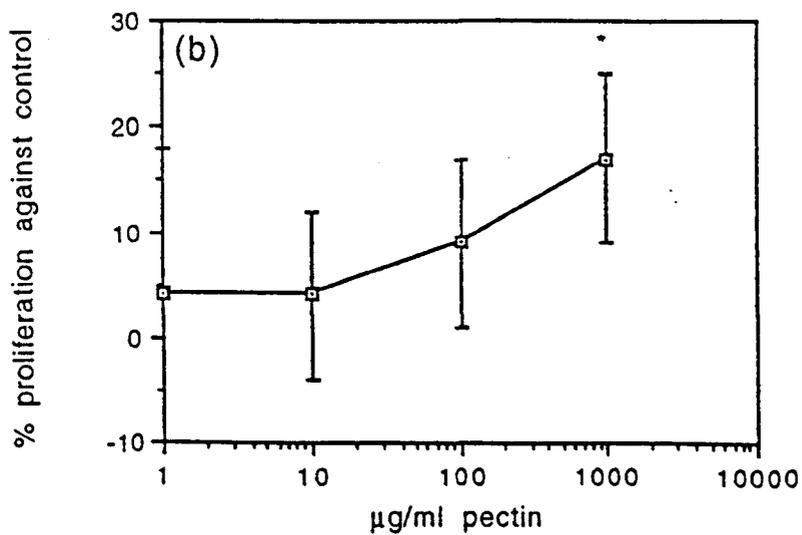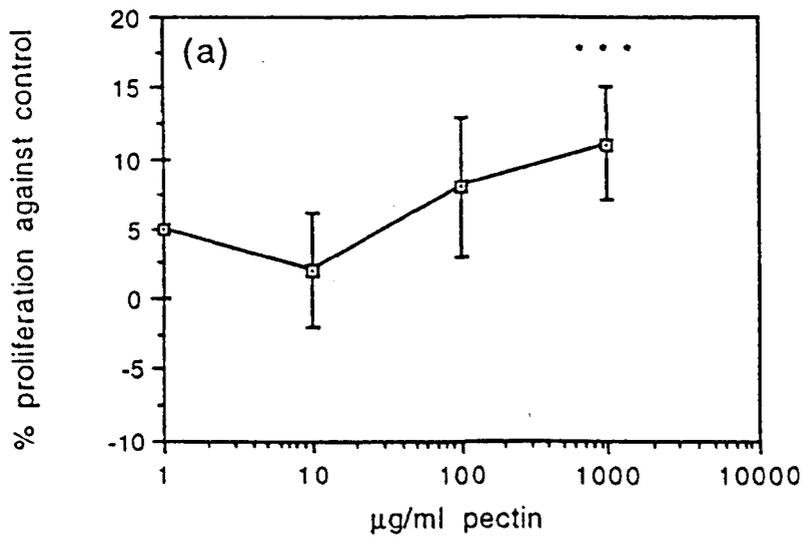
Figure 4.27   The effect of increasing concentrations of pectin on the proliferation of (a) L929 fibroblasts incubated for 1 day and (b) BAEC endothelail cells incubated for 2 days. Data are the mean ± SEM of six replicates. Significant differences are indicated by *P < 0.05 and ***P < 0.001.

FIXATIVE	EFFECT ON PECTIN
Methanol	Gel formation
1% Glutaraldehyde in HBSS	No gel formation
1% Formaldehyde + 1% $CaCl_2$ in water	Gel formation

Table 4.2 :   Effect of different fixatives on pectin gelation

The results indicate that only high concentrations of pectin used in this study had any effect on cellular proliferation. The results also show a similarity to those shown by ORC (2) with L929 and BAEC cells.

Pectin is a relatively cheap and easily produced polysaccharide and, therefore, the need for high concentrations to have any possible effect in an *in vivo* wound healing situation may not be a particular financial disadvantage. Further studies, however, were carried out on pectin fragments to discover if they had any effect on cell proliferation as seen with other polysaccharide fragments

4.10 The Effect of Degraded Pectin on Cell Proliferation

Degraded pectin, produced by alkali hydrolysis (see section 2.1.6) as previously described in Chapter 3, was tested for its effect on the proliferation of L929 fibroblasts and BAEC endothelial cells. Over a concentration range of 1-1000 µg/ml, no significant effect on proliferation was measured in these cell types. The results shown by degraded pectin (Fig 4.28) indicated that breakdown of the molecule resulted in the loss of its effect on proliferation of cells. This effect may be caused by the molecule having a different conformation to the undigested pectin after treatment with alkali, or perhaps the receptors on the cell surface do not recognise the pectin fragments.

4.11 Discussion

Due to the known *in vivo* effects (McPherson *et al*, 1988) and *in vitro* effects of heparin demonstrated in this study, it was used as a comparator to which all the polysaccharide digests were compared (section 4.5). Comparing the proliferation results of heparin to
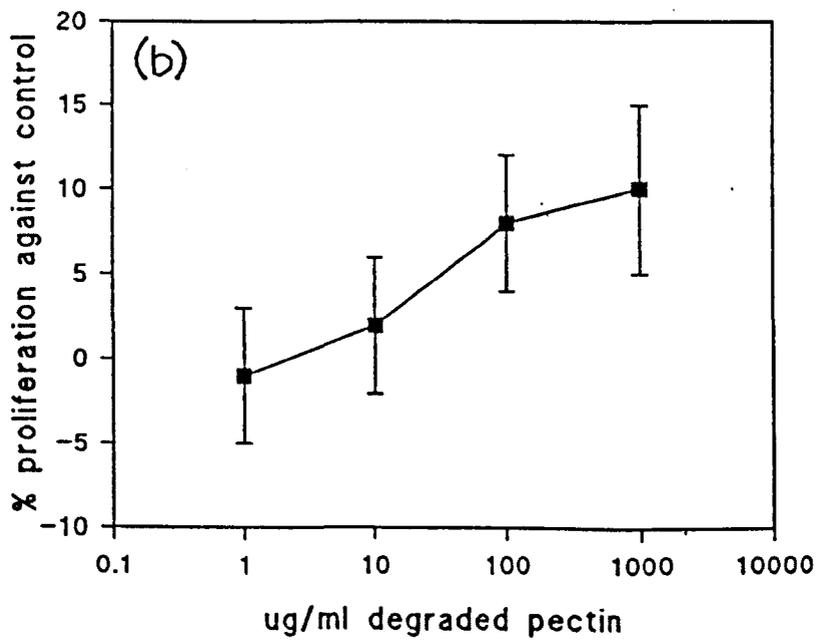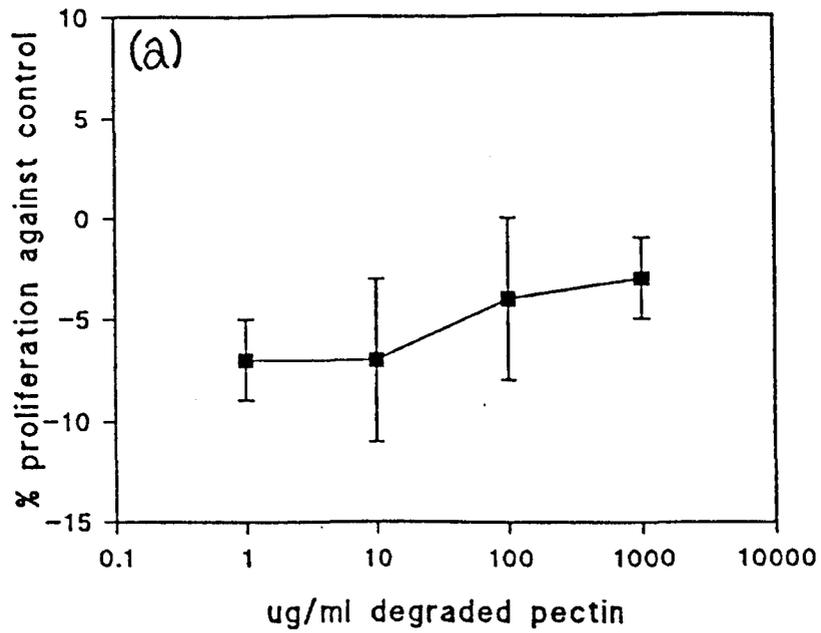
Figure 4.28   The effect of increasing concentrations of degraded pectin on the proliferation of (a) L929 fibroblasts and (b) BAEC endothelial cells. The results are expressed as the mean ± SEM of six replicates.

the other polysaccharides tested, only ORC (1) showed similar characteristics, as it inhibited L929 cell proliferation, however ORC (1) stimulated 3T3 cells unlike heparin. Previous evidence from West & Kumar (1989) suggests that inhibition can be due to the size of the molecule. They found that smaller oligosaccharides, in their case HA, were required in higher concentration to cause inhibition than the larger fragments. They also suggested that high concentrations of polysaccharide may also cause inhibition and this was evident with undigested HA at concentrations above 400μg/ml. Wright *et al*, (1989) also reported that cells which have been growth arrested are more sensitive to growth inhibition and this was been demonstrated with heparin in smooth muscle cells. The inhibition shown by heparin and in this study may be due to its excessively high concentrations when incubated with the cells. This may have been worsened by the cells being in a state of growth arrest before their addition. In the case of ORC, the explanation is more difficult. In both L929 and BAEC cells, the inhibition shown by ORC 1 may be due to the same factors as discussed above for heparin, however, ORC 2 (the bigger of the two fragment ranges produced) showed stimulation of all cell types. With the Human Dermal Fibroblasts and 3T3 cells, both ORC fragments stimulated proliferation, therefore, these cells have been able to either internalise the ORC without detriment to themselves or have bound the ORC to receptors on their surface. It is, therefore, possible that cells have different tolerances to each other for the concentration of polysaccharide added and the size range of the sample.

From the results, it is impossible to predict if any oligosaccharide may have a similar *in vivo* effect on wound healing as heparin, as the mechanism of action may be different,

therefore, the following chapter investigated the differences in the chemoattractive ability of the oligosaccharides to cells. This would give an indication if the oligosaccharides had further similarities to heparin.

CHAPTER 5

CHEMOATTRACTION OF CELLS TO POLYSACCHARIDES

5.1   Introduction

In wound healing, it is imperative that cells migrate in to the wound area from the periphery and begin the process of healing. In order for this to occur, factors must be present at the wound site which attract the cells by a chemical signal and cause them to migrate into the wound area. Chemoattraction of cells toward a chemical signal is well documented for leukocytes (Forrester & Wilkinson, 1981) and fibroblasts (Albini & Adelmann-Grill, 1985; Postlethwaite *et al*, 1987).

The objective of this chapter was to investigate the ability of the polysaccharides and fragments produced in Chapter 3 to stimulate the *in vitro* migration of fibroblasts through a 8μm polycarbonate filter in a 48 well Boyden Chamber. The results of these studies would, in conjunction with the proliferation data in Chapter 4, allow a choice of oligosaccharide that might have the best *in vivo* possibility in terms of wound stimulation.

The assay was carried out as described in Method 2.4.6. Each sample was tested in triplicate and each experiment carried out in duplicate. Results were expressed as the mean ± standard error of duplicate experiments (Figs 5.1 & 5.2).

5.2   Chemoattraction of L929 Fibroblasts to Heparin

Heparin is a sulphated polysaccharide with a similar structure to HA and CS, and has
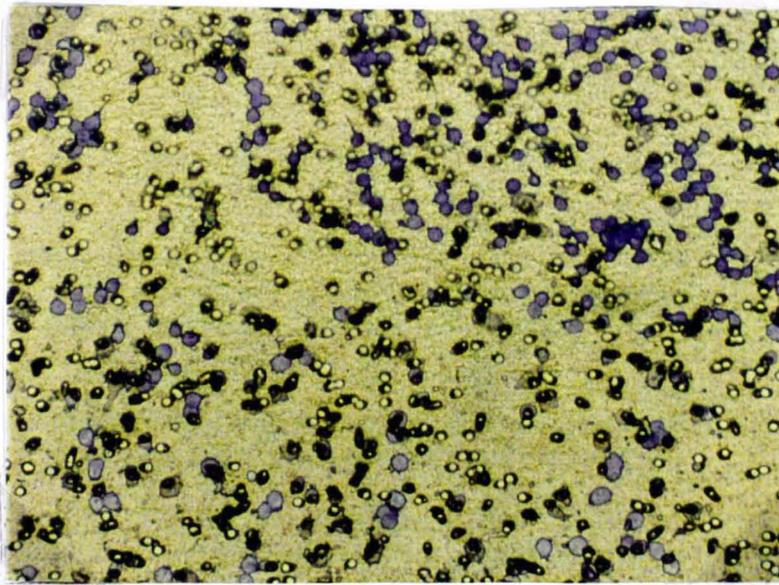
Figure 5.1   :   L929 fibroblasts which have chemotactically moved
through an 8µm polycarbonate filter. Stained with
Leishman's stain, magnification x10
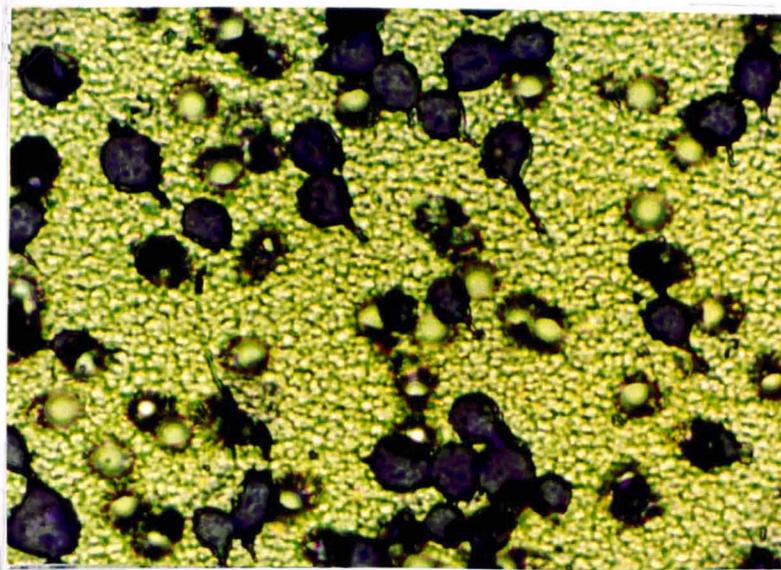
Figure 5.2   :   L929 fibroblasts which have chemotactically moved
through an 8µm polycarbonate filter. Stained with
Leishman's stain, magnification x40

been shown to encourage the invasion of fibroblasts into a collagen/heparin matrix when implanted subcutaneously in rats (McPherson *et al*, 1988). Heparin therefore was tested first for its direct effect on the migration of fibroblasts *in vitro* to try to compare with previous findings by other reporters *in vivo* (McPherson *et al*, 1988). Four concentrations of heparin from 0.1-100 μg/ml were tested. Controls containing Dulbecco's Modified Eagle's Medium (DMEM) alone and a positive control of 10 pg/ml TGFß per well (Postlethwaite *et al*, 1987) were used. The results showed (Fig 5.3) significant stimulation of migration ($p<0.05$) at 0.1-10 μg/ml, with the effect tailing off as the concentration of 100 μg/ml was reached. The negative charge of the heparin molecule does not seem to effect its ability to attract fibroblasts. This result suggests that heparin may have a role at low concentrations, at encouraging fibroblast migration and this may be relevant in the wound situation where application of heparin may influence recruitment of fibroblasts into the wound area to deposit collagen and remodel the wounded area together (Lynch *et al*, 1989; Morgan & Pledger, 1992). Comparing the chemoattraction data of heparin to the proliferation data in Chapter 4, it is clear that migration of L929 cells is not significant when the concentration of heparin goes above 100 μg/ml which correlates with the inhibition of the proliferation seen above the same concentrations. This suggests that concentrations lower than 100 μg/ml may be useful *in vivo*.

In vivo studies were carried out with the heparin to assess its effect in the rat wound healing model and the results of this will be discussed in Chapter 6.
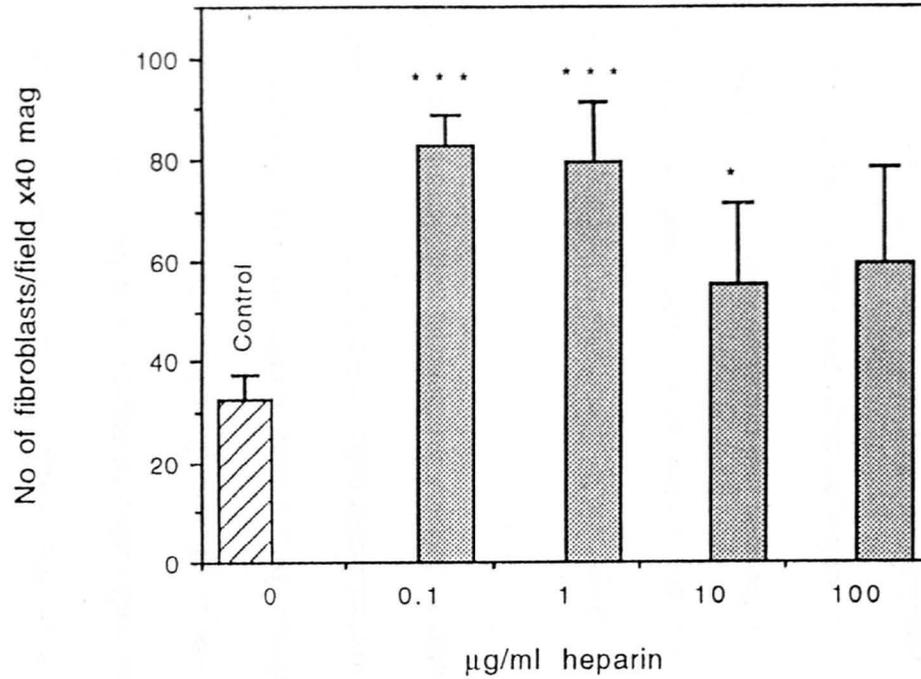
Figure 5.3 : The effect of increasing heparin concentrations on the migration of L929 fibroblasts. Cells were counted in 20 unit fields at x40 magnification in triplicate. Results are the means ± SEM of 60 replicates. Significant differences are indicated by *P < 0.05 and ***P < 0.001.

5.3 Chemoattraction of L929 Fibroblasts to HA and Its Oligosaccharides

As shown in Figure 5.4, undigested HA with a MW of 828,000 Da had no significant effect on the migration of L929 fibroblasts at the concentrations tested. It has been reported previously that undigested HA also inhibits leukocyte locomotion (Forrester & Wilkinson, 1981). In the *in vivo* wound healing situation, the inhibitory effect of undigested HA would be undesirable as migration of leukocytes to the wound site is necessary at the initial stages of wound healing when an inflammatory response is mounted. Previous evidence from cancer studies has also demonstrated that a cell surface layer of HA can protect tumor cells from attack by cytolytic lymphocytes and so this may impede the cellular defence mechanism and permit the growth of tumors (McBride & Bard, 1979).

The results obtained for the migration of fibroblasts in this study (Fig 5.4) suggests that the presence of undigested HA at the wound site would not improve the normal migration of fibroblasts into the area and therefore would not be able to overcome any detrimental effect of HA on leukocyte migration and subsequent lack of granulation tissue formation. The proliferation data in Chapter 4 also shows no effect on proliferation of L929 cells by undigested HA, therefore, this sample was not included in the *in vivo* study.

When the 5 min and 24h HA digests were tested for their effects on the migration of fibroblasts, it was observed that they both had similar effects as the undigested HA sample (Fig 5.4). At the concentrations tested, there was no significant effect on cell migration through the polycarbonate filter, thereby suggesting that different size ranges
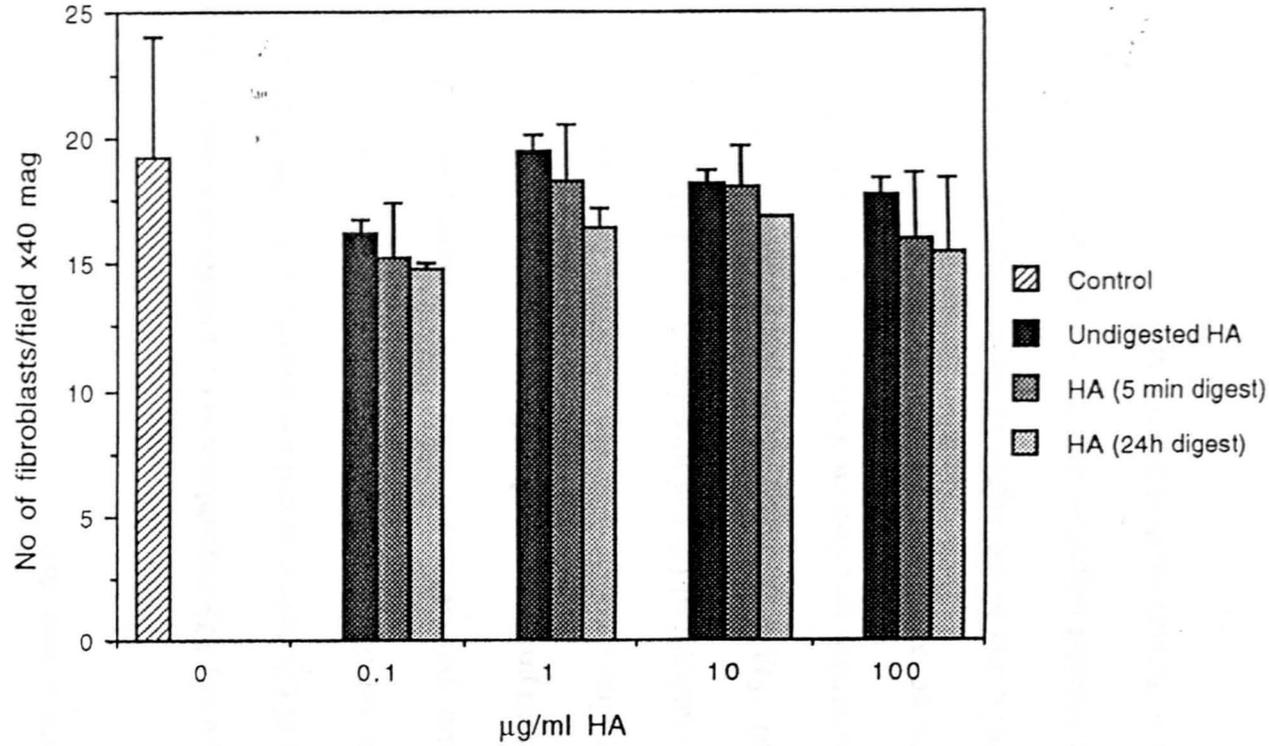
Figure 5.4 : The effect of increasing concentrations of HA and its oligosaccharides on the migration of L929 fibroblasts. Cells were counted in 20 unit fields at x40 magnification in triplicate. Results are the means ± SEM of 60 replicates at each concentration.

of HA oligosaccharides, including the undigested are not chemotactic for L929 fibroblasts *in vitro* . The results for the effect of HA on the migration of L929 cells correlate with proliferation results for the same cell line as none of the HA fragments tested were found to have any effect at all on this fibroblast cell line (see section 4.6) and so were not included in the *in vivo* study.

5.4 Chemoattraction of L929 Fibroblasts to CS and Its Oligosaccharides

Three different preparations of CS were prepared and molecular weights determined as described in section 3.6. The samples were then tested for their ability to attract L929 fibroblasts through an 8μm polycarbonate filter. The samples examined were undigested CS, a 5 min digested preparation and a 24h digested CS preparation.

Each digest was tested for chemoattraction at concentrations between 0.1 and 100 μg/ml. Like undigested HA, undigested CS had no effect on L929 fibroblast migration at the concentrations tested (Fig 5.5). Analysis of the oligosaccharide fragments prepared from CS also demonstrated no effect on fibroblast migration (Fig 5.5). CS has been reported also to have no significant effect on the migration of neutrophils (Forrester & Wilkinson, 1981), due to the highly charged nature of the molecule. Its lack of effect therefore on fibroblast migration may be due to repulsion of the charges on the fibroblast cell surface with those on the CS molecule.

In comparison to the proliferation results (Chapter 4), CS (5 mins) and CS (24h) had no effect on L929 cell migration but stimulated proliferation of these cells in culture. Perhaps higher concentrations of CS fragments would be needed to stimulate L929 cell
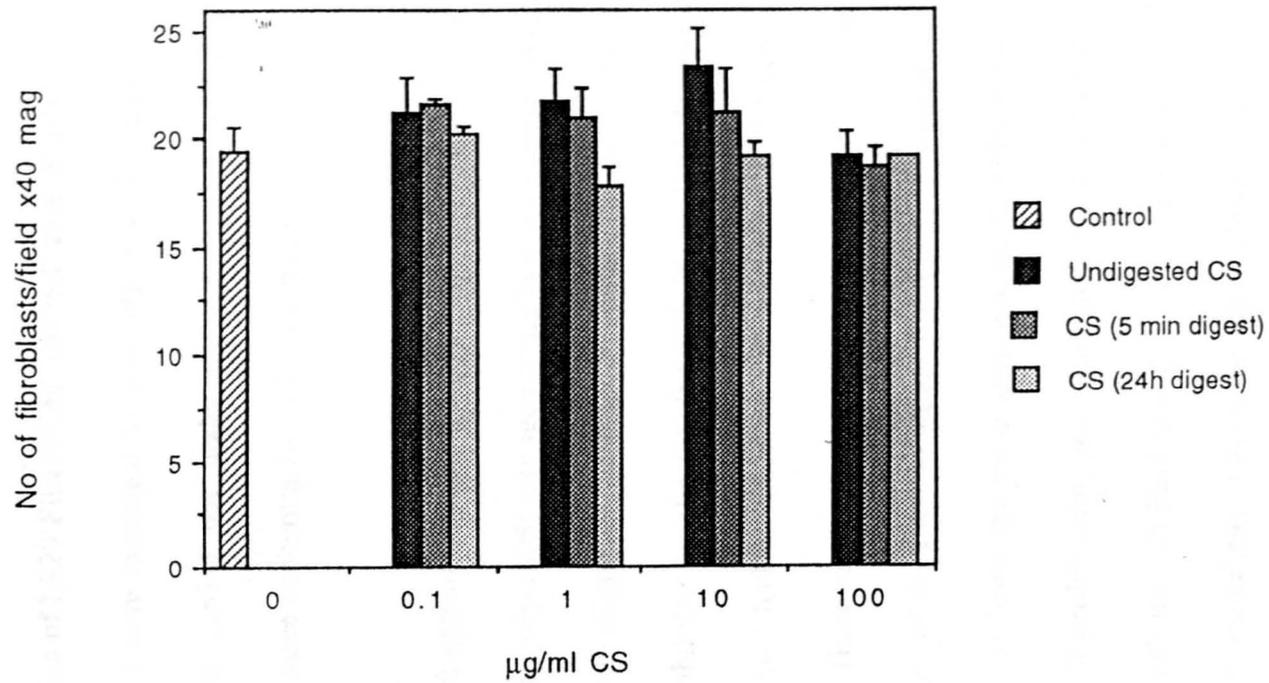
Figure 5.5 : The effect of increasing concentrations of chondroitin sulphate and its digests on the chemoattraction of L929 fibroblasts through a 8μm polycarbonate filter. Cells were counted in 20 unit fields at x40 magnification in triplicate. Results are the mean ± SEM of 60 replicates.

migration or simply, the CS fragments may not be chemoattractive. Further investigation would be needed to assess the effect of higher concentrations, and so it was decided not to include CS or its fragments in the *in vivo* study.

5.5   Chemoattraction of L929 Fibroblasts to ORC Fragments

Both fragment sizes (1 & 2) were examined for their effect on the migration of L929 fibroblasts. The assay was carried out under the same conditions as described previously, however, only three concentrations of each fragment (1-100 µg/ml) were tested in this experiment.

The results for the ORC 1 fragment illustrate a similar effect to that shown by heparin (Fig 5.6). Significant migration ($p < 0.05$) was seen at the lowest concentration, however, as the concentration increased, migration decreased to control values at 10 µg/ml and at 100 µg/ml, migration was inhibited significantly ($p < 0.05$) by the ORC fragment.

The ORC 2 fragment however had no effect on cell migration at the concentrations tested as shown in Fig 5.7. These results indicate that a difference in molecular weight causes a change in the ability of the ORC to attract fibroblasts through the filter, with the smaller fragment (ORC 1) being the more attractive to fibroblasts. Like heparin, ORC (1) inhibited L929 cell proliferation and stimulated the migration of the same cells. These results, along with the cell inhibition data, indicate that ORC and heparin have comparable in vitro characteristics. Given the acknowledged role of heparin in wound healing *in vivo*, it strongly suggests that ORC (1) should be tested under similar conditions to those of heparin in animal models.
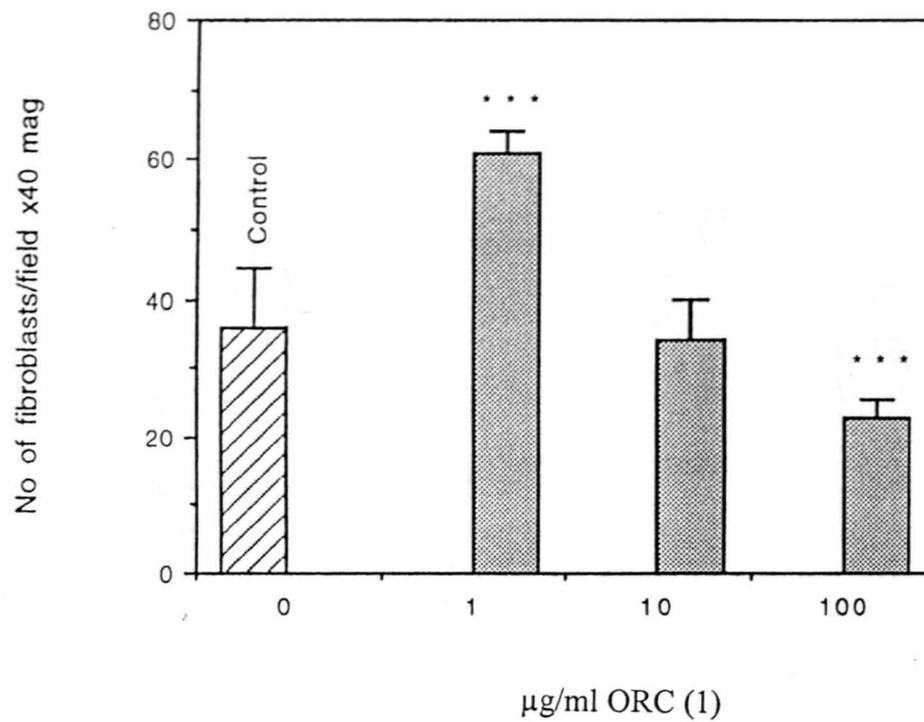
Figure 5.6 :   The effect of ORC (1) on the migration of L929 fibroblasts. Cells were counted in 20 unit fields at x40 magnification in triplicate. Results are the means ± SEM of 60 replicates at each concentration. Significant differences are indicated by ***P<0.001
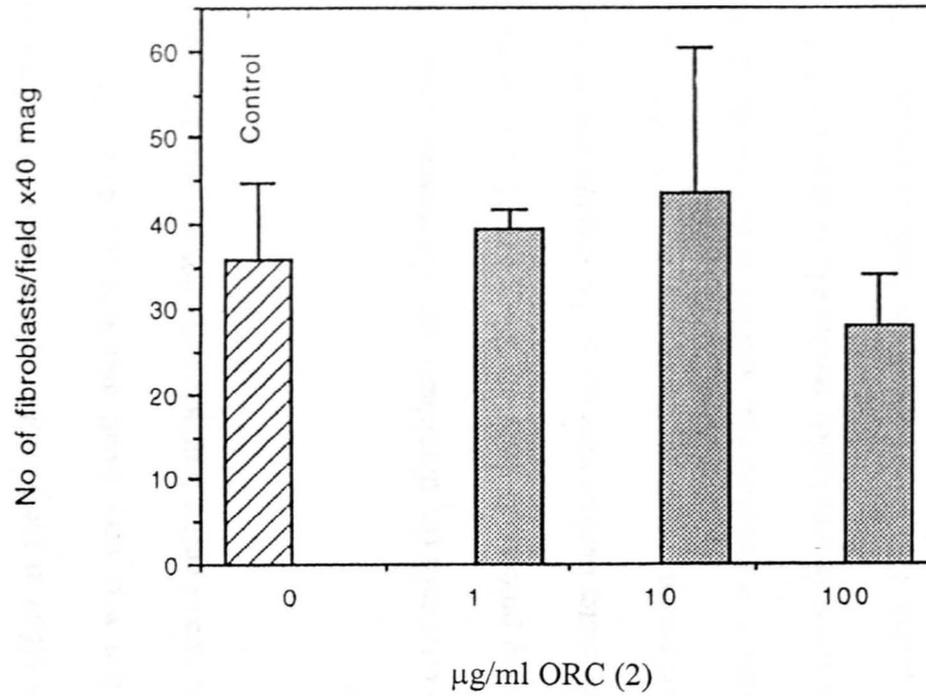
Figure 5.7 : The effect of ORC (2) on the migration of L929 fibroblasts. Cells were counted in 20 unit fields at x40 magnification in triplicate. Results are the means ± SEM of 60 replicates at each concentration. Significant differences are indicated by ***P<0.001

5.6 Chemoattraction of Fibroblasts To Pectin

Undigested rapid set pectin was tested *in vitro* for its chemoattractive properties. Four concentrations between 0.1 and 100 μg/ml were examined and their effect on cell migration is shown in Fig 5.8 Pectin stimulated the migration of fibroblasts at 1, 10 & 100 μg/ml, but had no effect at the lowest concentration at 0.1 μg/ml. However, the overall stimulatory effect was very small and was not dose related. Due to time constraints, the performance of pectin fragments was not investigated further.

5.7 Discussion

Chemoattraction of specific cells (ie. fibroblasts) into the wound healing site is the first step in the wound healing process. If therefore the speed of recruitment of these cells could be increased, then faster healing may occur. The results described above indicate that heparin, ORC 1 and pectin can attract fibroblasts, however, HA and CS appear not to in this study. Differences in structure may account in part for the variation in ability to attract fibroblasts as the monosaccharide residues in both HA and CS are joined by ß(1,3) bonds, whereas pectin, ORC and heparin (in part) are joined by 1,4 bonds. The structure of the molecules containing the ß(1,4) bonds only therefore may be more attractive to the fibroblasts and allow receptor binding to take place as the cells travel through the filter. Variations in charge are also a possible explanation for differences in chemoattractiveness, however, the more acidic polysaccharides did not seem to repel cell movement.

The results from both the *in vitro* cell proliferation and migration studies using ORC
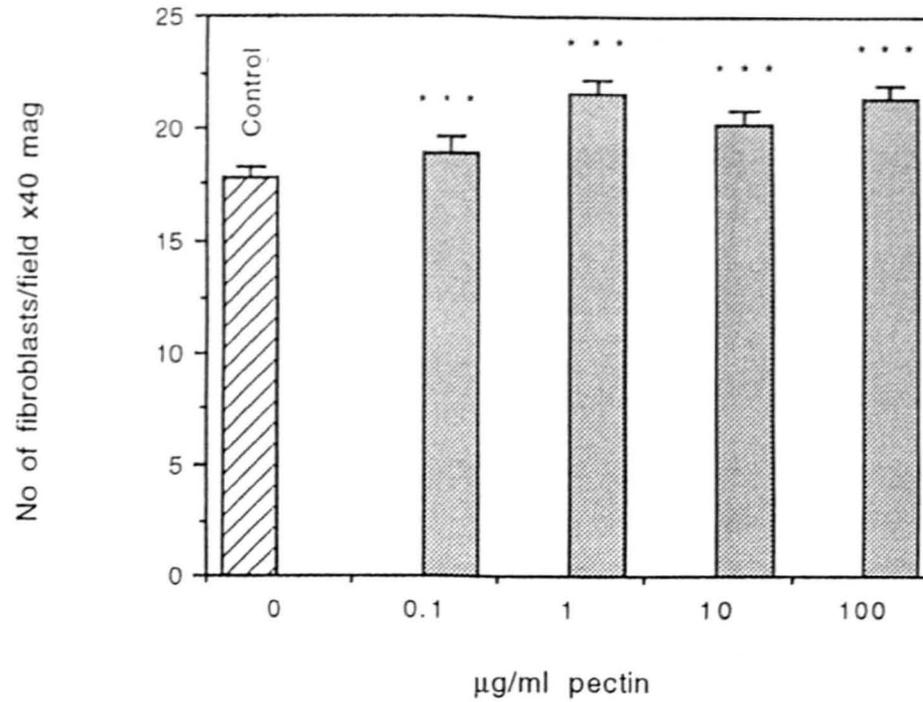
Figure 5.8 : The effect of pectin on the migration of L929 fibroblasts. Cells were counted in 20 unit fields at x40 magnification in triplicate. Results are the means ± SEM of 60 replicates. Significant differences are indicated by ***P < 0.001

were sufficiently encouraging to suggest a possible similar mode of action as heparin. For this reason ORC 1 was chosen for animal studies initially, however, ORC 2 was also included, as it expressed similar effects on proliferation as ORC 1 on different cell types and only differed in its effect on migration.

CHAPTER 6

6. *IN VIVO* ACTIVITY OF SELECTED POLYSACCHARIDES

6.1   General Introduction

A chronic animal model as such does not exist except in the case of the human and for practical and ethical reasons other models must be found. Impaired models do exist, however, these were not readily available and the appropriate animal licence and expertise were also not available. For this reason a simple acute subcutaneous rat model was chosen. Although this model has its limitations it was hoped that differences between controls and "active" agents could be identified in this model. The rat subcutaneous model (method 2.5.2) was constructed by implanting subcutaneously a biologically inert matrix made from polyvinyl alcohol (PVA) into the belly of the rat followed by injection of the active factor into the matrix. The rat subcutaneous model described above has been used successfully in previous studies to assess the potential of certain growth factors to affect wound healing (Davidson *et al*, 1985).

From the previous chapters described thus far in this thesis, it was apparent that ORC oligosaccharides had similar *in vitro* effects to heparin both in proliferation and migration studies and so this chapter describes the action of heparin and ORC on the rat subcutaneous implant model. Future studies may look at the other oligosaccharides produced, however, due to time constraints and limits to the number of animals available, these studies were not possible at the time.

6.2 Introduction to the Study

In this study, heparin and ORC (1) & (2) were chosen for *in vivo* in the rat subcutaneous implant model. Heparin was the first polysaccharide tested for its effect on the healing response in an animal model as previous evidence (McPherson *et al*, 1988) suggested that addition of heparin to a collagen suspension which was subsequently injected into guinea pig dermal wounds, produced an infiltration of fibroblasts and elicited the formation of granulation tissue as compared to the collagen alone. This study used an inert matrix to inject the potential active oligosaccharide into as the effect of the oligosaccharide on its own was to be assessed first before including it within a collagen matrix.

The *in vitro* study documented in Chapter 4 supported previous *in vitro* results (Ferrao & Mason, 1993; Westergren-Thorsson *et al*, 1991). This study will attempt to confirm previous *in vivo* results (McPherson *et al*, 1988) using a different animal model.

ORC (1), like heparin, inhibited the proliferation of L929 fibroblasts and was chemotactic to L929 cells and so it was felt that since its *in vitro* effects were similar to heparin, it may behave like heparin *in vivo*. ORC 2 was also included as it expressed similar effects on proliferation of certain cells as ORC 1 and only differed in its effect on migration.

6.3 Rat Subcutaneous Implant Model

The rat subcutaneous implant model (method 2.5.2) was used as it is easy to carry out

and assess the results. Another advantage is that both the control and the test sample can be assessed on the same animal. Other models, such as the pig full-thickness wound are available, however, it is expensive to set up this kind of experiment and trained staff are needed to carry out the surgery. The pig model does provide a better chronic wound model as full-thickness skin sections are cut out, whereas in the rat, the test samples are placed under the dermis between the panniculus carnosus and the body musculature.

The rat subcutaneous model used in this study gives an indication of the invasion of cells such as fibroblasts in the inert PVA sponge, as well as any vascularisation taking place. The degree of inflammation caused by adding a foreign substance can also be detected by the number of white blood cells present. The pig model, however has benefits in that the degree of granulation and re-epithelialisation can be observed in the healing wound. In this study however, this initial aim was to discover if recruitment of cells in to the PVA matrix could be aided by the test oligosaccharides. Future work could look at the effect of any successful samples in conjunction with collagen in the rat model or in the pig model.

After completion of the experiments carried out as described in Method 2.5.2, the PVA sponges were removed, wax-embedded and sectioned according to Method 2.6. Each sample was sectioned in triplicate and stained with haemotoxylin to visualise cell nuclei (blue-black colour) and eosin to visualise cell cytoplasm and connective tissue (pink)(method 2.7.1). Microscopic analysis of the samples was undertaken to assess the degree of inflammation by the presence of monocytes, neutrophils and macrophages (Method 2.8).

6.4   *In Vivo* Activity of Heparin

Three concentrations of heparin (1, 10 & 50 µg/ml) were used in this study and each concentration was injected into 5 rats for comparison at day 7 and day 10 after wounding. Low concentrations of heparin (1,10 & 50 µg/ml) were used in this study as previous results using amounts of heparin up to 30% w/w of the matrix resulted in severe haemorrhaging in the rats (personal communication K. Broadley). Data from Chapters 4 & 5 suggested that heparin at concentrations less than 10 µg/ml would be the most effective to use, however it was thought that during injection into the rat, some heparin may be lost into surrounding tissue and so higher concentration were included to take this into account. The results (Table 6.1; method 2.8) indicated that treatment with the 1 ug/ml sample of heparin had no effect on granulation tissue formation at Day 7, but had an overall inhibitory effect at Day 10 by visual analysis, however, statistics show that neither day 7 or 10 were significantly different from each other (p = 0.5). The 10µg/ml sample however demonstrated visually a stimulation of granulation tissue formation at Day 10 where there was no apparent effect at Day 7. Statistical analysis confirmed a significant difference between the two days (p = 0.05). The 50 µg/ml injection of heparin demonstrated visually a convincing stimulatory effect on the formation of granulation tissue at both 7 and 10 days, although neither day was significantly better than the other by statistical analysis (p = 0.34). The histology slides were independently assessed by two other observers and their results are included in Table 6.1. (also see Fig 6.1). The photographs in Figure 6.1 clearly show a difference between the control sponge and that injected with 50µg/ml heparin. Two control

Day of sacrifice & concentration of heparin	No. of animals	Observer No.1 Ratios			Observer No.2 Ratios			Average ratios of Observers			Effect on wound healing
		Pos	Neg	Zero	Pos	Neg	Zero	Pos	Neg	Zero	
7 days at 1 µg/ml	4	1	3	4	4	1	3	2.5	2	3.5	No effect
10 days at 1 µg/ml	5	3	5	2	3	5	2	3	5	2	Inhibitory
7 days at 10 µg/ml	4	2	4	2	1	2	5	1.5	3	3.5	No effect
10 days at 10 µg/ml	5	6	2	2	5	1	4	5.5	1.5	3	Stimulatory
7 days at 50 µg/ml	5	4	2	4	5	2	3	4.5	2	3.5	Stimulatory
10 days at 50 µg/ml	4	6	1	1	6	0	2	6	0.5	1.5	Stimulatory

Table 6.1 :   Histology results for heparin at 1, 10 & 50 µg/ml which was injected subcutaneously into rats with PVA sponges. Results are expressed as positive, negative or zero ratios after comparison of control values to experimental samples. Positive ratios showed better granulation tissue formation than controls, negative ratios were the converse and zero ratios were no different to controls.
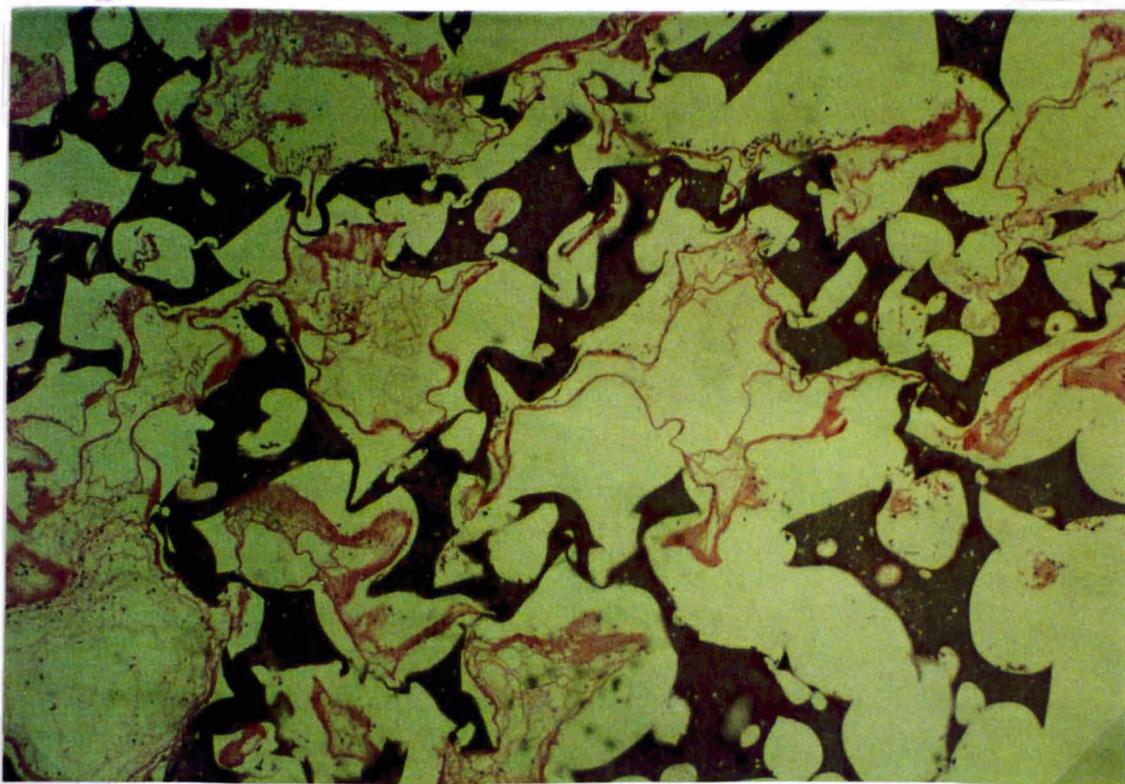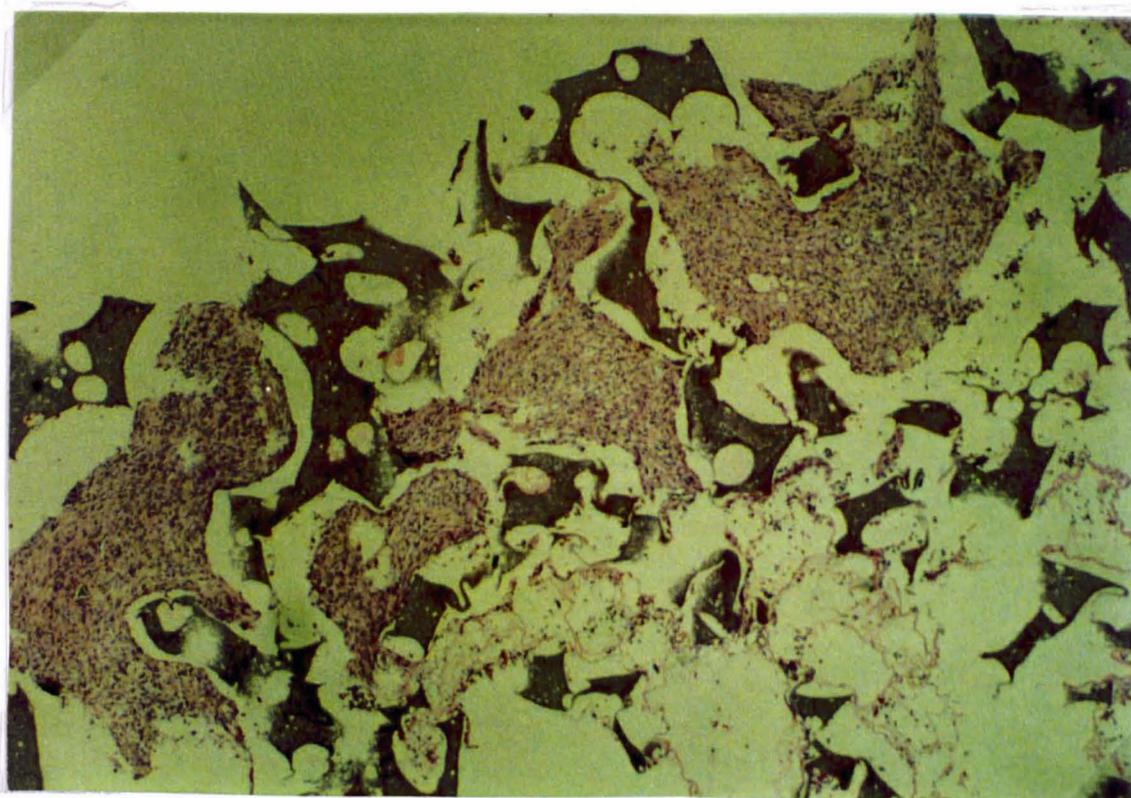
Figure 6.1 : Influence of heparin on the formation of granulation tissue *in vivo*. (a) PBS and (b) heparin at 50 µg/ml were implanted in the rat subcutaneous model and harvested at 10 days post-implantation as described in the Methods. Magnification x10.

photographs and 2 test photographs were taken per animal and this is an example of the results obtained.

It is difficult to quantify these results as the analysis was subjective, however an overall impression can be formed on the ability of the polysaccharides to enhance the formation of granulation tissue. Future work may investigate the ability of Image Analysis to carry out the analyses in a quantitative fashion which would allow the method to be validated and therefore reproducible.

6.5   *In Vivo* Activity of ORC Fragments 1 & 2

6.5.1   Introduction

ORC fragments, derived from the Johnson & Johnson product, Surgicel® were also tested on the rat subcutaneous implant model as they demonstrated comparable *in vitro* characteristics to heparin(excluding ORC 2 which was used as a comparator to ORC 1 (see chapter 4). Sponges injected with ORC (1) were unfortunately sectioned in the wrong orientation by Stirling Royal Infirmary and so could not be analysed histologically. ORC (2) at concentrations of 1 and 10µg/ml were chosen based on the proliferation results (chapter 4). The use of ORC *in vivo* was not expected to cause any adverse effects on the animal (Daniels *et al*, 1981), as it both a haemostat and an adhesion prevention barrier. The doses used here are clinically much lower than when used in these indications.

6.5.2 Results

The histology results obtained for the ORC 2 fragment by two independent assessors indicated that there was a high degree of inter animal variation (Table 6.2). This was particularly obvious in the 1μg/ml Day 7 and the 10μg/ml Day 10 results. Indeed in the latter data set, two of the five animals exhibited a distinct inhibitory effect whilst another two indicated the fragment to be stimulatory. There was also an obvious intra animal variation, although this was not as distinct as the variation between animals. In many of the animals, there were differences in the extent of cellular invasion between the sponges implanted at the top of the rat and those at the bottom, although this observation was not restricted to either of these areas. Visual analysis claimed the 1μg/ml sample of ORC (2) to have an overall stimulatory effect on granulation formation, however, statistical analysis of results obtained at day 7 and day 10 proved there was in fact no difference to control levels (p = 0.34).

6.6 Immunostaining of PVA Sponges

6.6.1 Introduction

In order to improve subjective assessment of 'newly' formed granulation tissue on a gross basis, it is believed that the ability to assess the cell types present in these tissues would give a better insight into the response to the agents added into the sponges (eg) inflammatory versus matrix components (i.e) collagen and fibroblasts. For this reason immunostaining of the sectioned sponges was carried out.

No method existed previously to immunostain wax-embedded sections of PVA sponges and so this had to be developed in this study. A range of antibodies at different dilutions

Day of sacrifice and concentration of ORC (2)	No of animals	Observer No.1 Ratios			Effect on wound healing
		Pos	Neg	Zero	
7 days at 1µg/ml	4	5	3	0	Stimulatory
10 days at 1µg/ml	5	2	3	5	No effect
7 days at 10µg/ml	4	3	3	2	No effect
10 days at 10µg/ml	5	4	5	1	No effect

Table 6.2 : Histology results for ORC (2) at 1 & 10µg/ml which was injected subcutaneously into rats implanted with PVA sponges. Results are expressed as positive, negative or zero ratios after comparison of control values to experimental samples. Positive ratios showed better granulation tissue formation than controls, negative ratios were the converse and zero ratios were no different to controls.

were employed to discover the optimal concentration for detection of the above components (methods 2.7.3 & 2.7.4)

6.7   Detection of Cells by Immunocytochemistry

Immuncytochemical analysis was carried out on sections of PVA control samples and PVA + 50 µg/ml heparin sections (as in Figure 6.1).Various dilutions of the primary antibodies (rabbit anti-laminin (Serotec), MCA 341 (Serotec) for monocytes and macrophages & MCA 276 (Serotec) for endothelial cells) were added. The appropriate secondary antibodies which were labelled with FITC were added after a suitable incubation period. For each sample a control containing the secondary antibody only was also included (method 2.7.4).

The controls containing the secondary antibody alone however demonstrated a high background staining and this posed difficulties for picking out fluorescent cells due to the non-specificity of the secondary antibody. Further studies would involve determining ways to reduce the non-specific binding. One method could to block the sections with non-immune serum from an animal, such as sheep, before antibody staining in order to block some of the non-specific binding sites. Optimising the antibody dilutions used for these sections could also be developed as time was limited in this study for that specific purpose. Fluorescence levels detected in this study were very low with exposure times of up to 2 minutes. This suggests that binding of the antibodies to the cells was poor and so further development of incubation times and methods for improving the permeability of the cells to the antibody could be carried out

to optimise this technique.

6.8   Conclusions

The result of the subcutaneous implant study indicated that at concentrations of 10 and 50ug/ml, heparin did stimulate the formation of granulation tissue within the PVA sponge. This effect was also observed by McPherson *et al*, (1988) in the guinea pig dermal wound model using collagen suspensions containing heparin at 300ug/ml of suspension. This study, however, demonstrated an effect on granulation tissue formation at much lower concentrations of heparin than McPherson used, but this may be due to the different sources and molecular weight of the heparins used or even the difference in species used for the study.

McPherson injected the heparin in conjunction with collagen suspension. Previous work he carried out suggested that injecting less than $30\mu g/ml$ of heparin would have no effect on cell invasion and injecting more than 3mg/ml heparin would cause haemorrhaging in the animal. It is likely therefore, that this study could have investigated higher concentration of heparin without having a detrimental effect and may even have given better cell invasion that shown at $50\mu g/ml$ heparin.

The ORC (2) results were so variable that no conclusions could be made unless the experiment was repeated. The trend demonstrated by the results, however, suggested that the ORC had no effect on granulation tissue formation at the concentrations tested.

Given additional time, it would have been interesting to take this study further by

looking at different doses of the polysaccharides and also looking at impaired animal models such as the diabetic mouse model. It would also have been exciting to investigate the *in vivo* effects on the other polysaccharide fragments prepared, such as chondroitin sulphate as the 5 min digest in particular had a significant effect on the proliferation of cells *in vitro*. Future work might also entail validating the method of histological assessment by using image analysis software.

CHAPTER 7 : CONCLUSIONS

A number of studies both *in vitro* and *in vivo* have suggested a possible role of oligosaccharides in wound healing processes (West *et al*, 1985; McPherson *et al*, 1988; West & Kumar, 1989; Westergren-Thorsson *et al*, 1991; Ferrao & Mason, 1993), however little is still known about the possible effects of these and other oligosaccharides on cells that are directly involved in the wound healing process. The present study has investigated the actions of several different intact GAGs, together with oligosaccharides generated from the intact molecules, both on cells *in vitro* and in animal models. The study also included the assessment of some GAGs previously investigated by other groups in order to compare them to those isolated in this study.

The aim was to select a bioactive oligosaccharide which could assist in, or stimulate the healing of chronic wounds. The main thrust of these studies was to assess the bioactivity of the oligosaccharides per se, although sometime was spent on the delivery of these moieties from PVA sponges in animal models.

Previous work by West & Kumar (1989) showed that HA oligosaccharides with a size range between 3 and 16 disaccharide units in length stimulated the proliferation of BAEC cells. This effect was also seen in this study with HA (5min) fragments with a average size range of 5 disaccharides and with CS (5mins) with an average size range of 10 disaccharides. Proliferation may have been induced by the modulation of other growth factors already present in the system or perhaps due to the synthesis or

formation of the extracellular matrix being affected (West & Kumar, 1989). West & Kumar also presented evidence for a hyaluronate receptor on BAEC cells and showed these cells were able to endocytose both low and high weight HA, however, the specificity of the endothelial cell receptor reported in this study may differ from that reported by West & Kumar in that it is also might be specific to chondroitin sulphate, a phenomenon also reported by Laurent *et al*, (1986).

L929 fibroblasts were stimulated to proliferate by CS (5min) and CS (24h) digests , however the other HA & CS samples had no effect on this cell type. Human dermal fibroblasts were also stimulated to proliferate by CS samples, however, one HA digest, HA (5min), also had a significant effect on this cell type. Stimulation may result from the induction of growth factors or inactivation of growth inhibitors present in the serum or those produced by the cells themselves (Westergren-Thorsson *et al*, 1989).

The differences observed in stimulatory activity on similar cells isolated from different sources may be due to the number of specific receptors on the surface of the cells (Culty *et al*, 1992) and the affinity of the receptors to the size of the oligosaccharides added exogenously (West & Kumar, 1989). Although not investigated in this study, another contributing factor may be that L929 cells are transformed cells, whereas the human dermal fibroblasts used in this study were normal primary cells. The state of the cell can effect its reaction to exogenously added GAGs as shown by Matuoka *et al*, (1984)

.

Heparin had an inhibitory effect on the proliferation of all cell types tested in this study, however the degree of inhibition was dependant on the concentration added. At

concentrations above 10µg/ml, inhibition was evident. Suppression of growth may be due to the heparin acting directly on the cells, however, it may also activate growth inhibitors or prevent growth factors from binding to cell receptors. Previous findings (Westergren-Thorsson et al, 1991) suggested that the inhibition shown by heparin may be due to the high content of sulphate and L-iduronate in the molecule., however ORC (1), an unsulphated molecule, also inhibited various cell types in this study. On closer examination of the structure of heparin and ORC (1), it is apparent that both molecules contain ß(1, 4) links as opposed to the ß(1, 3) links seen in HA and CS. This may provide one reason as to why these GAGs produce different *in vitro* results from HA and CS.

The cell types employed in this study were chosen because they were readily available and easy to grow, however, in order to obtain results which would be directly comparable to the *in vivo* situation, it would have been useful to look at primary cells isolated from rats (ie) rat skin and wound fibroblasts and rat endothelial cells. Rat wound fibroblasts were cultured during the study, however further work was required to optimise growth conditions before using them in a screening assay. Human dermal fibroblasts were employed in their place as they were primary cells and their results from the *in vitro* studies could more readily be compared to any future clinical trials in humans

The migration assay was applied in this study to try and identify oligosaccharides which would stimulate the migration of fibroblasts but may not have had any effect on the

proliferation of these cells *in vitro*. Attraction of wound repairing cells into the wound area is obviously vital for repair to take place, however the results demonstrated that only heparin and ORC (1) and pectin had the ability to do this *in vitro,* while HA, CS and ORC (2) did not. Previous findings also show heparin to be chemotactic for capillary endothelial cells (Azizkhan *et al*, 1980) within the same concentration range studied in this thesis.

The common feature between the stimulatory GAGs is again the presence of ß(1,4) links. This suggests that alignment and structure of the molecule is important in the chemoattraction of L929 cells by GAGs. The results also demonstrate that migration is highest when low concentrations of heparin and ORC (1) are used. When compared to the effect of these concentrations in the proliferation assay, it appears that no effect on cell proliferation was seen at these low values. These results indicate that the concentration of GAG added is important to achieve stimulation of migration and to prevent inhibition of proliferation.

Pectin both stimulated L929 cells to both proliferate and migrate. The migration was enhanced at all concentrations tested, however more work is required to investigate the optimal concentration for migration. Within the range tested, a dose response was not evident. Due to these effects, pectin has the potential to be a cheap and readily available polysaccharide to use in the treatment of wounds, however due to time constraints in this study, *in vivo* studies could not be carried out to measure its effect.

The animal studies undertaken were limited due to time. Based on *in vitro* results, only

two GAGs were investigated - heparin and ORC. The *in vivo* studies proved problematic in that the results were subjective and could not be easily quantitated, however, the difference between no effect and a positive effect in some cases were very obvious in the amount of fibrin and granulation tissue laid down.

The addition of heparin (10µg & 50µg/ml) caused a significant increase in the amount of granulation tissue laid down within the PVA sponge, with the 50µg/ml heparin causing a significant increase at Day 7 as well as Day 10. Heparin is also known to potentiate angiogenesis *in vivo* (Taylor & Folkman, 1982), however no staining of endothelial cells was undertaken in this study to confirm this finding. Currently, heparin co-adminstered with bFGF (an endothelial cell mitogen), is being studied for its angiogenic effect in healing wounds as heparin is known to potentiate the effect of bFGF *in vitro* (Thorton *et al*, 1983). The addition of growth factors in conjunction with oligosaccharide fragments therefore may be an area of investigation for future work.

In relation to the *in vitro* results demonstrated by heparin, the proliferation of fibroblasts was inhibited as also demonstrated by Ferrao & Mason, (1988) and Westergren-Thorsson *et al*, (1991), however there was no effect on endothelial cells at these concentrations (E. Lorimer, personal communication). Migration was stimulated by 10µg/ml of heparin but there was no effect at 100µg/ml heparin. It seems likely that the presence of higher concentrations of heparin may be needed *in vivo* as the injection of the sample may not all be absorbed by the PVA sponge causing leakage into the surrounding tissues. If this is the case, then an injection of 50µg/ml of heparin may result in only 2µg being absorbed (100µl injected = 5µg heparin of which 3µg may be

lost), which is equivalent to the *in vitro* results shown by the 10µg/ml heparin (2µg heparin). The problem, however, with injecting large amounts of heparin into the animal is of course the risk of haemorrhaging due to the anti-coagulant activity of heparin (Barrowcliffe *et al*, 1992).

The results of injecting ORC (2) into PVA sponges in the animal model were inconclusive and further investigation is required. Comparing the *in vitro* results of ORC (1) to those of heparin, one might expect ORC(1) also to show *in vivo* stimulation of granulation tissue formation, however, due to an error in sectioning the ORC(1) samples, this experiment could not be completed. It would be recommended that this study is repeated in future work, as ORC has the potential to be a cheap and readily available polysaccharide to use in the treatment of wounds.

The results indicated that inhibition of fibroblast proliferation *in vitro* by heparin did not affect the ability of the cells to be chemoattracted and this was supported in the *in vivo* animal model. It was thought initially that stimulation of proliferation would be an essential requirement for an oligosaccharide to possess in order for it to be bioactive *in vivo*, however, heparin has shown that inhibition of a particular cell type is not necessarily detrimental to its *in vivo* effect.

Several significant findings have emerged from the study : selected oligosaccharides fragments generated from HA and CS stimulated proliferation of various types of fibroblasts and endothelial cells *in vitro*, however they did not have any effect on the

migration of fibroblasts; ORC (1) and heparin both inhibited L929 proliferation at concentrations of greater than 100μg/ml and stimulated the migration of L929 at concentrations of less than 1μg/ml; pectin stimulated the proliferation of fibroblasts and endothelial cells and stimulated the migration of L929 cells; while heparin encouraged granulation tissue formation in wounded rats *in vivo*.

Although the *in vitro* results of both proliferation and migration indicated that several oligosaccharides may have a role to play in the process of wound healing, it is clear that translating these results directly to the animal situation and further into potential clinical efficacy is not an easy task.

I believe the first step of identifying a number of potential candidates for wound stimulation has been completed, but there are obviously a great deal of animal studies required to identify a "true" wound stimulating factor for use in the clinic and hospital.

CHAPTER 8 : FUTURE WORK

8.1 Oligosaccharides

The *in vitro* and *in vivo* studies described in the previous chapters were carried out using crude enzymatic digests of the various polysaccharides. Future work should investigate large scale production of selective sizes of fragments. Small amounts of pure fragments were obtained in this study, however there was not enough produced to undertake both *in vitro and in vivo* screening. Investigation of selective sizes would give important information about the size ranges necessary to observe *in vitro* effects on cell proliferation and cell migration of various cell types and granulation tissue formation etc. in animal models.

8.2 Additional *In Vitro* Studies

The *in vitro* studies discussed in this thesis relied on the cell types which were readily available and easy to grow. Future studies should look at specific cells from humans as this is the final destination for the commercial product. For the purposes of wound healing studies, it would be beneficial to have a source of fibroblasts from a human wound, however, these may be difficult to obtain. It would also be useful to look at the effect of oligosaccharide fragments on human endothelial cells. As the *in vivo* model in this study was based on the rat model, different rat cells could also be sourced. This study looked at rat wound fibroblasts initially but they did not grow well in culture and were omitted from subsequent investigations. More work into the optimal culture conditions would be necessary in order to use these routinely in proliferation studies.

Another area of investigation may be to look at the addition of growth factors in conjunction with oligosaccharide fragments, as previous evidence using heparin in conjunction with bFGF has been shown to be angiogenic *in vitro* (Thorton *et al*, 1983).

8.3   Animal Models

The aim of this thesis was to produce a collagen sponge containing a bioactive oligosaccharide fragment. Due to time constraints, no *in vivo* studies were carried out using these collagen sponges to assess the effect of the fragments, however initial studies were carried out on inert PVA sponges. Future work should investigate the addition of collagen, as it is important to assess how the collagen and fragments interact independently and together *in vivo*.

Future work may involve carrying out *in vivo* studies on other types of animal models such as the pig excisional wound model (Paresio, 1994) or impaired wound models. The pig model is a suitable model as the healing more closely resembles that of humans. Such models have been used extensively to test wound dressings (in house ; Paresio, 1994), however, the most appropriate model would be full or partial thickness wounds to better simulate an impaired wound and this could be carried out possibly using diabetic pigs.

8.4   Histological Evaluation

This thesis briefly investigated staining the different cell types present in sections of implanted PVA sponges impregnated with heparin. More work is needed to develop the methods to determine what cells are being recruited into the wound as this can give

important information regarding the inflammatory response and presence of granulation tissue in the wound. Several antibodies are available to cells and extracellular matrix molecules involved in the wound healing process, therefore work is needed to optimise the concentrations of antibodies which will give adequate staining of the antigens concerned. Several methods of visualising the cells could be developed further such as, immunostaining & immunofluorescence.

Further work is also necessary on the visual analysis of the histological sections. In this thesis, it was a subjective assessment using a grading scale of 1-5, however, it may be possible to develop a method via an Image Analysis system to count specific cell numbers or calculate percentage areas of granulation tissue observed. This would give quantitative analyses of the sections which could be validated for others to use in the future.

8.5   ORC and Chondroitin Sulphate

Johnson & Johnson now have patents for collagen sponges containing ORC, CS and ORC fragments. This now gives scope for developing the work already carried out on these oligosaccharides into a commercial product that has intellectual protection.

Currently, a clinical trial is being set up in which collagen sponges containing ORC are being tested. Following the results of the trial, it is hoped that the product will be launched commercially.

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