In vitro mucopolysaccharide metabolism of epithelial tissue cells

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In memory of my Father who would
have continued to be
indulgent of my
frustrating and protracted
attempts at this rewrite
and to my
Mother
so many miles away who has
continuously hoped for
a completed
rewrite.

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DECLARATION.

I declare that this thesis contains no uncited material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge, contains no material previously published by any other other person, except where due reference is made in the text. Further, due reference has been made to my research contributions which have been submitted, accepted for publication or published in the refereed scientific literature. Relevant publications appear as an appendix.

Many of the results have been presented to meetings of the International Association of Dental Research (Australian Division), The Australian Society for Medical Research and at several International Connective Tissue Symposia, the references to which are cited as abstracts in the Bibliography.

OLE W. WIEBKIN,

Adelaide, South Australia. March, 1983.

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I am indebted to Mrs. Renee Thonard for her support and affection while typing the original thesis, and later to Miss Anne McArdle and Mrs Helen Muir, who have greatly contributed to the rewrite by patient and diligent typing of the drafts. My grateful thanks are extended to them.

In the interim, between the first submission and this one, all the above have continued to show friendship and interest in my work.

The period spent in the Dental School, between 1967 and 1970 was a very happy one. The work was carried out during the tenure of an University of Adelaide Postgraduate Scholarship, further support for the research was provided by University Research Grants and the National Health and Medical Research Council.

Since the depressing loss of my manuscripts of a complete rewrite, and of the raw data following the sinking of my houseboat home in 1974, I have made several attempts to rewrite. This ultimate attempt has only been achieved through the gentle persuasive encouragement of my wife, Sue. She has tolerantly shared my frustration at a job unfinished. Without her, this rewrite may not have been completed.

IN VITRO MUCOPOLYSACCHARIDE METABOLISM OF EPITHELIAL CELLS

SUMMARY

Intercellular protein/polysaccharide matrices have been implicated in the regulation of diffusion and transport mechanisms in biorheology, biocements and in the maintenance of tissue integrity.

Biochemical identification of macromolecular polysaccharide corresponding to proteoglycans (mucopolysaccharides) in an amnion cell line, and evidence of de novo synthesis have been achieved. Primary cell cultures of disaggregated human gingival epithelium and short term gingival slice incubations revealed that these cells were also capable of synthesising intercellular proteoglycan.

Techniques to localize such materials both within intercellular sites and as retained intracellular components have been developed. They include modifications of conventional histochemistry and autoradiographic methods. The specificity of the incorporation of radioactive precursors during 'pulse' incubation has been improved by utilisation of controlled precipitation with detergents such as cetylpyridinium chloride, together with sequential elution with increasing salt solution.

Some preliminary resolution of the biosynthetic events which are involved in elaborating the sulphated polysaccharides in both amnion and gingival epithelial cells also relied on detergent precipitation techniques, followed by cellulose acetate electrophoresis, paper chromatography and high voltage electrophoresis. Amnion cells grown to different degrees of monolayer development have been assayed for uronic

acid and sulphate as well as for protein associated with cetylpyridinium chloride precipitable material.

Cells of fragmented human gingivae were fractionated according to the size of the cell clumps that passed sequentially through nylon filters of decreasing gauge. This procedure provided cells ranked with respect to their intercellular surface contact. Uronic acid, sulphate and associated protein was assayed on representative culture of these categories. Following one interpretation of the data presented in this thesis, an hypothesis has been advanced that the degree of sulphation of intercellular matrix macromolecules which contain chondroitin sulphate reflects the degree of the cell-cell interface contact.

The addition of hyaluronate to gingival-epithelial cell cultures inhibited both proteoglycan synthesis and secretion similarly to that previously described by the author for chondrocytes. The implication that the initial mode of action of hyaluronate on gingival epithelial cells was due to passive binding at the cell surface was tested with fluorescently labelled hyaluronate. This material would bind to epithelial cell surfaces and was shown to be indistinguishable from unlabelled hyaluronate with respect to biological activities (namely ~~proteoglycan~~ inhibition of biosynthesis of proteoglycans by chondrocytes).

Associative and dissociative extractions of proteoglycans from separated gingival epithelium and connective tissue provided material which was fractionated on Sepharose 2B-CL gel chromatography. Reassociation of a connective tissue proteoglycan fraction in the absence

of extraneous hyaluronate resulted in the identification of a species of macromolecule, distinct from a fraction extracted from the epithelium. This latter fraction had the capacity to interact with hyaluronate.

The results emphasise the importance of the intimate extracellular matrix in the regulation of the maintenance of tissue integrity. Not only does the amount (total uronic acid) appear to be important, the composition (sulphate/protein levels) and conformation (molecular size and extractability) of the intact proteoglycan are arguably fundamental in determining whether tissue is susceptable to degradative disease processes. Macromolecular interactions within extracellular matrices are thus regarded as arbiters of structural as well as functional tissue integrity.

CHAPTER I.

INTRODUCTION.

The *in vitro* metabolism of epithelial mucopolysaccharides was investigated in order to elucidate some aspects of the maintenance of tissue integrity in the gingivae, a tissue which undergoes excessive mechanical abuse and also plays host to a spectrum of potentially pathogenic biota.

The work described in this thesis was originally undertaken between 1967 and 1970, being supported by a University of Adelaide Postgraduate Research Scholarship. However the interpretations of some of the data are based on more recent understanding of the chemistry and functions of proteoglycans, resultant upon the increased emphasis on proteoglycan research throughout the world, but particularly from work done by the author since 1970 (1971-77) in the Biochemistry Division of the Kennedy Institute, London, U.K. and more recently in the Department of Oral Biology.

Although the history of interest in tissue components has been variously reviewed (see Review citations) a brief outline of the development of appropriate investigations is included for completeness.

The Chemistry of Schleimstoff.

To test and verify predictions about the structure and function of living tissue, investigators have dissected bodies, isolated tissues and extracted components. After making intellectual and rational classifications, anatomical and chemical structures

have been related to function.

NICOLAS THEODORE DE SAUSSURE (1835) recognised "Schleimstoff", subsequently called mucin, as an entity chemically different from the other compounds of the body.

Later SCHERER (1846) described the precipitation of mucin with cold acetic acid and by using MULDER'S (1839) methods for determining carbon and nitrogen values of protein, he was able to show that these values were significantly lower in mucin and that they contained low amounts of sulphur. EICHWALD (1865) discovered that the hydrolysis of mucin with mineral acids yielded reducing sugars and that purified mucins were free of phosphorus and protein. Sulphate was released by acid hydrolysis. During the latter part of the 19th century, isolations of mucin from cartilage yielded a species of polysaccharide, chondroitin sulphate (ChS) (KRUNKENBERG, 1884; MORNER 1889; SCHNEIDERBERG, 1891). Preceeding the expanding knowledge on the structure of mucins and their residues, ROLLET (1858) had considered a functional relationship, hypothesizing that the mucin acted as an interfibrillar cement. He had noted that the tensile strength of skin was much reduced as a result of mucin extraction.

The mainfold roles of the mucin-like components of tissue are now more clearly understood. The present report attempts to show further the importance of these substances in the actual dynamic maintenance and control of tissue integrity. The models which were adopted were epithelium and 'epithelial-like' cultures.

Mucopolysaccharide defined.

The term mucin has been superseded by a variety of nomenclature. MEYER (1938) introduced 'mucopolysaccharide' (MPS) to describe hexosamine containing polysaccharides of animal origin, occurring either in the pure state or as a protein salt. The prefix'muco' was chosen to denote the relationship of these materials to that of mucous - a physiological expression of a viscous secretion.

However, in vivo MPS's are rarely found as a mucous secretion alone. Connective tissue from both vertebrates and invertebrates owe much of their functional capacity to the MPS. For example, the flexibility and toughness of cartilage, as measured by creep modulus, have been attributed to macromolecular aggregates of MPS content associated with the fibrillar structural moiety, collagen, as opposed to collagen itself, (KEMPSON, MUIR, SWANSON & FREEMAN, 1970). Apart from the structural supportive role that MPS appears to play between fibres and cells of connective tissue (SCOTT, 1975) these chemical species are probably involved in important physiological and pathological responses of the body (DORFMAN, 1958). Examples of physiological roles for proteoglycan are, Ca^{++} and other electrolyte control; in water binding capacity; in the control of extracellular fluid composition; as lubricants and more specifically, in the highly specialized structures of the eye; (ie. the maintenance of a stable transport medium).

The term mucopolysaccharide has been confused with both protein polysaccharides of high protein or peptide content, more accurately defined as glycoproteins, and with glycolipids. Despite this confusion, the general nomenclature has been mainly restricted to

those heteroglycans that contain uronic acid and hexosamine residues. Consequent on the chemical specificity of these molecules, the term MPS is largely replaced in the biochemical literature by the term glycosaminoglycan (GAG).

Since the details and evidence supporting the molecular model for GAGs and their linkages to proteins have been reviewed (RODÉN 1965, 1970; LINDAHL & RODÉN, 1972; MATHEWS, 1975), a brief description should suffice, together with emphasis on special biosynthetic events.

The Glycosaminoglycans and Proteoglycans.

GAGs are classically straight chained polysaccharides, usually consisting of repeating disaccharides. The most typical GAG repeating units which have been isolated from the intercellular mammalian tissue and purified by proteolysis are tabulated in Table I-1. The MW of ChS chains of cartilage is about 2×10^4 (LUSCOMBE & PHELPS, 1967) whilst skeletal keratan sulphate (KSII) has an average MW of $4 - 8 \times 10^3$ (HASCALL & RIOLO, 1972; HOPWOOD & ROBINSON, 1975). Various estimates for the MW of hyaluronic acid (HA) (BALAZS, 1974; LAURENT, 1977) and for heparin (Hep) (HORNER, 1971) range up to several millions. With the exception of HA, GAGs are sulphated and *in vivo* they are covalently linked at intervals to a protein core resulting in total average MW of about 4×10^6 (HASCALL & RIOLO, 1972); these macromolecules being called proteoglycans (PGs) or PG subunit Fig. I-1. The linkage region contains xylose and galactose.

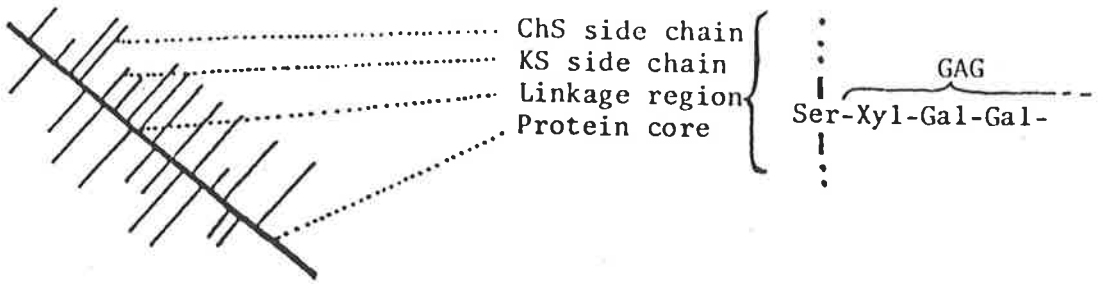


Fig I-1. Schematic representation of a Proteoglycan (PG) macromolecule
Ser,serine;Xyl,xylose;Gal,galactose;ChS,chondroitin sulphate;KS,keratan sulphate.

The term acid mucopolysaccharide (AMPS) will be used in this review where precise chemical nature is not identified e.g. histology; while the terms glycosaminoglycan (GAG) and proteoglycan (PG) will be used where chemical composition is significant.

BIOSYNTHESIS OF PROTEOGLYCANS.

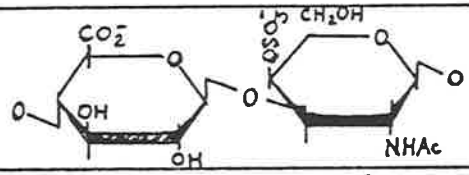
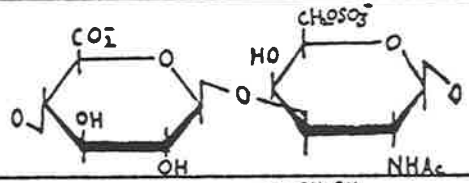
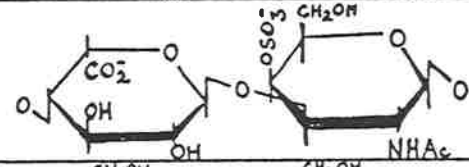
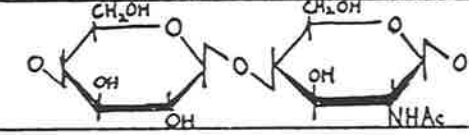
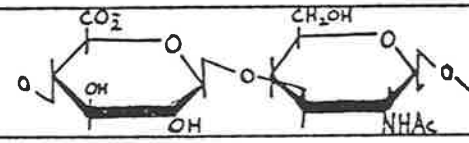
The events in the biosynthesis of PGs are:
(i) protein core synthesis,
(ii) protein-carbohydrate linkage synthesis,
(iii) carbohydrate chain formation, and
(iv) sulphation

The PROTEIN CORE.

BAXTER & MUIR (1975) have fractionated protein 'cores' from those dissociated PGs which have been extracted from hyaline cartilage.

The amino acid analyses, the hydrodynamic size and the GAG chain involvement, suggest that there are at least two classes of PG-protein 'cores' in cartilage. Nevertheless, both appeared to contain alanine as the N-terminal amino acid residue. The proportion of glutamic acid increased significantly with the hydrodynamic size.

Table I-1. Typical saccharide repeating units of glycosaminoglycan chains.

POLYSACCHARIDE		RESIDUES	
Chrondroitin Sulphate A (Ch-4-S)		D-glucuronic acid,2 acetamido-2-deoxy-4-Osulpho-Dgalactose.	
Chondroitin Sulphate C (Ch-6-S)		D-glucuronic acid,2 acetamido-2-deoxy-6-Osulpho-Dgalactose	
+Chondroitin Sulphate B Dermatan Sulphate	DS	L-iduronic acid,D-glucuronic acid, 2-acetamido-2-deoxy-4-Osulpho-D galactose.	
Kerato Sulphate (Keratan Sulphate)	KS	D-galactose,2-acetamido-2-deoxy-6-Osulpho-D glucose.	
Heparatan Sulphate (Heparan Sulphate)	HepS	D-glucuronic acid,2-deoxy-2-sulpho-amino-amino-D-glucose (with O & N-sulphate) 2-acetamido-2deoxy-D-glucose.	similar to Heparin: variability in sulphation and acetylation
+ Heparin	Hep	D-glucuronic acid,L-iduronic acid 2-deoxy-2-sulphamido-Dglucose (with O-sulphate & N-sulphate)L-iduronic acid.	see page 31.
Hyaluronic Acid	HA	D-glucuronic acid,2 acetamido-2-deoxy-D-glucose.	

+ periods of disaccharide containing either L-iduronic acid or D-glucuronic acid occur along the molecule.

Individual protein cores of the PGs can accommodate both KSII and ChS and the linkages were thought to be at threonine and serine amino acid residues respectively (SENO, MEYER, ANDERSON & HOFFMAN, 1965; TSIGANOS & MUIR, 1967 and HEINEGÅRD & GARDELL, 1967).

The molar proportions of xylose to serine (destroyed on alkaline β-elimination) were equivalent in most fractions, indicating that serine residues were attached to a xylose of the ChS. The ratio of serine to threonine residues appeared to be similar in all fractions, but the fractions of smallest hydrodynamic size contained less KS than those of the larger size, implying that in the former, the KS chains were shorter than in the latter.

Glycine, glutamic acid, serine and proline were the predominant amino acids, each consisting of between 10% and 15% of the total number of residues. The amino acid sequence around those serine and threonine residues which could act as xylose acceptors in GAG chain initiation, were important, since neither free serine, nor those not originally linked to ChS could initiate GAG formation. Also, serine residues in proteins other than PGs did not act as xylose acceptors (BAKER, RODÉN & YAMAGATA, 1971). WUSTEMAN & DAVIDSON (1975) have isolated ChS linkage containing polypeptides. They found two non-glycosylated serine residues in the region of the peptide which contained four substituted serines. They confirm the importance of the amino acid residues, glutamic acid and glycine previously noted by MATHEWS (1971) as an

invariant sequence near to those serine residues which are destined to be glycosylated. The glycine had been shown to be adjacent to the C-terminal of serine (KATSURA & DAVIDSON, 1966; JOHNSON & BAKER, 1973). They also showed that there are eight amino acids between the residues which are involved in the linkage (Fig I-2).

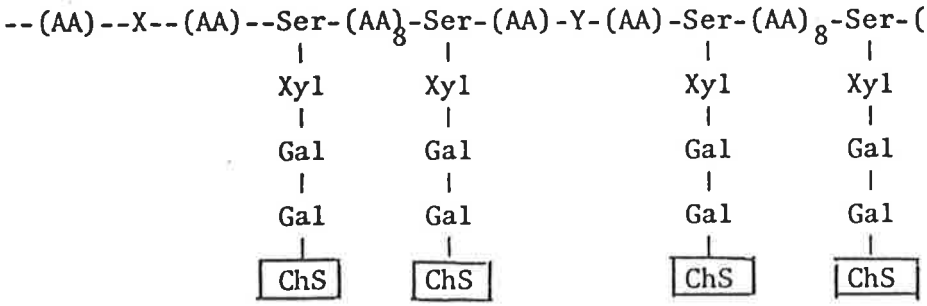


Fig I-2. Schematic representation of the arrangement of chondroitin sulphate chain off the polypeptide core of proteoglycan. At least one of the linking serines may be terminal following trypsin and chymotrypsin digestion (it may be terminal in native material). Chondroitin chains may be clustered at the carboxyl terminus.
(AA),sequence of amino acids;Ser,serine;Xyl,xylose; Gal,galactose;ChS,chondroitin sulphate.

Antigenicity of PG is due to the protein moiety (HIRSCHMAN & DZIEWIATOWSKI, 1966). The smaller PGs differ from the larger by reacting directly with antibody, whereas larger PGs must be pre-digested with hyaluronidase before they will react. (BRANDT, TSIGANOS & MUIR, 1973). The smaller PGs have fewer antigenic determinants than the larger PG. These determinants are common to all PGs and are situated at the GAG linkage regions. Radioactive isotope tracer experiments have shown that such PGs are not precursors, nor degradation products of the majority of PGs. Different PG pools have been identified on other criteria such as

half life, degradability, pinocytotic susceptibility etc. (See GALLAGHER 1977). The protein cores of other PGs may also be characteristic of their tissue e.g. the multichain form of Hep depends on a unique polypeptide core containing only serine and glycine in equimolar proportions (HORNER 1977).

GAG CHAINS.

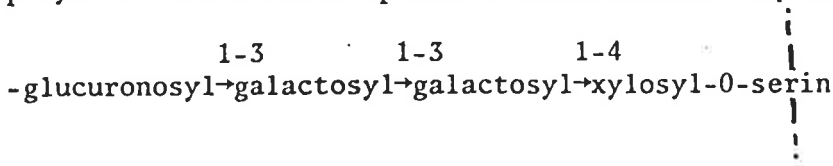
The biosynthesis of sulphated GAGs, reviewed elsewhere in considerable detail (e.g. STOOLMILLER & DORFMAN, 1969; RODÉN, 1970) commences with free glucose and a series of transglycosylation reactions. Energy is derived from nucleotide triphosphates during the formation of the nucleotide sugars.

Nucleotide triphosphate + glycoside-1-P.
⇅ Pyrophosphorylase
Nucleotide diphoshate- glycoside +PPi

LELOIR (1951) first demonstrated uridine diphosphoglucose and subsequently other intermediate metabolites of sugars from natural sources were isolated.
For example, uridine diphosphate -D-glucuronic acid (DUTTON & STOREY, 1953; SMITH & MILLS, 1954; STOREY & DUTTON, 1955) and uridine diphosphate-N-acetyl glucosamine (CABIB, LELOIR & CARDINI, 1953).

Where appropriate, acetylation of glucosamine-6-P through acetyl - Co A preceded uridine attachment as precursors of ChS (PERLMAN, TELSER & DORFMAN, 1964); of HA (SCHILLER, SLOVER, DORFMAN, 1964) and of Hep (SILBERT, 1962, 1963).

Stepwise transfers of the monosaccharide units from their nucleotide sugars to the non-reducing terminals of the nascent GAG chains are initiated at a polysaccharide-protein linkage region. The linkage region for ChS (-4-&-6-) and Hep-like-polysaccharides has a specific trisaccharide sequence as follows:



(RODÉN & SMITH, 1966; HELTING & RODÉN, 1968). Although serine is the major amino acid in the protein core of most PGs, threonine has been proposed for KSII (TSIGANOS & MUIR, 1967; HOPWOOD & ROBINSON, 1974) but the evidence has been contested by CHOI & MEYER, (1975). More recently AXELSSON & HEINEGÅRD, (1978) have indicated the involvement of asparagine residues in the linkage region of corneal KSI.

Several workers (TODO & SENO, 1970; HASCALL & RIOLO, 1972; HEINEGÅRD, 1972 and BAXTER & MUIR, 1975), have found considerable amounts of glutamic acid associated with KSII and they have suggested that glutamic acid or a derivative is involved in an alkali stable linkage between KSII and protein.

O-and N-LINKED OLIGOSACCHARIDES.

Some recent interest has focused on the consistent observations of small amounts of sugars other than those mentioned previously; mannose and fucose particularly. Hitherto, these were considered to be contaminants or some expression of microheterogeneity. However,

THONAR, & SWEET, 1979 and GREGORY 1981 have described both O-and N-linked oligosaccharides on the protein cores of cartilage PG and dermatan-PG respectively.

On the cartilage PG there are up to 50 chains of oligosaccharide with molecular weights ranging between 1200 and 2000. These sugars represent less than 3% of the total PG weight. The O-glycosidicly linked oligosaccharides occur throughout the molecule while in the cartilage PG, the N-glycosylamine - linked oligosaccharides are mainly situated on the hyaluronate-binding region (THONAR & SWEET, 1979; DELUCA, LOHMANDER, NILSSON, HASCALL & CAPLAN, 1980; LOHMANDER, DELUCA, NILSSON, HASCALL, CAPUTO, KIMURA & HEINEGÅRD, 1980).

CHONDROITIN (-4- and -6-) SULPHATE.

The sugar residues of ChS consist of D-glucuronic acid, 2-amino-2-deoxy-D-galactose, acetyl and sulphate. The -4- and -6-(or A and C) ChS molecular species are distinguished by their optical rotation and the solubility of their calcium salts in ethanol. The structural difference lies in the position of the sulphate ester in the hexosamine residue, i.e. in the -4- or -6-C position, (see Table I-1). This polysaccharide is distributed widely throughout connective tissues in mammals. Normally ChS is covalently linked to a protein core in the form of PG. However patients suffering from certain forms of mucopolysaccharidoses, a group of genetically inherited diseases, do present with free GAG chains excreted in their urine - for

example it might be ChS; Type VII hyperglycosaminoglycanuria, or DS/HS; Type 1H hyperglycosaminoglycanuria.

The polysaccharide polymers of ChS (Table I-1) are synthesized by the concerted action of six glycosyl transferases, the N-acetylgalactosamine residues being sulphated by either of the two sulphotransferases which are thought to be specific for the -4- and -6- position (RODÉN, 1970). This will be discussed later. These enzymes are associated with membranes of the endoplasmic reticulum, (ER).

Those enzymes which catalyse consecutive transfer steps appear to be located in adjacent positions in an organized multi-enzyme complex, probably formed as a result of specific interaction between individual enzymes (HOROWITZ & DORFMAN, 1968; STOOLMILLER, HOROWITZ & DORFMAN, 1972). By analogy, a number of complexes which catalyse multi-step processes have been isolated from membranes e.g. mitochondrial electron transfer and the Na^+ & K^+ ATPase of the plasma membrane (ATKINSON, GATENBY & LOWE, 1971).

Evidence for such a discrete multi-enzyme complex in polysaccharide systems is, as yet, only indirect. Although SCHWARTZ, DORFMAN & RODÉN (1975) have now reported specific interaction between enzymes catalysing the first steps in polysaccharide formation in ChS synthesis, GREBNER, HALL & NEUFELD (1966) had demonstrated the transfer of xylose from UDP-xylose, an endogenous donor in the initial step in linkage formation. BAKER, RODÉN & STOOLMILLER (1972) searched for suitable xylose acceptors. The protein core of cartilage PG obtained by SMITH degradation (periodate oxidation, followed by reduction with

borohydride and mild acid cleavage) was the best acceptor (Km= 0.064 mM as conc. for serine). Isolation of (^{14}C)-xyloserine from proteolytic digests of the reaction product indicated that xylose was indeed transferred to the serine residues.

The xylotransferase appeared to be unique among the chondroitin sulphate glycosyltransferases in being loosely bound to the membrane. It has been purified by STOOLMILLER et al., (1972) but SCHWARTZ, DORFMAN & RODÉN (1975) have further purified and characterized both its structure and its mode of action. The enzyme-substrate (protein core) interaction could only be dissociated by the substrate itself; however, the interaction between individual molecules could be dissociated by salt. The enzyme readily aggregated at low ionic strength forming cloudy precipitates.

The second reaction of ChS synthesis is the transfer of galactose from UDP-D-galactose to the xylose-protein which had been catalysed by the former enzyme galactosyltransferase I. There are several acceptors for the active sites of galactosyltransferase I which include D-xylose, O-β-D-xylosyl-L-serine, methyl and p-nitrophenyl β-D-xylopyranoside (RODÉN, 1970; OKAYAMA, KIMATA & SUZUKI, 1973; LEVITT & DORFMAN 1973). Galactosyltransferase I is tightly bound to the membrane of the endoplasmic reticulum (ER) by ionic and hydrophobic interactions, van der Waals forces and hydrogen bonding.

The isolation and purifications of the other glycosyltransferases are still being actively pursued. By a combination of detergent and alkali solubilization HELTIN (1971) isolated two galactosyltransferases from a Hep producing mastocytoma, which were analogous to the enzymes involved in ChS biosynthesis. Subsequently SCHWARTZ et al., (1975) considerably increased the efficiency of the isolation by varying the detergent component (from Tween 20 to Nonidet P-40) and by replacing the alkali with an ionic salt concentration gradient.

This enabled separation of N-acetylgalactosaminyltransferase from the other galactosyltransferases which included galactosyltransferase II (UDP-D-galactose : 4-0- galactosyl-D-xylose galactosyltransferase), the enzyme which added the next galactose to the carbohydrate linkage region.

Consequent on the multi-factor isolation procedures, together with affinity separation techniques, SCHWARTZ et al. considered the concept of the multi-enzyme complex as a mechanism for GAG side chain assembly. Nevertheless, it is not yet possible to formulate a precise description of the in vivo relationship between all the individual glycosyltransferases. There does appear to be specific interaction between the xylosyltransferase and the galactosyltransferase I which may be of physiological importance. However, the chain initiating xylosyltransferase, being of greater solubility, may direct or transport the core protein from a point of release, the polysome, to the exact location of galactosyltrasferase I. Further sequential repeated addition of the galactose, uronic acid and hexosamine to the GAG chain

may then occur at the membrane surface being catalysed by discrete individual enzymes.

HELTING & RODEN, (1968) had shown that glucuronic acid could be linked to the galactose, however they did not confirm that the glucuronosyltransferase could also catalyse the attachment of glucuronic acid to galactosamine. Accumulation of UDP-xylose will however inhibit UDP-glucuronic acid formation (NEUFELD & HALL,1965) by inhibition of UDP-glucose dehydrogenase activity. The alternate monosaccharides glucuronic acid and galactosamine are then progressively added to the growing chains. Subsequently the growing chains are sulphated. The determination of length of the GAG chains, or the overall PG size is not well understood. However in the final chapter the relationship of chain type, PG size and metabolic pool will be discussed. HORWITZ (1972) has shown that the ratios of ChS-glycosyltransferase activities vary from one segment of the ER to another. These enzymes involved in the formation of the carbohydrate-protein linkage region are located largely in the rough ER while the polymerizing enzymes are more evenly distributed throughout both the rough and the smooth ER. In other cell free systems, using microsomal preparations, TELSER, ROBINSON & DORFMAN,(1966) had shown that appropriate single sugars could be added individually to non-reducing ends of exogenous oligosaccharide acceptors which had been derived from ChS. However these oligosaccharides did not act as "primers" for polymerization in the formation of long Ch chains.

SILBERT (1964) & PERLMAN et al. (1964) utilized endogenous primers from microsomal preparations, as distinguished from oligosaccharide acceptors, to effect polymerization of polysaccharide chains. The chains were constituted of N-acetyl chondrosine repeating units and were larger than standard Ch-4-S (SILBERT, 1964). They appeared to be linked to protein. More recently, RICHMOND, DELUCA & SILBERT, (1973) further investigated the endogenous microsomal material as primers for polysaccharide polymerization. They also discussed the likelihood of concurrent sulphation.

The high levels of sulphated residues are characteristic of the PGs (excluding HA). Inorganic sulphate incorporation is one of the most important diagnostic functions of AMPS metabolism: prominent reviews on sulphation of the MPS's are available (DZIEWATKOWSKI, 1951, 1961, 1962; ROBINSON & DORFMAN, 1969; GREGORY & ROBBINS, 1960). D'ABRAMO & LIPMAN (1957) first described 3'-phosphoadenosine 5' phosphosulphate (PAPS) as the direct donor in the sulphation of ChS. Subsequently PAP (^{35}S) together with soluble enzyme systems have been used to demonstrate the incorporation of sulphate into GAG acceptors (ADAMS, 1960; SUZUKI & STROMINGER, 1967; PERLMAN, TELSER & DORFMAN, 1964; MEEZAN & DAVIDSON, 1967; ROBINSON, 1969). Sulphation of AMPS is a two step reaction, the sequence being represented as follows:

$SO_4^=$ + adenosine triphosphate —(ATP sulphurylase)→ 3' phosphoadenosine sulphate + pyrophosphate

3' phosphoadenosine sulphate + adenosine triphosphate —(APS Kinase)→ 3' phosphoadenosine 5' phosphosulphate (PAPS) + adenosine diphosphate

Experimentally, the yield of sulphated products was very small. Normally, the bulk of chondroitin sulphating activity appeared to be particulate, occuring as microsomal sulphotransferases rather than as soluble enzymes (DELUCA & SILBERT, 1967, 1968).

RICHMOND, DELUCA & SILBERT (1973) have suggested that the primer molecule on which the ChS chain was built plays a role in determining the site of sulphation by being structurally programmed to lead to -4-or -6- sulphation.

These workers also submit evidence which implies that the process of sulphation occurred concurrently with the process of polymerization or immediately following polymerization. Indeed SUZUKI & STROMINGER (1967) had earlier discovered UDP-galactosamine-4-S in extracts of hen oviduct but, since they were unable to demonstrate further utilization of this compound, they concluded that sulphation follows polymerization.

HOROWITZ & DORFMAN (1968) postulated that polymerization occured as the growing polysaccharide chain moved along the ER system with sulphation occurring after the polysaccharide had moved to a separate site. SUZUKI, TRENN & STROMINGER (1961) reported the existence of three sulphotransferase activities, but MEEZAN & DAVIDSON (1967), commenting on the positioning of the sulphate, concluded that the cartilage sulphotransferases were probably non-specific. 4-sulphation occurred preferentially when the acceptor was a polysaccharide-protein complex while 6-sulphation occurred when free ChS chains were available. They further

suggested that fully formed non-sulphated chains do not subsequently acquire sulphate. However, others have shown sulphate transfer to pre-formed polysaccharide chains, demonstrating the ability of various compounds as $SO_4^=$ acceptors (see BÖSTRÖM & RODÉN in Amino Sugars IIB). ROBINSON (1969) isolated an enzyme which transfered (^{35}S)-PAPS to ChS and which had optimal pH and ionic strength requirements. The percentage of Ch4-S and Ch-6-S formed was pH dependant. He also showed that chondroitin had a high specific activity for conversion to ChS. In spite of the important effects which pH and ionic strength had on sulphation specificity, ROBINSON commented that the notion of an identical non-sulphated precursor giving rise to both Ch-4-S and CH-6-S is not impossible. Notwithstanding the cell free and extracellular systems used to investigate sulphation criteria, the physiological activity in fully formed AMPS is certainly an intracellular one.

However, the isolation by ROBINSON (1969) of a nonsulphated fraction possibly corresponding to chondroitin, suggests that polymerization and sulphation can be quite separate steps. Several workers, PERLMAN et al. (1964), SILBERT & DELUCA (1968) and DELUCA & SILBERT (1967) had shown that non sulphated MPS could be prepared in cell free systems in the absence of PAPS or PAPS synthesizing enzymes.

More recently DERGE & DAVIDSON (1972) suggested that sulphation was a pre-requisite for further polysaccharide formation, but the evidence of RICHMOND et al. (1973) did not entirely substantiate this view in reviewing the sulphation of Hep. However LINDAHL et al.

(1977) has reported evidence for the role of sulphation itself in the regulation of the biosynthesis of this particular GAG. Not only does N-sulphation dictate substrate recognition for the epimerase but it also appears to be necessary before O-sulphation is initiated. Of course the biosynthesis of Hep and heparan sulphate (Hep S) do present their own peculiarities; specifically their mechanisms for N-sulphation. Both these compounds have amino groups that are N-acetylated and N-sulphated. CIFONELLI & DORFMAN (1960) had proposed an acetyl-sulphate exchange and LINDAHL subsequently demonstrated the importance of N- deacetylase in regulating ensuing N-sulphotransferase activity. He also reported that PAPS stimulated N-deacetylation (see Heparin section). LINDAHL's evidence further emphasizes the orderly fashion in which these macromolecules are constructed. Much of the general review literature commonly depicts simplistic GAG chains as uniformly sulphated repeating residues within each polymer.

WASTESON & LINDAHL (1971) have shown that sulphate density is lower in the vicinity of the carbohydrate-protein linkage region. LINDAHL conceived that the polysaccharide portion immediately adjacent to the protein core may be relatively inaccessible to the sulphotransferases. In spite of uncertainty over the sequence of events during sulphate incorporation the mechanism of rapid sulphation with rapid polymerization of ChS (RICHMOND et al., 1973) could be achieved by a "complex" of enzymes intimately organized to complete the total assembly and sulphation of ChS.

The general consensus of opinion on the mechanism of sulphation of most species of polysaccharide is that sulphated oligosaccharide or nucleotide precursors are not polymerized.

HYALURONIC ACID.

The lack of any single, comprehensive review on HA biosynthesis reflects the relatively limited understanding in this area. HA has been isolated from a large number of biological sources some of which are tabulated below, (Table I-2).

Table I-2. The Distribution of Hyaluronic Acid from Biological Sources

Source	ref.	
Vitreous humour	Meyer & Palmer	(1934)
Connective tissues	Asboe-Hansen	(1950)
Umbilical cord	McClean & Hale	(1941)
Embryonic pig skin	Loewi & Meyer	(1958)
Human serum	Deutsch	(1957)
Cock's comb	Boas	(1949)
Rous chicken sarcoma	Warren et al.	(1949)
Rabbit's ovum	.. .. do	(1957)
Walls of veins	Moore & Schoenberg	(1957)
Other parts of arteries	.. .. do	(1957)
Synovial fluids	Balazs et al	(1959)
Aerobacter aerogenes	Warren	(1954)
Pseudomonas aeruginosa	Jensen	(1957)
Streptococcus pyogenes	Pierce & White	(1954)
Cartilage, laryngeal	Hardingham & Muir	(1973)

HA is unique amongst the GAGs in that it is normally unsulphated and is essentially free from protein. Therefore while the synthesis of some of the other GAG side chains appears to be dependent on the concerted events of a multi enzyme packet to produce chain initiation at the appropriate amino acid on the protein core, our understanding of HA biosynthesis remains obscure.

HOPWOOD & DORFMAN (1977) isolated from the incubation of microsomal preparations from a rat fibrosarcoma, but butanol-soluble components containing radio active label derived from UDP-(^{3}H) glucosamine and UDP-(^{14}C)-glucuronic acid. Further study revealed the presence of a lipid-pyro-phospho-GlcNAc-GlcA compound. These authors raised the possibility that this compound may be an intermediate in the synthesis of HA as well as in Hep & HepS which themselves contain GlcUA-GlcNAc disaccharide sequences. Furthermore, trace amounts of tetra and hexasaccharide linked to lipid have been identified together with the lipid-GlcUA-GlcNAc. This latter material may serve as an acceptor for glucuronic acid. Such lipid mediated systems are not without precedence (ANDERSON, MATSUHASHI, HASKIN & STROMINGER, 1965; WAECHTER & LENNARTZ, 1976; TURCO & HEATH, 1976). Following the identification of xylosyl-phosphoryl dolicol (LELOIR, STANELONI, CARMIATTI & BEHRENS,1973) and N-acetyl glucosaminylpyrophoshporyl dolicol (MOLNAR, CHAO, IKEHARA, 1971; GHALAMBOR & JEANLOZ, 1974) in various mammalian tissues, a role for these polyprenols may be to act as acceptors for HA chain initiation or as lipid mediated donors of the sugars. Indeed in other unpublished studies by the Author, lipid analyses of organic solvent extracts (methanol/chloroform) from cells and from culture media of suspension and monolayer cultures of chondrocytes which had been selected from quantitatively high and quantitatively low HA producers, demonstrated different lipid components on thin layer chromatography according to their levels of HA production*. Subsequently, OKAYAMA, TAKAGAKI, KAJI & TOOLE, (1977) have shown HA-lipid associations in fractions from cell surface preparations, but not in secreted HA. Indeed there may be alternative biosynthetic pathways.

*indicates reference to appendix.

Evidence against the lipid component being either a saccharide donor or acceptor in GAG synthesis is the finding that tunicamycin, an inhibitor of lipid-pyrophosphate-GlcNAc synthesis (TKACZ & LAMPEN, 1975) showed little inhibition of total HA synthesis in cell free preparations of a rat sarcoma (HOPWOOD & DORFMAN, 1977), in cultured rat glial cells (HORWITZ & DORFMAN, 1977) nor in cell free preparations of Group A streptococci (SUGAHARA, SCHWARTZ & DORFMAN, 1977). BAXTER & FRASER, 1980, BAXTER, FRASER & HOLMS (1978), have recently reported that HA does contain small amounts of amino acid, and saccharides other than the fundamental constituents of its polysaccharide chain. (TOOLE et al. (1980) have published important confirmation of this observation in a separate system).

These workers are aware of the difficulty of analysis of HA since the appearance of minute proportions of saccharides and amino acid residues may be contaminants of other tissue components from which the HA was initially extracted. However, both fucose and leucine have been shown to incorporate into purified HA. Moreover, the degree of incorporation of these components differs from the incorporation of acetate, glucosamine and glucose in response to stimulation of HA synthesis by dibutryl cyclic adenosine monophosphate. Thus the minor components may play a specific role in both the structure and the biosynthesis of mammalian HA. Nevertheless, HA synthesis by Streptococci may not be dependent upon concomitant protein synthesis, (STOOLMILLER & DORFMAN, 1967, 1969) but such an anomolous synthesizing system may prove to be an adaptive specialization.

An attractive alternative proposal for HA synthetic sequences was described by STOOLMILLER & DORFMAN (1969). They postulated the existence of an intracellular membrane or secondary template into which appropriate synthesizing enzymes were inserted. This insertion would proceed in an orderly fashion following synthesis at the ribosome. These authors cite the evidence which shows that UDP-substrates and Mg^{++} stabilize activation of ChS polymerase by 4%n-butanol (TELSER, ROBINSON & DORFMAN, 1966). Such data are consistent with the idea that Mg^{++} and the UDP- moiety stabilize spatial arrangements of the specific enzymes. However these spatial arrangements of the enzyme may not be relevant to the translational mechanism.

Apart from the complex biochemical events which result in the synthesis of HA intracellularly, two other enigmas exist.

Firstly how does a highly hydrophilic molecule with a molecular weight in excess of 6×10^6 become secreted?

Second, in some tissues, PGs, synthesized concomitantly with HA, have the ability to interact with HA to form even larger maromolecular aggregates; how is this prevented intracellularly? Indeed the in vitro synthesis of PG by cells which synthesize both HA and a PG which will interact with HA, can be specifically regulated by exogenous HA in the culture medium, TOOLE, 1973; WIEBKIN & MUIR, 1973a,b; WIEBKIN, HARDINGHAM, & MUIR, 1975a; SOLURSH, VAERWYCK & REITER, 1974) possibly by a mechanism associated with specific cell surface binding (WIEBKIN & MUIR, 1975). Indeed LOWTHER, (1978) speculates that HA will itself regulate PG synthesis at the site of formation of protein GAG linkage. Lipid mediated synthesis and intracellular transport are attractive modes of action.

KERATAN SULPHATE (Kerato-suphate).

Different keratan sulphates (KS) have been isolated from corneal stromae and from hyaline cartilage. Unlike other GAGs, KS possesses D-galactose in place of uronic acid. Two classes of KS have been described and are more commonly referred to as KSI or corneal kerato-sulphate and KSII(cartilage kerato-sulphate) or skeletal kerato-sulphate.

The basic structure of the repeating disaccharide is as shown (Table I-1) but sulphation can occur to a variable extent at the C-6 positions of both the N-acetylglucosamine and the galactose residues.

There is a molar excess of galactose over N-acetylglucosamine; these extra galactose residues may be linked as side branches from the KS chain(BHAVANANDAN & MEYER, 1968) while other sugars appear to be component parts of the KS structure e.g. fucose, mannose, sialic acid and N-acetylgalactose (GREGORY & RODEN, 1961; MATHEWS & CIFONELLI, 1965; SENO et al, 1965; BRAY, LIEBERMAN & MEYER, 1967). They may occupy terminal positions or side branches off the main KS chain. The two types of KS should be regarded as distinct PGs. Although the GAG side chains are similar, the structural conformation and constituent parts are different (BETTLEHEIM & PLESSY, 1975; AXELSSON & HEINEGÅRD, 1975; 1978).

About one third of the PGs extracted from bovine corneal slices consist of a fraction rich in KS and small amounts of associated

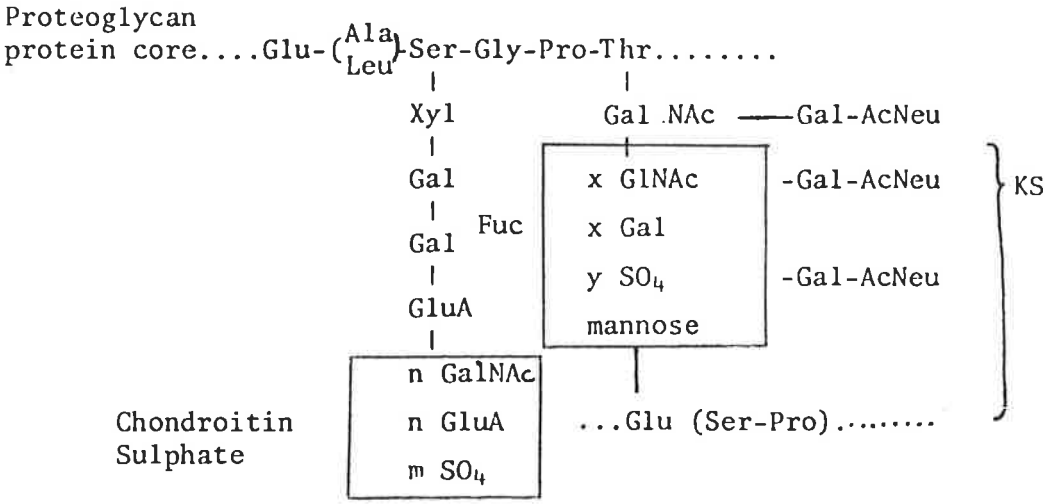
oligosaccharides but no galactosaminoglycan (AXELSSON & HEINGÅRD, 1975). Such KS has not been identified in any other tissue. In comparison, KS from cartilaginous tissues (including nucleus pulposus) is attached to the protein core to which ChS is also covalently bound (HARDINGHAM & MUIR, 1972, 1973a, 1973b, ROSENBERG, PAL & BEALE, 1973).

Extensive biochemical analysis has attempted to identify the structural differences between KS-PGs isolated from corneas and skeletal cartilage (AXELSSON & HEINEGÅRD, 1978). Corneal KS-PG contains monomers with MW about 72,000 and additional polydispersed larger molecules but with the same chemical composition. The analyses indicate that these PGs consist of 45% protein, 30% KS and 10-12% oligosaccharide with the remainder being counter ions. The MW of the protein core is approx 32,000. The total MW of KS chains would be 22,000 and the oligosaccharides 8,000. There appear to be interchain disulphide bonds. From rough estimates based on the MW of the chains (9,000-19,000), (LAURENT & ANSETH, 1961; GREILING & STUHLSATZ, 1966) the average KS-PG would contain only 1,2 and 3 chains, but since there is a wide variation in MWs of both PG and KS chains some molecules would have several chains.

The oligosaccharide structures associated with the KS fractions have MWs of between 700-1,000 and AXELSSON estimates that there may be up to 12 oligosaccharide chains in the average molecule. These oligosaccharide chains have not yet been proved unequivocally

to be covalently linked to the PG. The PG monomers form aggregates possibly promoted by a large molecular weight fraction of the KS-PG preparation. Indeed aggregation may only be an artifact of the *in vitro* preparation.

HOPWOOD & ROBINSON, 1974, propose a structure for KSII-PG (with ChS) as follows:-



values x=24; y=31; m=25; n=28

AcNeu, N-acetylneuraminic acid; GalNAc, galactosamine; GlNAc, glucosamines. GluA , glucuronic acid.

In contrast AXELSSON & HEINEGÅRD state that the KSI-PG show poly-dispersity and heterogeneity. Glutamine (or glutamic acid) appears to be among the predominant amino acids of KS-PGs and RODÉN (1956) found that the glutamine stimulated the *in vitro* incorporation of (^{35}S)-sulphate into KS in pig *nucleus pulposus.* Variation in the content of fucose

and sialic acid in KS isolated from tissues of various age and origin may reflect natural heterogeneity of KS composition introduced during its biosynthesis or it may stem from hydrolysis of labile sugars during isolation and purification. KS has not been shown to be present in epithelial tissue.

DERMATAN SULPHATE (Chondroitin sulphate B).

Dermatan sulphate (DS) has been detected in skin, lung tendon, heart valve, aorta, spleen, brain, ligamentium nuchiae, and in urine from patients with various mucopolysaccharidoses (Types I, II, III and IV). Its schematic structure is a hybrid molecule with repeating units of iduronic acid and galactosamine-4-SO_4 (Table I-1) interspersed with D-glucuronic acid containing periods.

Three alternative pathways for the formation of the unusual hexuronic acid moiety have been proposed (FRANSSON, 1973).

Pathway 1 proposes the formation of UDP-iduronic acid from UDP-glucose via UDP-idose.
Pathway 2 depicts a direct epimerization at C5 of UDP-glucuronic acid to form UDP-iduronic acid (JACOBSON & DAVIDSON, 1962). Any precedent for UDP- glucuronic acid- 5'-epimerase in the biosynthesis appears to be unique, especially since there is no evidence for the involvement of the nucleotide sugar in GAG biosynthesis; RODEN & DORFMAN, (1958) had shown that glucose -6- (^{14}C) was incorporated into the carboxyl group of L-iduronic acid.

Pathway 3 assumed the C5 epimerization of hexuronic acid moieties may take place on the polymer without scission of the carbon skeleton e.g. bacterial alginate C5 epimerization of D-mannuronic acid to L-guluronic acid on the polysaccharide (HAUG & LARSEN, 1971).

Further, L-iduronic acid is formed by epimerization at C5 of those glucuronic acid residues previously incorporated into the GAG chain during in vitro biosynthesis of Hep by microsomal fractions of a mouse mastocytoma (LINDAHL, BACKSTRÖM, MALMSTRÖM & FRANSSON, 1972). However, LINDAHL (1977) does not consider this epimerase to be similar to the epimerase involved in DS synthesis. (^{3}H)-C5-chondroitin appears to be analogous to the DS epimerase substrate, but it does not liberate tritium on incubation with mastocytoma epimerase.

FRANSSON, 1973, cites strong evidence which shows that fibroblasts in cultures can synthesize and secrete a product that contains very little, if any, D-glucuronic acid. Some of the L-iduronic acid moieties of this polymer are subsequently transformed to D-glucuronic acid again without cleavage of the DS chains. This epimerization appears to take place extracellularly and is catalysed by an enzyme derived from fibroblasts.

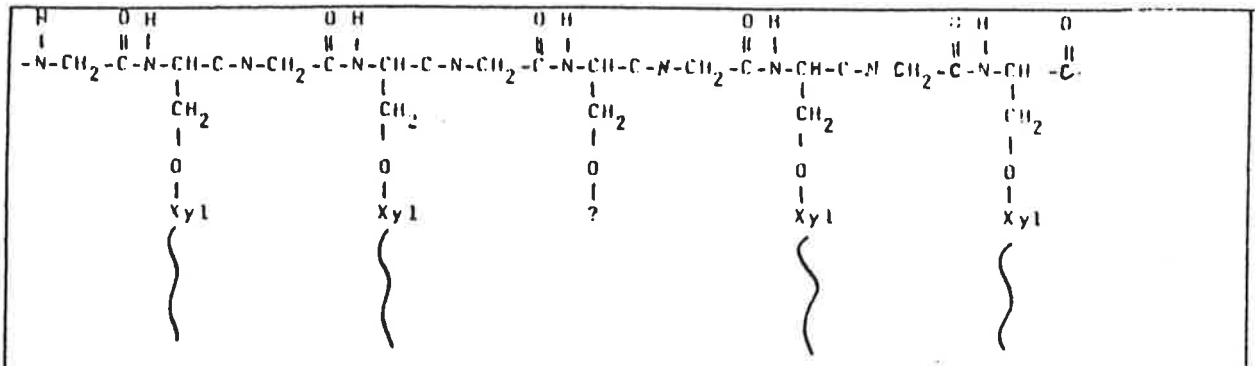
HEPARAN SULPHATE, THE HEPARIN-LIKE POLYSACCHARIDES AND HEPARIN.

Heparan sulphate (HepS) first reported by JORPES & GARDELL in 1948, was considered to be a discrete polysaccharide species and was not thought to represent one end of a spectrum of molecules with Heparin (Hep) at the other although they are chemically very similar (Table I-1).

The detailed structures of Hep and Hep S have not been as well documented as have those of the other GAGs and it remains unclear whether the two kinds of uronic acid α-L-iduronic and β-D-glucuronic acid, are randomly distributed or assembled in clusters as shown for DS. Iduronic acid units have been found in highly sulphated regions of the polymer of both Hep and HepS. (HÖÖK, LINDAHL & IVERIUS, 1974).

Structural studies of Hep S suggested that such regions contain both N-& O-sulphate groups while the N-acetylated glucosamine residues are largely devoid of O-sulphate.

Hep is found intracellularly in mast cells, and basophilic leucocytes (RILEY, 1959) while Hep S is a ubiquitous cell surface molecule (KRAMER, 1971). Characteristically both molecules have protein linkage regions containing serine but the actual core structure of Hep is unknown. Both HORNER (1977) and LINDAHL (1977) present strong evidence for a polypeptide core of serine and glycine



Tentative structure for rat skin heparin. The structure of the individual chain is:

$-Xyl-(Gal)_2-Glc\ UA-(GlcNAc-UA)\ 1{:}2-Glc\ NSO_3-(UA-GlcNX)_a-(UA-Glc\ NX_b-UA-Glc\ NX)_c$

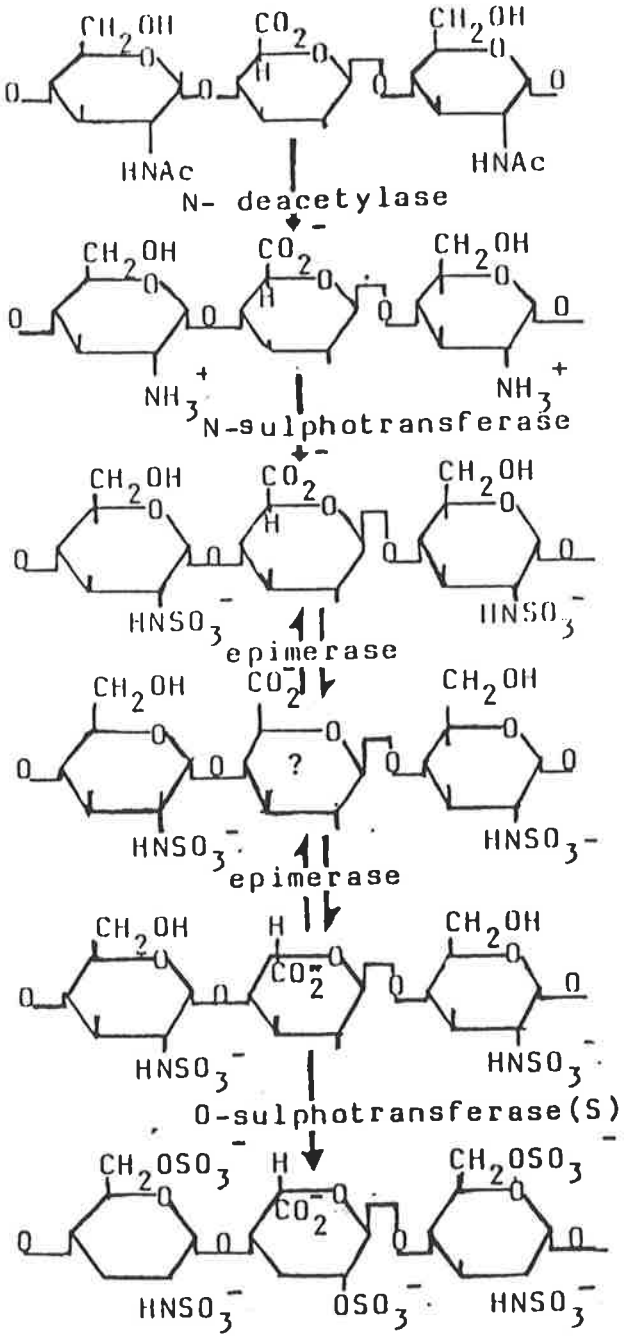
UA, unspecified uronic acid (D-glucuronic or L-iduronic); GlcNAc, D-glucosamine with acetylated amino group; $GlcNSO_3$-, D-glucosamine with sulphated amino group; GlcNX, D-glucosamine residue with either sulphated or acetylated amino group.

Each polysaccharide chain M. Wt=60,000-100,000. Xylose joins the chains to - $(Ser-Gly)_n$-; a maximal amount of 1 unsubstituted out of 3 serine residues may be inferred, (ROBINSON, HÖÖK, ÖGREN & LINDAHL, 1978)

SILBERT (1975) has synthesized Hep-like polymers derived from a mouse mastocytoma in cell free systems, a reaction sequence which was initiated by polymerization of N-acetylglucosamine and glucuronic acid. These were derived from UDP sugar precursors. Then, by step wise addition of the monosaccharides, non-sulphated chains were formed. They showed resistance to testicular hyaluronidase, characteristic of Hep. Subsequent sulphation occurred in the presence of PAPS. Recent studies by LINDAHL et al., (1977) and his co-workers have revealed important biosynthetic relationships between the mechanisms of N-and O-sulphation (sulphamino-groups and ester sulphate groups respectively) and the C5-epimerization reaction which leads to the formation of L-iduronic acid units.

Although the overall sulphation reaction was rapid (30 secs) it did not involve the whole pool of molecules available. The reaction was successively repeated by passing a limited number of molecules through the pathway leading to sulphation until the pool of non-sulphated precursors was depleted.

The formation of N-sulphated groups required the deacetylation of GlcNAc; deacetylation can occur in the absence of PAPS but may be stimulated by PAPS.



The tentative reaction sequence leading to the formation of a fully sulphated heparin tri-saccharide segment.
The structure of the intermediate formed during uronic acid epimerisation is unknown; a C5 carbonium ion, carbanion or radical are all conceivable as is Δ 4,5 unsaturated species.

N-sulphation and O-sulphation did not occur simultaneously, the latter depending on the former. C5 epimerization of the glucuronic acid unit which had been previously incorporated into the polymer also appeared to be related to the sulphation process.

Indeed, LINDAHL states that deacetylation represents a key step in the biosynthetic regulation which provides the proper structural configuration for the subsequent series of reactions involving N-sulphation, uronic acid epimerization and O-sulphation which finally completes the Hep molecule. The level of activity of deacetylase may determine whether a given precursor polymer may be formed into Hep or HepS. HepS should, however, not be regarded as a metabolic precursor of Hep. Instead, the two types of polysaccharide should be regarded as end-products of separate, although functionally related, biosynthetic pathways.

PHYSICO-CHEMICAL STRUCTURE AND MOLECULAR FUNCTION.

The quarternary structures of PGs are complicated by:

a. the constituent repeating saccharide units of the GAGs and the ratios of GAG species on any one protein core.

b. the sugar configuration and repeating unit linkages together with the degree of O- and N-sulphation.

c. the variability of amino acid sequences in the protein cores and their tertiary structures.

d. the frequency and spatial distribution of the O-glycosidic linkage of the GAGs attached to the protein core at serine or threonine residues.

e. the GAG chain lengths and the ratios of long to short lengths on any one protein core.

f. the relation of similar and different neighbouring PGs to one another as a function of local concentration in tissues.

g. the relation with other structural components e.g. collagen, glycoprotein.

h. ionic concentration.

i. hydration.

LAURENT (1968) has lucidly described the effect and importance of steric exclusion in a model three component system consisting of (i) the solvent (ii) the globular protein (iii) the high molecular weight polysaccharide*. PGs in tissues can be regarded as concentrated solutions and will behave with the characteristic properties of concentrated polysaccharide solutions (PRESTON & SNOWDEN, 1973; PRESTON, 1977). The molecular exclusion effects are important in the regulation of the extracellular protein distribution between various tissues or intra-tissue sites. Larger plasma proteins should be excluded from the polysaccharide containing compartments to a greater extent than smaller ones. This phenomenon should therefore prevent dilution of protein over large extracellular connective tissue spaces thus acting to confine the higher molecular weight (active) substances to the vascular system e.g. albumin: γ-globulin is higher in joint fluid than in plasma. Indeed as early as 1939, ROPES, BENNETT & BAUR, when considering colloidal osmotic pressures of synovial fluid, reported higher osmotic pressures than could be accounted for by the contribution of HA and serum proteins alone.

Exclusive phenomena causing a decrease in solubility of other macromolecules could also cause precipitations of fundamental biological importance. Examples of these precipitations are collagen fibre formation (WOOD, 1960), mineralization of bone (WEIDMAN, 1963), or urate precipitation in gout, (LAURENT, 1964). In vivo macromolecule transport may occur through polysaccharide compartments, with the polysaccharide acting as a seive. The rate of transport through a tissue will be somewhat variable since the intercellular matrix is not homologous, there being

local quantitative (and qualitative) differences in polysaccharide in the tissue.

Recent studies by PRESTON (1977) have investigated the interactions between polysaccharides and other substances. Two particularly important statements are emphasized a) Polysaccharides markedly retard the diffusion of various small e.g., monosaccharides, sodium ions etc., b) At low concentrations of polysaccharide translational migration of linear molecules, such as DNA, is retarded. The retardation is consistent with end-on motion. At higher concentrations of the "network" polymer, very marked enhancement of migration rates can be observed.

Many in vitro model studies have been made to elucidate the complex relationships of polysaccharides with other biological moieties (ARNOTT, GUSS & WINTER, 1975; BLACKWELL, SCHODT & GELMAN, 1977; WINTER, SMITH & ARNOTT, 1975 etc.). The in vitro biological relevance is sometimes difficult to interpret but several examples are evident. Interactions of GAGs with acid soluble calf skin collagen indicated that the presence of the polysaccharide increased the thermal stability, changing the melting temperatures from 38^{o} to 46^{o}C. One interpretation of that effect is that the presence of the polysaccharide induced the formation of aggregates which have a higher thermal stability than isolated tropo-collagen molecules (GELMAN & BLACKWELL, 1973, 1974).

In the case of Hep-protein aggregates high charge density could induce conformational changes in the charged regions of the protein thereby affecting biological activity. Indeed LAURENT

(1977) claimed that in unpublished work of BJÖRK that there was a conformational change in anti-thrombin when bound to Hep.*

The previous resumé of the chemical structure of the GAGs indicates that the saccharide components are diagnostic of the species of molecule. ARNOTT etal.,(1975) suggests, however, that the GAGs, with alternate 1-3 and 1-4 glycosidic linkages, provide a variety of helical structures with marked family resemblances despite their different carbohydrate constitutions, their sulphate and other substitutions. He emphasizes that linkages appear therefore to be their dominant conformational determinants.

Substantially the same backbone conformations are observed for Ch-6-S as for DS clearly showing that α-L-iduronate and β-D-glucuronate have the same C1 chair conformation. In other circumstances this residue can also assume the C1 chair. The conformational flexibility of α-L- iduronic acid may be biologically significant, e.g. in the case of similar ambivalent furanode rings of nucleic acids (ARNOTT, GUSS, HUKINS, DEA & REES, 1974).

Various experimental techniques are available for investigating the PG structures. Physical methods include circular dichroism, X-ray fibre diffraction and nuclear magnetic resonance.

Recently SCOTT & TIGWELL (1978) have published a comprehensive discussion on the shapes of glycosaminoglycuronans in solution by methods using periodate oxidation. They too remark that L-iduronic acid can take up different conformations depending on the polymer's enviroment.

In summary, the conformational structures of the GAG chains have close similarities; the structure for HA being characteristic. (Fig. I-3)

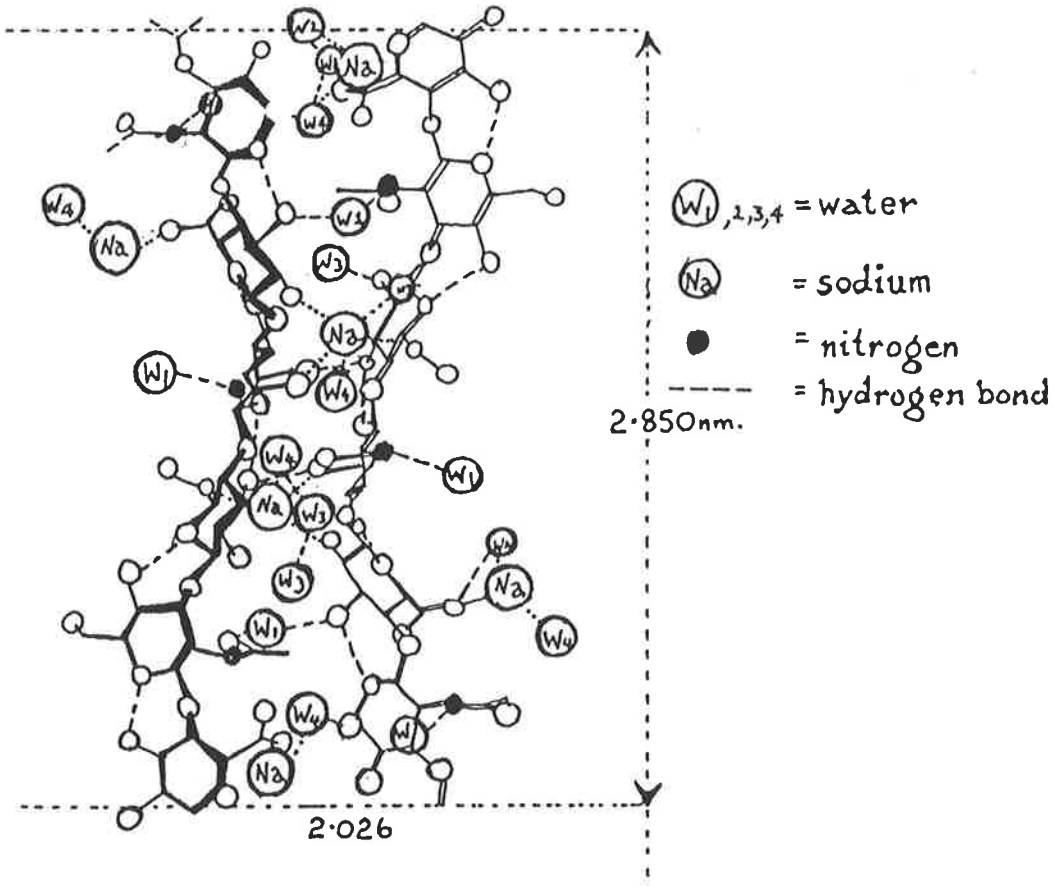


Fig I-3. Molecular conformation of the hyaluronic polyanion.

In the condensed state, HA is most commonly observed as a 3-fold fully extended helix or a 4-fold compressed helix. (DEA, MOORHOUSE, REES, ARNOTT, GUSS & BALAZS, 1973; ATKINS & SHEEHAN, 1973 and GUSS, HUKINS, SMITH, WINTER, ARNOTT, MOORHOUSE & REES, 1975). In fact these two conformational parameters have greater similarity than might be expected. A distortion of the conformation angle at the 1-4 linkage region exists in the 4-fold form. Both structures exhibit left handed chirality and the chains are packed in an anti-parallel manner; they are stabilized by both a network of intra-and inter-molecular hydrogen bonds and by O...Na...O (Fig. I-3) bridges. The inter molecular interaction appears to be greater in the 4-fold structures.

Since HA would mimic the conformational structure of Ch-6-S, WINTER et al.,(1975) have speculated that the biosynthetic regulation of ChS synthesis by cells incubated in the presence of HA (WIEBKIN & MUIR, 1973a; SOLURSH et al, (1974) may be consistent with a mechanism of competitive inhibition. Ch-6-S can exist in a 3-fold helical configuration with a pitch similar to that of HA. It differs from HA only in the axial disposition of the hydroxyl group at C_4 on the hexosamine and in the presence of covalently bound sulphate. However, these authors omit to mention that the in vitro regulation of PG synthesis by HA only occurs in those cultures which sythesize PGs specifically capable of interaction with HA, eg chondrocytes (WIEBKIN & MUIR, 1977a,b,) or epithelial cells (WIEBKIN, BARTOLD & THONARD, 1979). Moreover, there is

increasing evidence that the regulation of PG synthesis effected by HA requires, in the first instance, the interaction of HA at the cell surface (WIEBKIN & MUIR,1975a). Further, a special spatial orientation of the HA carboxylate anion appears to be required for HA : PG interaction (CHRISTNER, BROWN & DZIEWIATKOWSKI, 1977). Since the de novo secreted PG will be in an inspissated form, any such interaction with HA would tend to restrict subsequent conformational changes of the newly formed macromolecular aggregates at the cell surface.

By invoking the previously mentioned concept of compartmental exclusion of other molecules by polysaccharide matrices, the molecular enviroment of the cell under these conditions would have a profound influence on biosynthetic regulation. Other macromolecular aggregates such as those formed by DS-interaction (CÖSTER & FRANSSON, 1977) may prove to be equally important at the cell surface in biosynthetic regulation. (FRANSSON & CÖSTER, 1978).

TOOLE, JACKSON & GROSS (1971) were the first to describe HA mediated inhibition of cell-cell association in certain cultures of developing somites. They also described a positive correlation between this inhibitory effect and the absence of HA synthesis. BALAZS & DARZYNKIEWICZ (1973) have reported several effects of HA as an immobilizing agent of lymphomyeloid systems, as an inhibitor of mobility and multiplication of non adherent fibroblasts, in the survival of skin allografts and

on lymphocyte cytotoxicity. To date, the evidence is not available to relate these functions to the molecular interaction referred to above. Indeed they may be mediated through the mechanism of mutual identity between the similar molecular conformations of chemically distinct molecular species as suggested by WINTER et al. (1975).

THIS THESIS - LIMITS OF STUDY AND AN HYPOTHESIS.

The intercellular material of epithelial tissue appears to contain predominantly polyanionic polysaccharides. Since tissues such as gingival epithelium undergo extensive mechanical and enzymatic (from oral biota) abuse, an initial characterization of types of intercellular macromolecule was envisaged. More important however, was the hypothesis that non vascular tissue such as epithelium, and gingival epithelium in particular may rely on the maintenance of its tissue integrity by an autoregulatory (a feedback) mechanism of its PG synthesis.

The following chapters of the present report describe observations identifying PGs associated with cell surfaces and at cell-cell interfaces. The evidence indicates that there are significant differences in the PGs of epithelial cultures under different states of in vitro development. The significance of some of these differences undoubtedly implies structural involvement and more important do imply that PG interactions exhibit a direct effect on the regulation of PG biosynthesis.

PGs appear to be the major intercellular components of epithelium. Although the described study has been restricted to epithelial PG,

the author has relied extensively on reference to his own and other's work in the areas of research of connective tissue PG.

Two epithelial sources have been investigated. Firstly an amnion cell line which readily formed monolayers and which showed contact inhibition at full confluence and second, epithelial cells obtained from human gingivae at the time of surgery.

The data obtained from the amnion cell line was of a fundamental nature. Nevertheless it provided an indication of the metabolic events which might endow gingival epithelium with its remarkable resistance to loss of tissue integrity. Even a healthy oral cavity is subjected to massive enzymatic and mechanical abuse.

The in vitro studies on gingival epithelium developed along two distinct lines; one which was analytical and one which was descriptive (tissue component localization of MPS). However, both approaches revealed that the biosynthetic products secreted into the medium or environment of the cells were important in regulating the cells' biosynthetic behavior. Indeed, some of the concepts alluded to in this thesis, have been supported and confirmed by more recent evidence which has shown that the interaction of intercellular substances

and the configuration accessibility of those substances (PRESTON & SNOWDEN, 1973) can be highly specific. They are often significant in biosynthetic control amongst a wide range of tissue cells and at various stages of biological development (WIEBKIN, 1969; WIEBKIN & THONARD, 1969; TOOLE, JACKSON & GROSS, 1972; NEVO & DORFMAN, 1972; TOOLE, 1973; WIEBKIN & MUIR, 1973a,1975.,1977; SOLURSH et al, 1974; KOSHER, LASH & MINOR, 1973; and others).

CHAPTER II

DEMONSTRATION OF MUCOPOLYSACCHARIDES IN EPITHELIAL TISSUE.

A. Histochemical Identification of Epithelial Proteoglycan.

1.0 INTRODUCTION.

Using histochemical technique, MPS have been localized in a variety of human tissue, for example synovial membranes (CASTOR, 1959), cartilage (STOCKWELL & SCOTT 1967) and in other connective tissues (MANLEY & KENT, 1963; QUINTARELLI 1968). The epithelium of the bladder (MONIS & ZAMBRANO, 1968) and of the human gingiva (THONARD & SCHERP, 1962) have also been shown to contain MPS. By using specific enzyme elimination procedures the latter histochemical studies indicated that the quantitatively major component of the intercellular substances was MPS. The tissues forming the basis of these and many similar studies were non-specifically fixed and sectioned prior to the application of appropriate dyes.

Other studies employing histochemical and autoradiographic techniques, have shown that avian and human cells of epithelial origin synthesize MPS in-vitro (LANGKAMP, PLATT & THONARD, 1968; HENNING, WIEBKIN & THONARD, 1970; SISCA, LANGKAMP & THONARD, 1971). The cells in these experiments were also fixed non-specifically before staining. Although the specificity of the MPS stains employed has been increased by the use of polycationic detergent fixatives together with critical electrolyte elution, (SCOTT, 1960; WIEBKIN & THONARD, 1981) or by polysaccharidase elimination, there nevertheless remains

the possibility that staining due to acidic lipids, to nucleic acids, or to tissue treatment artifact may account in part for the described results.

More precise chemical identification of materials extracted from epithelial sources has now also been made. These findings will be described in the following section together with some observations on morphological localization by histochemical and autoradiographic techniques of the partially characterized components.

2.0 MATERIALS AND METHODS.

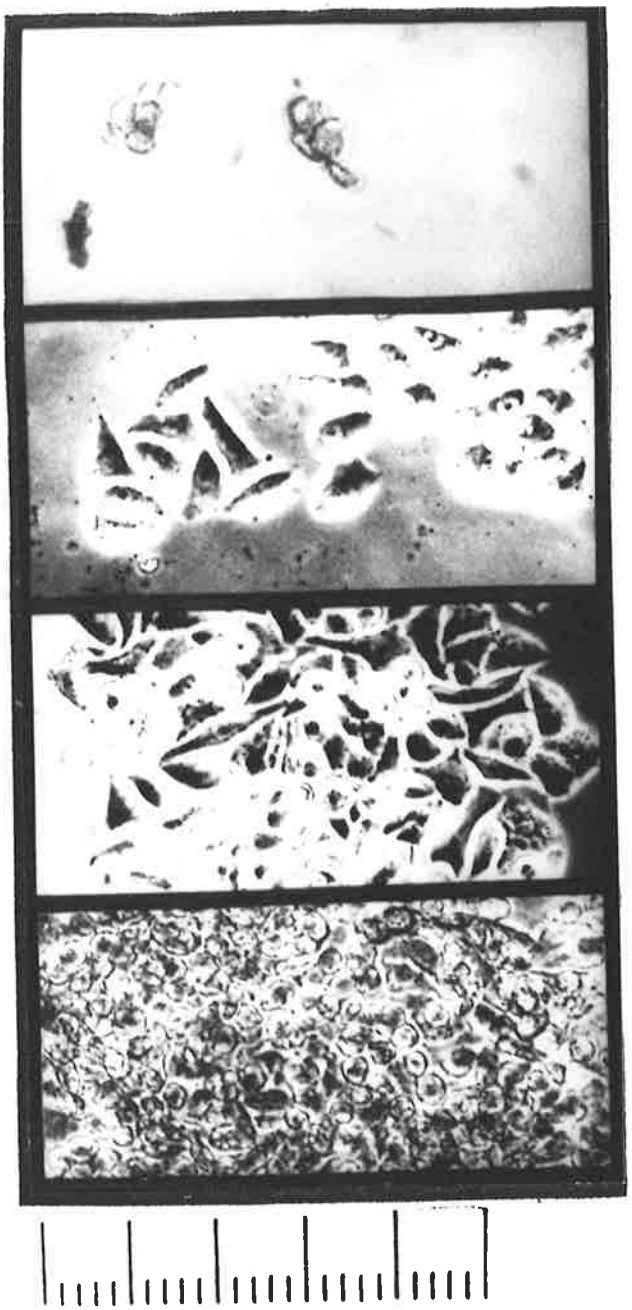
2.1 Epithelial Sources.

2.1.1 Amnion cell line.

Human amnion AV-2 heteroploid continuous cell lines* were obtained from the Commonwealth Serum Laboratories, Melbourne Australia. They were supplied in Medium 199* supplemented with 20% foetal calf serum (FCS). Subcultures were made into 100ml flat bottomed flasks in Medium 199 containing 8% bovine serum, (BS). Cultures were grown to different stages of monolayer confluence (Fig.II-1) before harvesting for biochemical assay, but in no case were they harvested less than 96 h after subculture. At 48 h and at 24 h before quantitive assay, medium changes were made. Several coverslips (Assistant No.1 (22 x 8mm)) were placed in the culture flasks and retrieved for morphological examination. In some experiments serum was omitted completely prior to harvesting.

2.1.2 Human gingival epithelium.

Small pieces of human gingival tissue, 2-3mm thick were obtained from gingivectomy specimens from periodontally affected patients. The gingivae had been subjected to presurgical preparation and appeared to be clinically free from inflammation.



Ranking

a +

initial inoculation
taken to the glass

b ++

clones develop
cells grow in size

c +++

clones approach
confluence, cells large

d ++++

complete confluence
cell size confined by
numbers

scale: 1 small division=5u

Fig.II-1 Conditions of Confluence Human amnion AV-2 heterploid continuous cell cell cultures were grown to different stages of confluence in Medium 199 8% BS. The grades of culture were (a) +, isolated cells, (b) ++, small clones, (c) +++, early adherent surface coverage (d) ++++, fully confluent, dense coverage.

The pieces of chopped gingivae were initially washed in Hanks' Balanced Salt Solution (BSS)* and incubated at 37°C in Medium 199 containing penicillin (200 units/ml); streptomycin (100 units/ml; and amphotericin B (4 µg/ml) together with 10% BS; pH was maintained at 7.4 with $NaHCO_3$.

2.2 Cell Preparation.

2.2.1 Intact Gingival Preincubations.

Excised tissue pieces were incubated at 37°C for 2-3h in Medium 199 containing antibiotics as described above but supplemented with only 4% BS. They were then transferred to Petri dishes for experimental incubations in which pH was maintained at 7.4 by flushing with CO_2 when required.

2.2.2 Cell Suspension Cultures.

Excised gingivae were collected as described previously washed three times in sterile Hanks' BSS and placed into 2-5ml of a disaggregating solution (Trypsin/EDTA).

Disaggregating solution.

E.D.T.A.,1.0g; NaCl 8.0g; KCl 0.2g; Na_2HPO_4, 1.15g; KH_2PO_4 0.2g; made up to 1000ml (WILT et al, 1964). Trypsin was added to this solution to 0.25% w/v filtered through to 0.2µ Gelman membrane* and adjusted with 2.8% $NaHCO_3$ to pH 7.3 - 7.4.

Disaggregation.

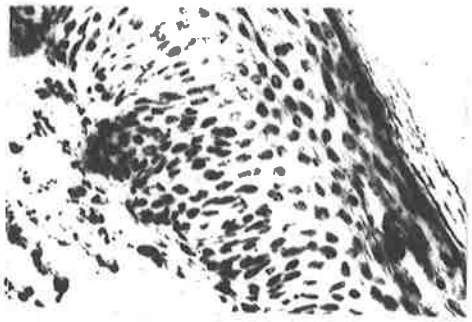
Very gentle agitation at 37°C for 15 min liberated some superficial cells which were discarded. The bulk of the

remaining tissue pieces were removed with forceps to 2-5 ml fresh disaggregating solution, and gently shaken for a further 10 min. At this stage the tissue pieces were again transferred to a Petri dish containing Hanks' BSS with antibiotics (as above) and 0.2% glucose at 37°C. The epithelium was easily detached from the underlying corion by dissection using fine forceps and a corneal scalpel with a bent tip. This separation resulted in minimal contamination of the epithelium with connective tissue cells. Histological evidence of the separation of epithelium from the underlying dermis of gingivae is shown in Fig.II-2. A further 15 min treatment of the epithelial sheets in disaggregation solution resulted in a "fragmentary suspension" of cells. These cells were initially grown in Medium 199, with 15% BS but could be variously maintained for periods up to 27 days, by which time experiments had been completed. Only cells from foetal sources would consistently attach to culture vessels and spread.

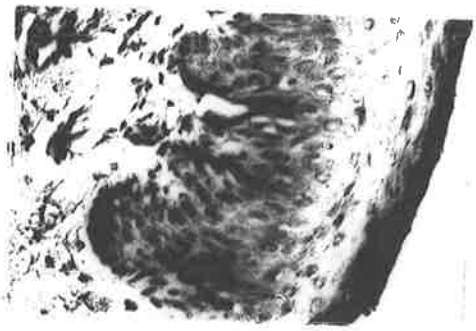
Fractionation of cell clumps by filtration.

Single cell suspensions were separated from small aggregated clumps by filtration (Fig.II-3).

The filters consisted of a series of three nylon meshes . The 50μ nylon mesh prevented all but single cells from passing through. Thus cells were divided into four categories depending on their retention by filters.



Gingival Pieces were washed in Hanks' BSS and placed into Trypsin/EDTA Solution at 37° for 15 min.



The tissue was replaced with fresh EDTA/Trypsin and incubation continued for a further 10 min.

The tissue could be micro-dissected in Hanks' BSS with forceps and corneal scalpel.

Further disaggregation of the epithelial tissue resulted in a "fragmentary suspension" of cells.

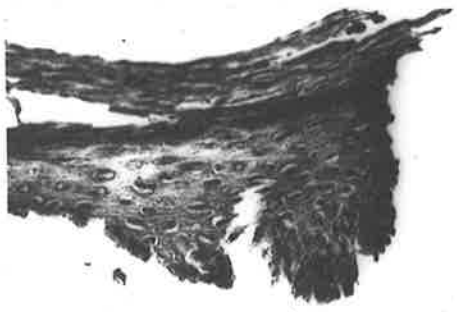


Fig.II-2 Gingival epithelial preparation. Epithelium was removed from the underlying connective tissue following sequential EDTA/Trypsin tissue disaggregation.

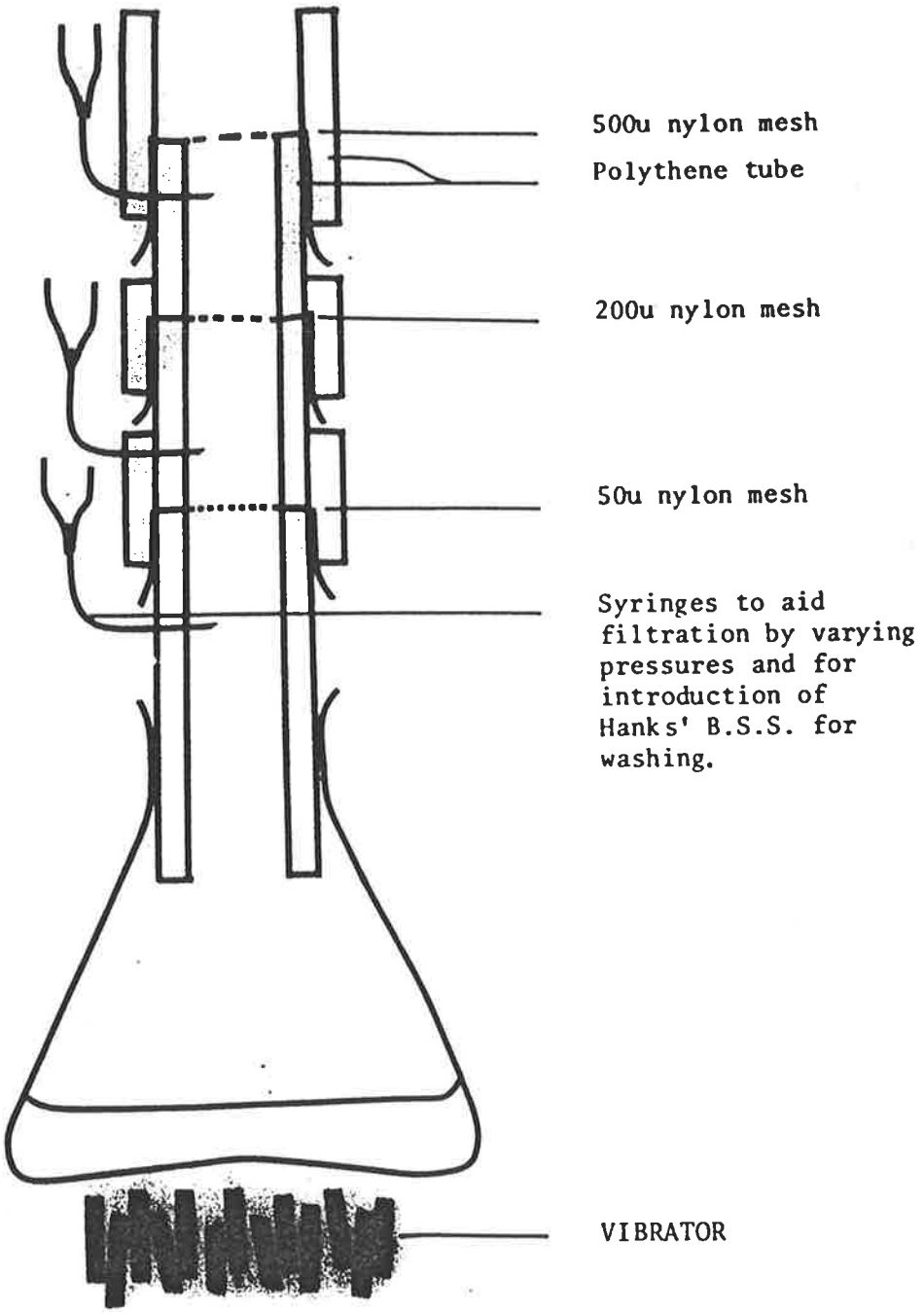


Fig.II-3 Filter for disaggregated gingival cells.
Gingival epithelial "fragmentary suspension" cells were filtered according to cell clump size.
a. Category I consisted of single cell suspension.
Category II contained small clumps of cells.
Category III contained larger clumps of cells.
Category IV contained large clumps of squame-like cells.

Cells in Categories I (single cell suspension), II (50μ nylon mesh) and III (200μ nylon mesh) were round, lucid and they appeared healthy. The largest clumps retained by 500μ mesh, Category IV, appeared to be morphologically different from the other culture fractions in that they often contained large squame-like cells. In general, these cells persisted in this form throughout the culture and probably originated from the outer epithelial layers.

Culturing.

Viability was tested by trypan blue exclusion (HOSKIN, MAYNELL & SAUNDER, 1956; BLACK & BERENBAUM, 1964).

Suspension cultures contained between 5×10^5 and 1×10^6 cells/ml as counted in a haemocytometer. The total number in the cultures, containing large clumps of cells was extrapolated from a function of the dry weight. To establish this function, representative cultures were disaggregated to suspensions of single cells which could be counted and then dried *in vaccuo* over $CaCl_2$ and weighed. A standard curve was obtained for each category and the dry weights of each experimental culture tested was read off the representative standard curve, providing an approximate cell number .

In all experiments the medium was replaced 24h prior to harvesting with Medium 199 containing only 8% BS. Since the gingival cultures were derived from non sterile tissue,

a battery of antibiotics was necessary.

Amphotericin B was thought to be the least compatible with tissue culture. It disrupts cell membranes by binding irreversibly to surface steroids (LUBIN, 1967) and produces graded leaks to small molecules (LAMPEN, 1966). Such modifications affect protein synthesis (LUBIN & ENNIS, 1964). Amphotericin B was therefore removed within 18h of initial cell suspension preparation.

Subsequently, the use of Gentamycin and penicillin have normally been found to be adequate.

2.3 Histochemical Techniques.

Both ammion cell cultures, and gingival epithelium were investigated histochemically. Monolayers of amnion cells were retrieved as monolayers on "flying" coverslips from the 100ml flab culture flasks. Just prior to cell harvesting and culture disruption (for biochemistry), they were transferred to petri dishes containing Hanks' BSS to await microscopical investigation which was performed within 1h of removal from the flask. Small pieces of gingival tissue were fixed within 20 mins. of excision or directly from *in vitro* culturing conditions.

2.3.1 Fixation.

A series of fixation procedures was undertaken to evaluate their relative efficacies. The pieces of human gingivae which were used for this study were placed into one of various fixative

solutions for periods varying from 4h to 2wks. They were removed, dehydrated wax embedded, sectioned and stained by the Hale's or toluidine blue techniques. Two independent observers were employed to evaluate the staining intensity and localization on the gingival epithelial cells.

Optimal fixation for classical morphological characterization was obtained with 10% formalin 80% ethanol (THONARD & SCHERP, 1962), but cells fixed in 1% cetylpyridinium chloride (CPC) in 80% ethanol also appeared to localize intercellular material well, as evidenced by autoradiographic and histochemical staining. Cells fixed with CPC invariably required a short treatment (<5 min) with 0.5M NaCl prior to staining (particularly with toluidine blue) to partially dissociate the quaternary ion/anion complex.

2.3.2 Staining.

Toluidine Blue.

The methods of SCHOENBERG & MOORE (1958) were employed with minor modifications.

Stain:

Solutions of toluidine blue were prepared so as to attain a dye concentration of 1.5×10^{-4} M in phosphate buffer at an ionic concentration of μ=0.002 (MILLER & GOLDER, 1950).

Staining was carried out a pH 6.0 and pH 2.5. pH 6.0: 0.5M NaCl, 0.3ml; 0.5M Na_2HPO_4, 0.92ml; 4.0M NaH_2PO_4, 0.66ml; made up to 2000ml. pH 2.5: 5M NaCl, 0.32ml; 1M glycine in 1M NaCl, 1.06ml; 2N HCl, 0.49ml; made up to 2000ml.

Under these conditions spontaneous dye molecule aggregation is virtually eliminated and differential anionic dissociation of the AMPS's is possible.

The coverslips were fixed in one of the above fixatives and equilibrated in the appropriate buffer for 30 min. They were then stained at room temperature for 20 min and read or photographed wet.

Alcian Blue*

The use of this dye has been a nidus of contention. Although the stain requires the dissociation of the acidic groups of the macromolecules, it has been found that when the hydrogen ion concentration is too high, no dye binding occurs (QUINTARELLI, 1968).

The following procedure has been adopted as that which correlates most closely with those ascribed to other techniques.

Stain:

Alcian Blue 0.1 g.
3% acetic acid solution 100 ml.

The solution (adjusted to pH 2.5) was filtered and a few crystals of thymol added. The cell coated coverslips were equilibrated for no more than 3 min in 3.0% acetic acid and then stained for 3h with Alcian blue. A tapwater rinse and 3% acetic acid wash finalized the procedure before dehydrating and permanent mounting in Xam* (polystyrene mountant).

Sudan Black.

Stain:

A filtered saturated solution of Sudan black* in 80% ethanol.

The coverslips were fixed as described and rinsed in 70% ethanol and then immersed in the Sudan black at 60°C for 5-10 min. Dehydration and mounting in Xam followed.

Colloidal Iron Stain (HALES).

The poor permeability of intact fixed cells to colloids (of either charged state) will limit the availability of intracellular architectural components for dye binding. This criterion is valuable in interpretation of the histology of intact cells.

Consequently, unlike sectioned tissue, intact tissue culture cells on coverslips lend themselves to a staining procedure in which it is desired to emphasize the staining properties of the cell surface components.

Stain:

Preparation of the anion ferric oxide stock solution.
50 ml of 0.5M $FeCl_3$ (6.75g of $FeCl_3 . 6H_2O$ in 50 ml distilled H_2O were added fairly rapidly (dropwise stream) 600 ml of boiling distilled water). The resultant iron sol was dialysed against water ensuring that sufficient flacidity was allowed. A final concentration of 1.3g iron per litre H_2O was prepared using stannous chloride reduction in acid conditions and titrating with potassium dichromate*. 100ml of this solution was mixed with equal parts of 0.0012N HCl to provide the stock solution with a final pH of 3.5.

The "working solution" was prepared when needed by adding
10ml stock colloidal iron solution to
10ml glacial acetic acid and 20ml distilled water (Final pH of 1.8)
The "stain"
80ml 2% potassium ferrocyanide
160ml 1% HCl

Coverslips were equilibrated in 3% aqueous solution of acetic acid for 15 min then flooded for 10 min with the

"working solution" of colloidal iron stain and rinsed twice with water. Iron combination was visualized as a blue stain by acidic potassium ferrocyanide reduction for 10 min. Cells treated only with potassium ferrocyanide were used as controls.

The stained cells were mounted under Xam.

2.3.3 Digestion Procedures.

Pyridine digestion (to remove unsaturated fats).

Coverslips were digested in pyridine for 24h at 60°C and washed thoroughly before staining.

Hyaluronidase digestion.

Reagents:

1. 30 mls of H_2O.
2. 750 TRU* hyaluronidase (Testicular; EC4.2.99.1).

Coverslips were immersed in the hyaluronidase solution at 37°C for 12h.

Controls were immersed in hyaluronidase solution which had been inactivated in boiling water for 30 mins.

Ribonuclease digestion.*

0.2 - 0.5 mg crystaline ribonuclease (EC.2.7.7.16) per ml H_2O. Cells were treated at 60°C for 2-3 h or at 37°C 4-8 h. The pH was maintained at 6.7 with 1N NaOH or in 1N HCl (BRACHET, 1940).

Potassium hydroxide digestion (for RNA).

Coverslips and attached cells were incubated at 37°C for 16 h in 0.3N KOH. Controls were incubated in water.

B. Localization of Epithelial Glycosaminoglycans.

1.0 INTRODUCTION.

The histochemical methods described above were based on classical microscopical disciplines for localizing classes of molecule in tissues and in cell culture. However, incorporation of radioactive precursors by epithelial cells in short term incubations was followed in order to more precisely establish the metabolic capacity of the cells. More specifically, the localization of the radiolabel in macromolecules was visualized by autoradiography techniques described in this section did not rely on the capriciousness of stain affinities and binding capacities. It is recognized that primary cultures and freshly excised tissue can respond in short term incubation regimes differently from their in vivo metabolism.

2.0 MATERIALS AND METHODS.

Amnion cells were grown in 100ml flat flasks as described previously. The flasks contained "flying" coverslips (Assistant No. 1, 22 x 8) Medium 199, 8% FCS containing either 50 µCi/ml (^{35}S) -sulphate or 10 µCi/ml (^{3}H) - acetate.

Human gingival tissue was obtained as described previously. The pieces were chopped finely (1-2 mm^3), washed in Hanks' BSS and incubated in Medium 199 with 10% FCS + antibiotics. pH was maintained at 7.4 with $NaHCO_3$.

2.1 Amnion Coverslip Cultures.

Coverslips on which amnion cells were growing were immersed into Petri dishes containing pre-warmed medium in which radioactive precursor was included. They were incubated at 37°C for periods of time between 5 and 20 min. The coverslips were then washed three times in non-radioactive medium and further incubated in radioactive free Medium 199 at 37°C for periods between 2 and 150 min. A rapid wash in warm Hanks' BSS preceded fixing in 1% CPC in 80% ethanol for 30 min. Some coverslip cultures were fixed only in 1% CPC in 0.05M NaCl for 30 min. Such cultures were then soaked in 0.5M NaCl, 0.7M $MgCl_2$, 1.25M $MgCl_2$ or distilled water for 18h at 22°C.

All coverslips cultures were placed in 0.5M Na_2SO_4 or 0.05M sodium acetate after fixation to ion exchange the unbound inorganic radioactive precursors.

The edges of the coverslips were then attached to the edges of the microscope slides with cement and coated with melted Kodak NTB 2 nuclear tracking emulsion at 40°C in the dark. The slides orientated with the coverslips protruding uppermost, were left to drain and dry slowly in still air in the dark. The microscope slide provided for even drainage of excess emulsion from the bottom of the coverslip.

When dry, the coverslips were mounted onto slides and stored in light proof boxes for about 21 days at 4°C over $CaCl_2$. The coverslips were then developed in Kodak D19B for 2 min at room temperature, stopped in water, and fixed with Ilford

Acid fixer (Hypam). Faint counter/staining with light green and nuclear fast red* aided histological observation.

2.2 Gingival Epithelium.

The gingival tissue pieces were transferred from the initial Medium 199 to 90 mm Petri dishes containing 5-7 ml of fresh pre-warmed Medium 199 and either 50 μCi/ml (^{35}S)-sulphate or 10 μCi/ml (^{3}H)-acetate and were incubated at 37°C for periods between 5 and 25 min. pH was controlled with 5% CO_2 in air. Each dish contained no more than 5 pieces of tissue, all from the same source. The gingival pieces were then washed three times in Hanks' BSS at 37°C and further incubated for up to 1h in radio isotope free medium. Since the total weight of isotope added to the 'pulse' incubation was of the order 10^{-4} m mols, adjustment of the 'chase' nutrient medium with equivalent radio isotope free salt was considered unnecessary.

Control gingival tissue incubations were performed with tissue which had been previously killed and fixed in either 10% formalin, 80% ethanol or had been freeze-thawed (x2). The purpose of the controls was to determine the relative fate of radio isotope in non metabolizing tissue under similar incubation conditions as described above. All tissue pieces were sectioned by cryostat at 6 μ, in an orientation which cut transversely through the epithelium. They were air dried under constant conditions of air flow and temperature.

In vivo incorporation and localization of (^{35}S) -sulphate and (^{3}H) -acetate into the gingivae, buccal mucosa and tongue of rats were also studied. Rats were injected intraperitoneally with either 10 μCi/gm body weight of (^{35}S) -sulphate or 4μCi/gm body weight of (^{3}H) -acetate and sacrificed 4h after injection. Oral tissues were dissected out and 6 μ cryostat sections were cut. All sections were slide fixed with either 10% formal-saline - 80% ethanol for 10 min, or with 1% CPC in 0.05M NaCl solution for 30 min at 25°C. Slides were then immersed in three changes (20 min) of 0.025M Na_2SO_4 or 0.01M Na acetate to displace unincorporated radioactive (^{35}S) - sulphate or (^{3}H) -acetate. Slides fixed in CPC were soaked in two changes of either 0.5M NaCl, 0.63M $MgCl_2$ or 1.25M $MgCl_2$ solution, all containing 0.1% CPC.

Slides were coated with nuclear tracking emulsion as described previously. Care was taken, when coating the slides, to dry them slowly since rapid differential drying of the tissue and emulsion can cause micro-cracking of the emulsion, often over intercellular locations, resulting in the development of false silver grains due to "pressure sensitivity".

The slides were then stored in light proof boxes as described previously, and eventually (after about 21 days) they were developed in Kodak D19B. Histological contrast was provided

by faint counter staining with light green and nuclear fast red.

C. Biochemical Identification of Epithelial Glycosaminoglycans.

1.0 INTRODUCTION.

Apart from the pioneering and elegant work of SCHULTZ-HAUDT (1958) very few attempts have been made to investigate biochemically the constituent GAGS of gingivae, particularly of human origin.

One pragmatic reason is that studies are limited by the inherently small amount of material generally available. Recently we have established that 0.07% of the dry weight of human gingival epithelium and 0.23% of the underlying connective tissue represents uronate (BARTOLD, WIEBKIN & THONARD, 1981). Since the work described in this thesis was carried out, two groups of Japanese workers (HIRIMATSU, ABE & MINAMI, 1978; SAKAMOTO, OKAMOTO & OKUDA, 1978.) and some others have isolated sufficient material from bovine and porcine gingivae, by isolating GAGS from more than 84 g of mixed tissue (epithelium and connective tissue together). This represents excision from about 300 specimens of gingivae. Other studies on human gingivae have employed whole gingivae i.e. a composite of epithelium and connective tissue.

In the studies discribed here on human material, only the gingival epithelium was used and the analyses attempted were compared with similar procedures on amnion cell

cultures. Even from these epithelial cell culture strains, very small amounts of intercellular uronate was available. Following attempts to prepare protein free GAGs, and to reproducibly measure hexosamine on "crude" extracts of PG , the only quantitative biochemical methods adopted were restricted to uronic acid, sialic acid and sulphate assays together with assays for associated protein. The availability of human gingivae which were essentially free from inflammation was limited since the practice of presurgical preparation was not widely regarded by periodontists as appropriate.

2.0 METHODS.

2.1 Preparation of Amnion Cells for Biochemical Assays.

The medium was poured off from adherent amnion cell cultures together with three washings of Hanks' BSS (5ml) and any detached suspended cells were spun down at 800g in bench centrifuge. The pellets of cells were washed (x 2) with 2 ml Hanks' BSS and retained for assay. The washings, pooled with their media, were stored at-10^{o}C. The remaining adherent cell cultures were ranked according to the degree of confluence as shown in Fig. II-1. Cultures in which adherent cells were sparsely spread across the flask surface were ranked +, while fully confluent cultures were ranked ++++. The cells were carefully scraped off with a rubber policeman and finally washed into

centrifuge tubes. Both suspended cells and "adherent" cells were counted in a Neubauer haemocytometer. Viability was established by the trypan blue exclusion test (HOSKIN et al, 1956, BLACK & BERENBAUM, 1967). Biochemical assays were performed on these cells and media.

2.2 Preparation of Gingival Epithelial Cells for Biochemical Assay.

Cells were prepared as described above and variously sized cell aggregates were separated from single cells by filtration, (Fig.II-3). Each cell separation was then centrifuged, washed and suspended in 1% cetylpyridinium chloride (CPC) in 0.1M NaCl and ultrasonically disrupted. The MPS extraction and precipitation from such cells and from their representative medium was achieved by the procedure described below.

2.3 Preparation for Mucopolysaccharide Isolation.

Cells were either treated with 0.33N NaOH prior to CPC preparation or they were directly suspended in approximately 1.5ml of CPC in Hanks' BSS before completely disrupting with an ultrasonic probe (Dawe Soniprobe, Type 1130A); microscopical investigation determined that complete cell destruction had been achieved. These CPC preparations (adjusted to a final concentration of 1%) were then incubated at 37°C for 30 min.

Both medium from the cultures and normal, unused control medium samples were made up to 1% CPC and incubated similarly.

All samples were then shaken with Keiselguhr* and centrifuged at 3680g in conical centrifuge tubes. The temperature was not allowed to fall below 22°C to ensure that the CPC remained in solution.

The supernatants were poured off and saved. The pellets were shaken with 2 ml of 0.5M NaCl and incubated at 37°C for 30 min. They were then centrifuged as before and the supernatants were removed and saved. Such a procedure was repeated twice and the supernatants pooled. The remaining Keiselguhr pellets were again eluted as described above, but with increasing magnesium salt concentrations; (ie. 0.7M $MgCl_2$ and 1.25M $MgCl_2$), were sequentially applied to the pellet.* The excess detergent in each of the three salt elutions was removed by subsequent precipitation with potassium thiocyanate (KORN, 1959). Following the removal of these precipitates some of the salt solutions were subjected to exhaustive papain/cysteine digestion; these, together with undigested samples, were dialysed against distilled water and the volumes reduced by vacuum dialysis.

Polycationic enzymes such as papain and trypsin were originally avoided in this study because of their strong affinity for polyanions such as AMPS (RINGERTZ & REICHARD, 1960).

All samples of any one series of assays were brought to a known volume for data calculation purposes. Salt elution fractions were compared by cellulose acetate electrophoresis with standard GAG & PG preparations. Uronic acid, protein, sulphate and sialic acid estimations were made on such fractions. Paper chromatography

on hydrolysates was also used.

2.4 The Assays.

2.4.1 Uronic Acid Estimation .

Essentially the technique of KNUTSON & JEANES (1968) was adopted.

Reagents:

1. Concentrated H_2SO_4 sp.gr.1.84; analar grade.
2. Stock borate solution; 24.7g; H_3BO_3 dissolved in 45 ml 4M KOH; solution diluted to 100 mls. with distilled H_2O to give a stable stock solution 4M with respect to $BO_3^{\equiv}$.
3. H_2SO_4 - borate reagent; 25 ml stock borate solution made to 1 litre with H_2SO_4. Final solution in 0.1M in H_3BO_3.
4. Carbazole reagent; 0.1% solution prepared by dissolving carbazole recrystallized from benzene, in absolute ethanol purified by the method of STARK (1950).

Test:

Six ml of H_2SO_4-borate was added to test tubes containing 0.7 ml of sample solutions. These tubes were cooled in an ice bath. After vigorous mixing they were placed into a boiling water bath for exactly 20 min. Following equilibration to 4^oC in an ice bath, the optical densities were read (HITASHI PERKIN-ELMER 139, UV-VIS) at 530 mμ through 1cm path length; then 0.2 ml carbazole solution was added to each tube and they were heated for exactly 10 min, cooled in ice and again read at 530 mμ at room temperature. The difference between the two readings for any sample was then read from a standard D-glucuronic acid curve*; standard : solutions of D-glucuronic acid were included amongst

the unknown estimations. The blank sample in all cases was 0.7 mls H_2O. Small amounts of protein, 1-5 µg, (ovalbumin) were added to glucuronic acid standards but there was no marked interference in chromophore formation.

Standard curves for the estimation of uronic acid in the presence of small amounts of protein have been prepared and appear as an appendix*.

2.4.2 Protein Estimation.

The technique of HARRIET, as described by KABAT & MAYER (1961) was followed.

Reagents:

1. Folin's reagent (Ajax chemicals).
2. 12.5% solution Na_2CO_3, anhydrous.
3. 0.1% solution of $CuSO_4.5H_2O$.

Test:

Aliquots of 2.0 ml of the unknown protein solutions were pipetted into tubes; 6ml of the bicarbonate solution and 1 ml of the copper sulphate solution were then added and stirred well. These were left for 1h at room termperatures. Freshly prepared Folin's reagent was diluted 1:3 in H_2O and 1 ml added slowly to each tube with constant stirring. The reactions were read after 30 min at 750 mµ. against a blank of water to which the above reagents had been added.

Standard curves were prepared against ovalbumin*

2.4.3. Sulphate Estimation.

Because of the very small quantities of sulphate to be measured, the turbimetric methods adopted by most workers could be not be applied. The method of ANTONOPOLOUS (1962) was found to be successful and sensitive if fresh benzidine reagent was stored for no longer than a week.

Reagents:

1. 98 - 100% formic acid.
2. 95% ethanol.
3. Benzidine reagent: 0.5% solution of benzidine in 95% ethanol kept in the dark.
4. Amyl alcohol.
5. Acetone/ethanol mixture 1:1 (v/v).
6. 1.ON HCl.
7. 0.5% w/v thymol in 2N NaOH.
8. 0.1N sodium nitrite.

Test:

1.3 ml of each of the samples, adjusted to 25% formic acid, by volume, were hydrolyzed in sealed tubes in a boiling water bath or autoclaved at 120°C. Although ANTONOPOLOUS suggested that 24h was required, for minimum digestion at least 36h were necessary for complete hydrolysis. 0.3 ml aliquots of the hydrolysates were pipetted into 15 ml conical centrifuge tubes; 0.5 ml 95% ethanol, 0.2 ml benzidine and 0.5 ml amyl alcohol were then added in each tube. These were shaken vigorously and allowed to stand at 0°C for 1h.

The tubes were then centrifuged at 6,000 rpm in swingout heads on a BTL bench centrifuge and the supernatant gently discarded. A mixture of 1 ml acetone/ethanol and 0.05 ml amyl alcohol was used to wash the pellets and the tubes were centrifuged for a futher 6 min. The supernatants were again discarded and final washings of 1 ml of acetone/ethanol were made which were then centrifuged. The pellets were dissolved in 3.0 ml HCl, 2.0 ml distilled water and diazotized for 3 min with 1 ml of 0.1N sodium nitrite. Finally, 5 ml of alkaline thymol were added. Immediately an orange/red colour developed and the samples were read at 505 mμ against a blank control of water that had been subjected to the entire process. All glassware for this technique was boiled in formic acid prior to use. Up to four estimates were made on each sample if sufficient material was available.

Standard curves of sodium sulphate were frequently prepared because of the need to use fresh benzidine; however, the curve remained constant within 0.5%*.

2.4.4. Sialic Acid Estimation.

Washed cells, obtained from the monolayers, were mixed with Keiselguhr and CPC, subjected to ultrasonic disruption, centrifuged and the supernatants stored. The cellular extracts were eluted from precipitated detergent complex with 1.25M $MgCl_2$. The supernatants and CPC eluents were dialysed against water and then treated at 80°C for 2h with a final

concentration of 0.4N H_2SO_4 (1h hydrolysis is not sufficient). The H_2SO_4 was precipitated by equivalent barium hydroxide. All these aqueous fractions were concentrated by lyophilysing, and determinations against a standard N-acetyl-neuraminic acid curve* were made according to AMINOFF (1961).

2.4.5 Chromatography. (Sugars).

The solvent system used for sugar separations on paper chromatography was:

Ethyl acetate - 12
Pyridine - 5
Water - 4

Hydrolysis of polysaccharides was achieved with 6N HCl at 100°C for 18h under N_2.

2.4.6 Electrophoresis.

The method of BROOKHART (1965) was used on starch gel and modified * for cellulose acetate but the method described in Chapter III was used in the later stages of this study.

3.0 RESULTS AND DISCUSSION.

3.1.1 Histology- fixation.

The dental research literature contains many reports of histological investigations of gingival tissue and most of these reports utilize "standardized" staining techniques.

Despite the plethera of observations only a few studies have implied that the epithelial intercellular material contained AMPS , (THONARD & SCHERP 1962; COHEN 1968) nowhere was it established that the cells themselves were capable of synthesizing it.

Some of the conflicts of histological interpretation, even as recently as PEDLER'S (1979) contribution to literature, are due to insufficient attention being paid to the fixation of relatively soluble polysaccharides.

Thus as a preliminary study to the subsequent histochemical investigations cited in this thesis, the relative efficency of a series of fixation protocols was determined on sections of gingivae.

After 3 days of formal saline fixation, with or without ethanol of human gingival, sections stained with toluidine blue demonstrated limited metachromasia at pH 3.5; longer than 4h fixation in formal saline/ethanol was required before any metachromasia was observable.

The results of COHEN (1968) confirm and amplify the data expressed in Table II-I, by agreeing that the formal saline/ethanol fixative

is the most effective in demonstrating AMPs with affinity dyes.

Although CPC is satisfactory for fixing tissue prior to autoradiography, low and extremely low pH (< 1.5) does not allow solubilization of the fixative-tissue component complex (QUINTARELLI, 1968). In an incidental series of preliminary tests, PGs which had been fixed on filterpaper with CPC, were only marginally dissociated from the detergent in the low pH and ionic concentrations of the staining solutions of toluidine blue. Thus reassociation of the dye resulted. The detergent complex had acted as an anionic blocker, and dye binding and molecular alignment as demonstrated by metachromasia was minimal.

Following the staining procedures for establishing dissociative and associative conditions necessary in demonstrating the substrate on filter paper, we cannot necessarily assume by analogy, similar conditions within the tissues. Indeed the use of CPC as a fixative prior to dye binding (histological staining) was unreliable in these studies. Other areas of histology based on irrational fixation techniques may also produce equivocal interpretations. Furthermore, the imprudent use of prolonged or inappropriate fixation as well as decalcification must pose doubts on the validity of conclusions drawn from several studies, a point of issue being demonstrations of the presence of AMPS in the epithelial attatchment of gingivae to teeth, (CIMASONI & HELD,1963;

Table II-1. Intensity of staining following treatment with different fixative for different times.

Fixatives		Intensity of staining					
		Hale's Colloidal Iron Stain				Toluidine Blue in Gingival Epithelial Intercellular Substance Metachromasia	
		Intercellular Substance	Nuclei	Cytoplasm	Connective Tissue	pH = 3.5	pH = 6.0
1% TCA	4 hours	-	++	-	-	++	+
in 80%	8 hours	-	++	-	-		
Ethanol	3 days	-	+	-	-	-	-
	2 weeks	-	-	-	-		
5% CPC	4 hours	-	++	-	-	+	++
in 80%	8 hours	-	++	-	-		
Ethanol	3 days	-	+	-	-	+	±
	2 weeks	-	-	-	-		
1% CPC	4 hours	+	++	+	±	++	++
in 80%	8 hours	±	++	+	++		
Ethanol	3 days	-	+	-	-	-	±
	2 weeks	-	+	-	-		
10%	4 hours	+	+	+	++	++	++
Formal	8 hours	+	++	++	++		
Saline in	3 days	++++	+	-	++	±	+
80% Ethanol	2 weeks	+	-	-	±		
10% CPC	4 hours	-	+	-	-	++	++
in 80%	8 hours	-	+	+	++		
Ethanol	3 days	-	+	-	+	±	+
	2 weeks	-	+	+	++		
10%	4 hours	-	+	±	++	-	±
Formal	8 hours	-	+	±	++		
Saline	3 days	-	±	-	++		
	2 weeks	-	-	-	+	+	+

STALLARD, DIAB & ZANDER,**1965), require confirmation. Indeed the use of Alcian blue with "modified" acid fuchin (MOWRY,1978), for specifically demonstrating MPS or glycoproteins, when the initiating fixation chemistry at the cellular level is unknown, must also be questioned.

3.1.2. Histology - amnion cell line.

The histochemical techniques employed, consistently stained polyanionic material, some or all of which, depending on the stain and cell site studied, could be eliminated by treatment with agents which are considered to aid the identification of the stainable substrate. The results are summarized in Table II-2.

Treatment of cells with heat inactivated hyaluronidase or heat inactivated RNA-ase resulted in no discernable differences in staining reactions compared to those observed in untreated control cultures.

Cells stained by the Hale technique tended to show overall staining but definite concentration of the stain occurred in confluent cultures at cell interfaces. Similar stain distribution was noted with Alcian blue. Toluidine blue at pH 6.0 stained cells orthochromatically but metachromasia was observed scattered through-

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**footnote: In challenging Dr. H.Zander at The Eastman Institute, Rochester N.Y. in Jan 1971, he admitted to a belief that this paper should have been withdrawn from the literature.

out the cytoplasm and in the intercellular spaces of confluent cultures. At pH 2.5, to toluidine blue staining was much reduced and metachromasia was mainly limited to cell interfaces.

The Hale's histochemical technique restricts stain binding to the superficial cell surface of the intact cells; eg. amnion coverslip cultures; on account of the impermeability of plasma membrane to polyanionicly bound colloidal iron, (GASIC & BERWICK, 1963; GASIC, BERWICK & SORENTINO, 1968; CURRAN, CLARK & LOVELL, 1965).

Although basic dyes, particularly toluidine blue, have been used to visualize GAGs, intracellular nucleic acid usually has a greater affinity for cationic dyestuffs than do other polyanions. SCOTT & DORLING,(1973) cite that acridine which is very similar in size and shape to toluidine blue has been especially used to demonstrate RNA. Nevertheless, low pH will reduce binding to polycarboxylates and polyphosphates. Indeed, LANGKAMP, PLATT & THONARD,(1968) succesfully demonstrated AMPS in the intercellular material of human gingival epithelium with acridine orange.

In fact the phthalocyanin chromophore exactly fits the Watson-Crick base pair (guanine-cytosine) and intercalates and binds to nucleic acid. If bulky substituents are added, eg. S-methylene tetramethyl isothiouronium side chains on Alcian blue, such molecules can no longer intercalate. Alcian blue therefore, behaves like a coloured cetylpyridinium molecule, being displaceable from polycarboxylate and polyphos-

Table II-2. Degree of staining of amnion monolayers; before and after treatment of cells to remove substrate.

Stain	Control IF	Control CP	Control N	H'ase IF	H'ase CP	H'ase N	RNAase IF	RNAase CP	RNAase N	KOH IF	KOH CP	KOH N	Pyridine IF	Pyridine CP	Pyridine N
Hale	+++	+	±							++	-	-			
Alcian blue	+++	++	+	+	++	+				+	-	±	+++	+	+
Toluidine blue-pH 6.0	+++	++	±	+	+	+	++	+	±						
Toluidine blue-pH 2.5	+++	++	±	++	+	+	+++	+	±	+++	+	±	++	+	±
Sudan black	±	+	±	±	+	±							-	-	-

Cells were fixed in 10% formalin-80% ethanol. IF, intercellular interfaces; CP, cell cytoplasm; N, nucleus.

phate at lower salt concentrations than from polyester sulphates, (SCOTT & DORLING, 1965). For this reason Alcian blue is well suited to the intra and intercellular demonstration of non-nucleotide polyanions. Unfortunately only one Alcian blue molecule will bind to four substrate molecule sites, resulting in poor dye concentration on the tissue. On the other hand the metachromatic nature of dyes which conformationally bind such substrates as polyester sulphates, eg. toluidine blue, absorb certain wavelengths but emit at other wavelengths, often with increased intensity. Although they therefore express increased sensitivity they cannot obey Beer's nor Lambert's laws.

The present report emphasizes the value of the use of Alcian blue as a means by which the specificity of GAG substrates can be localized. Indeed the use of an electron dense atom, eg. Pt in place of the chelated Cu of Alcian blue is expected to be of use in future electron microscope localization.

Special note should be made of (a) the intercellular location of the polyanionic substrates detected; and (b) the polyester sulphate nature and non nucleic acid conformation of the molecules stained.

Material stained by Sudan black was in greater evidence in older cultures but this was generally restricted to the cytoplasm and occasionally to perinuclear regions. Only in older cultures (10-12 days) was there evidence of the intercellular substance staining with Sudan black.

Treatment of cells with testicular hyaluronidase reduced the intercellular metachromasia of toluidine blue at pH 6.0 but had little affect on that observed at pH 2.5. The effect of hyaluronidase treatment on staining by the Hale technique were not ascertained in the present study but SISCA et al. (1971) have reported that the majority of the overall staining and intercellular staining is abolished. In both studies, Alcian blue staining was never completely abolished by pretreatment of the cells with testicular hyaluronidase.

There may be several explanations for these observations. for example-

a. DS is hyaluronidase resistant.

b. PG conformations of fixed tissue contain cryptic enzyme cleavage sites.

Pyridine digestion had little or no effect on metachromasia of toluidine blue at pH 2.5 and equivocal reduction of overall staining due to Alcian blue. The evidence for the removal of fat, lipids and phospho-lipids was provided by the inability of Sudan black to stain cells following pyridine treatment. The use of CPC and/or ethanol in the fixation procedures also abolished much intercellular Sudan black staining. Potassium hydroxide pretreatment had little effect on the nuclear staining when the Hale and Alcian blue methods were used, and only marginally effected the Hale staining at the cell surfaces.

Alcian blue staining at cell interfaces was definitely reduced following KOH pretreatment. Decrease in cytoplasmic metachromatic granules was evident and no cytoplasmic staining

was observed in cells stained with Alcian blue or by Hale's procedure following KOH treatment.

KOH treatment would have solubilized RNA but it would also cause limited hydrolysis of PGs, resulting in scission of the GAG from the protein core (xylose-serine linkage). Since the tissue had been fixed with ethanol and the monolayers were morphologically complete (unlike histological sections) a proportion of the GAGs or their component parts possibly remained localized in situ.

RNA-ase effected a slight decrease in cytoplasmic staining with toluidine blue but did not reduce metachromasia of the intercellular materials.

3.2 Autoradiography (AMPS Biosynthesis and Transport).

3.2.1. Amnion cultures.

In an attempt to follow the intercellular MPS in the development of amnion cell cultures of various levels of confluence, monolayers were incubated for short periods in media containing (^{35}S)-sulphate or (^{3}H)-acetate. They were then transferred to radio isotope free media where they were further incubated. Autoradiographic studies were carried out on these cultures.

Sulphate incorporation.

In amnion coverslip cultures, the incorporation of (^{35}S)-sulphate was manifested as silver granules over perinuclear and cytoplasmicly confined locations within the first 9 min. Single, non-confluent cells consistently demonstrated early incorporation. The label was not reduced by testicular hyaluronidase digestion (10 TRU) of CPC fixed fully confluent cultures (Fig. II-4).

Cells incubated for 5 min in Medium 199 containing radio label and then for 30 min in isotope free medium, invariably demonstrated good localization in both confluent and non confluent cells. All cells retained a perinuclear label (PN) but confluent cells also showed more generalized cytoplasmic incorporation, (Fig.II-5). By 45 min (^{35}S)-sulphate was observed over the whole of the cytoplasmic area of both confluent and non confluent cultures. In planar view the area over the nucleus was less heavily labelled. The cytoplasm may be thinner over this area in monolayer cultures. On the other hand the thin cytoplasmic extensions (E) of the spreading cell were heavily labelled, (Fig.II-6). The observation can be interpreted, therefore as intracellular label being prepared for secretion in the regions of interface adhesion.

At this stage of synthesis and secretion, the label had not been localized over the entire perifery of the cell.

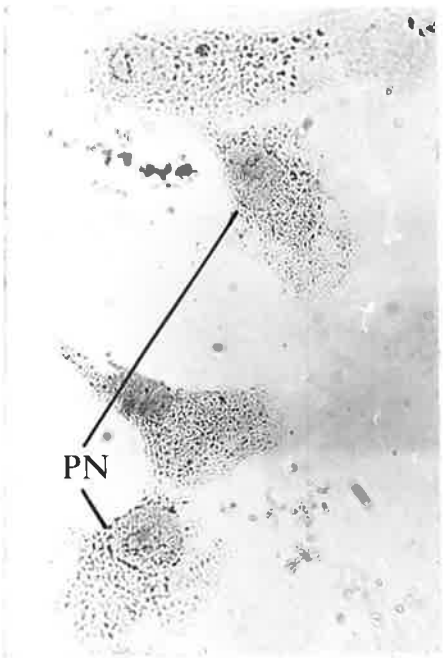


Fig. II-4

Autoradiograph of (^{35}S)-sulphate incorporated into cetylpyridinium chloride fixed material by amnion cells after 9 min of incubation in medium containing Na_2 (^{35}S) O_4 (PN=perinuclear label). (Mag x 1250)

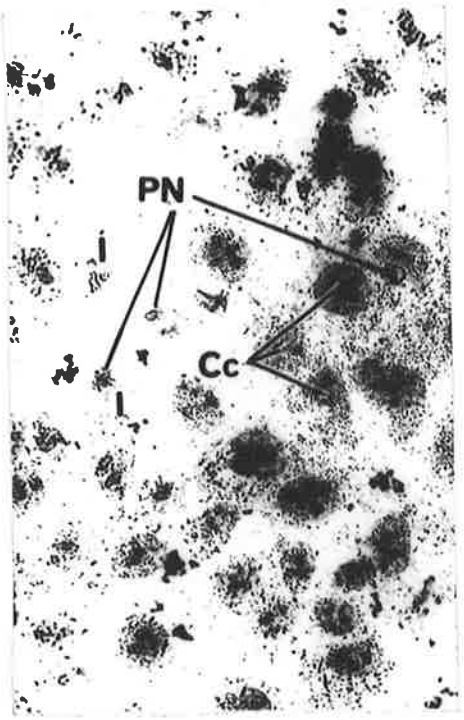


Fig. II-5

Autoradiograph of (^{35}S)-sulphate into cetylpyridinium chloride fixed material incorporated by prelabelled (5 min 'pulse') amnion cells after 30 min 'chase' incubation in radioactive free medium (PN=perinuclear label, Cc=cytoplasmically confined, I=intercellular label). (Mag x1250)

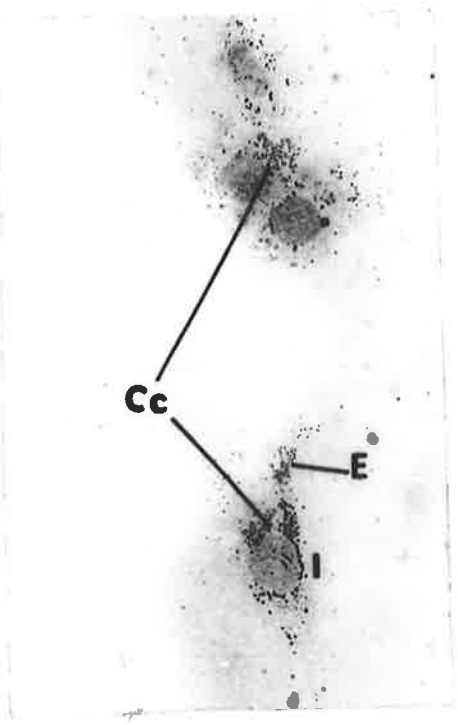


Fig. II-6

Autoradiograph of (^{35}S)-sulphate incorporated into cetylpyridinium chloride fixed material by prelabelled (5 min 'pulse') amnion cells after 45 min 'chase' incubation in radioisotope free medium.
(E=extension of cytoplasm).
(Cc=confined in cytoplasm).
(Mag x1250)

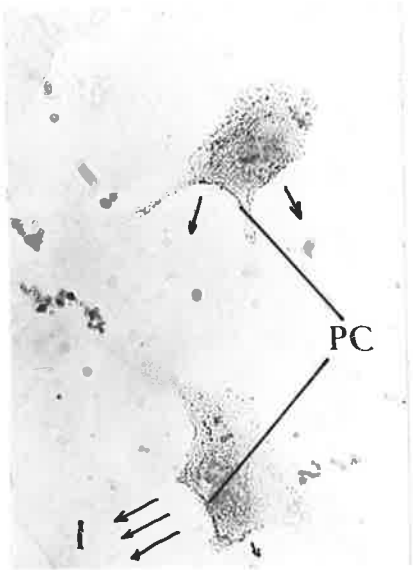


Fig. II-7

Autoradiograph of (^{35}S)-sulphate incorporated into cetylpyridinium chloride fixed material by prelabelled (5 min 'pulse' amnion cells after 60 min 'chase' incubation in radioisotope free medium.
(I=intercellular secretion, PC=pericellular).
(Mag x1250)

By the end of a 60 min 'chase' most of the label appeared to be at the perifery of the cell but discrete concentrations of label were evident, implying discrete ports of secretion for the macromolecules, (Fig.II-7). This material was susceptible to 1.25M $MgCl_2$ elution but the cytoplasmic label was not.

If non-confluent labelled cells were incubated for 120 min, CPC precipitable material appeared on the coverslip in locations several radii from the cells, (Fig.II-8.). The label was not readily washed away but it was susceptible to hyaluronidase and 1.25M $MgCl_2$. The label over cytoplasmic areas and at the cell perifery was neither susceptible to high molarity salt nor to hyaluronidase digestion.

The term "halo" has been nominated to describe the radio labelled area outside the observable boundaries of the cell in which a heavier concentration of label than background could be discerned, (Fig.II-8). It probably represents the sulphated moieties of the extrusions of detergent fixable macromolecules. The spatial restrictions imposed by neighbouring cells in completely confluent monolayers resulted in this label appearing as intercellular material, (Fig.II-9).

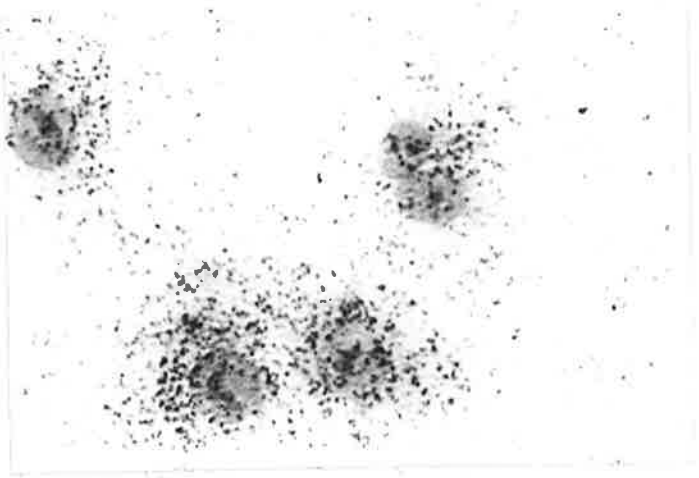


Fig.II-8

Autoradiograph of (^{35}S)-sulphate incorporated into cetylpyridinium chloride fixed material by prelabelled non-confluent amnion cells (+), (5 min 'pulse') after 120 min 'chase' incubation.
(Mag x1250)

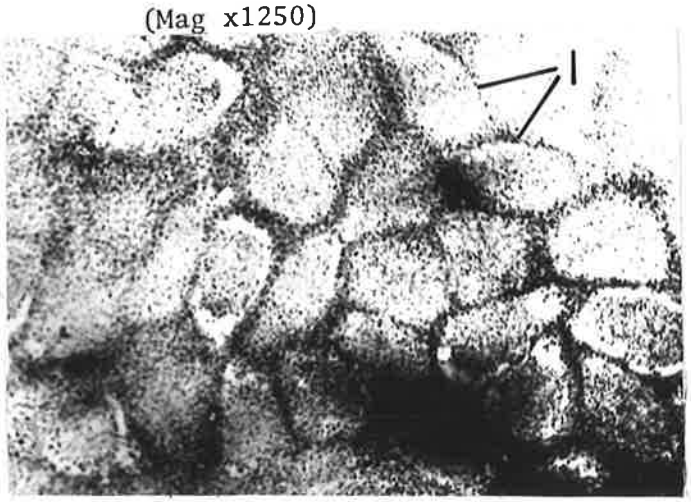


Fig.II-9

Autoradiograph of (^{35}S)-sulphate incorporated into cetylpyridinium chloride precipitable material by prelabelled (5 min 'pulse') fully confluent amnion cells (++++) after 120 min 'chase' incubation.
(I=intercellular).
(Mag x1250)

Acetate incorporation.

The results obtained from incubation of amnion cells in which (^{3}H) - acetate was incorporated do not entirely parallel those of the (^{35}S) - sulphate incorporation. The CPC precipitable (^{3}H) - acetate label was not evident in autoradiographs of cultures incubated for "chase" incubations over short periods ($<$ 15 min) after radio labelling. Moreover, extracellular (^{3}H) - acetate did not appear after 45min 'chase', (Fig.II-10). Nor were "halos" of non confluent cells evident after 2h but there was, nevertheless, some discrete label at the intercellular interfaces of adjacent cells, which could be eluted from CPC fixed preparations by all salt concentrations, (eg. Fig.II-11). Intra nuclear (^{3}H)-acetate was however observed at 2h.

These observations on autoradiographic studies of cultured amnion cells have been tabulated (Table II-3).

In summary then, following a short "pulse" labelling, non confluent cells synthesized a CPC precipitable product susceptible to low salt concentration elution. Those cells with intercellular interfaces appeared to incorporate (^{35}S)-sulphate into a product of higher charge and/or higher molecular weight, e.g. PG aggregates, Hep or HepS. Although the biochemical specificity of the incorporation of (^{3}H)-acetate into CPC precipitable PG is good, there are other biochemical pathways which utilize this precursor; for example, Acetyl Co A, followed by lipid anabolism, amino acid degradation and acetylation of sugars prior to glycoprotein synthesis.

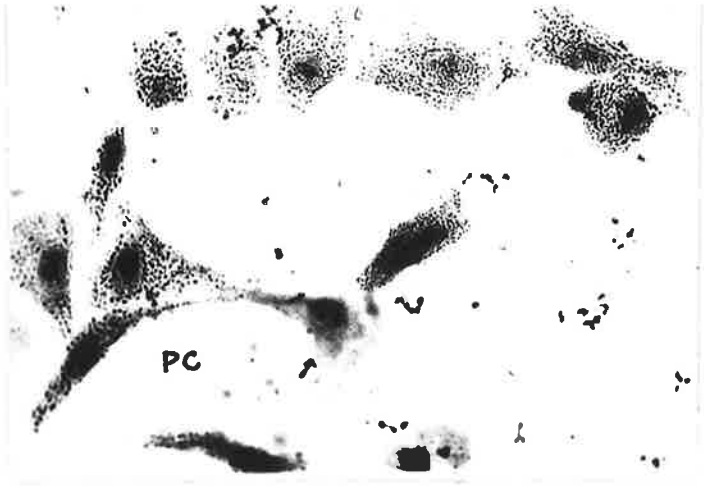


Fig.II-10

Autoradiographs of (^{3}H)-acetate incorporation into cetylpyridinium chloride precipitable material by prelabelled (5 min 'pulse') amnion cells after 45 min 'chase' incubation.
(PC=pericellular). (Mag x1250)

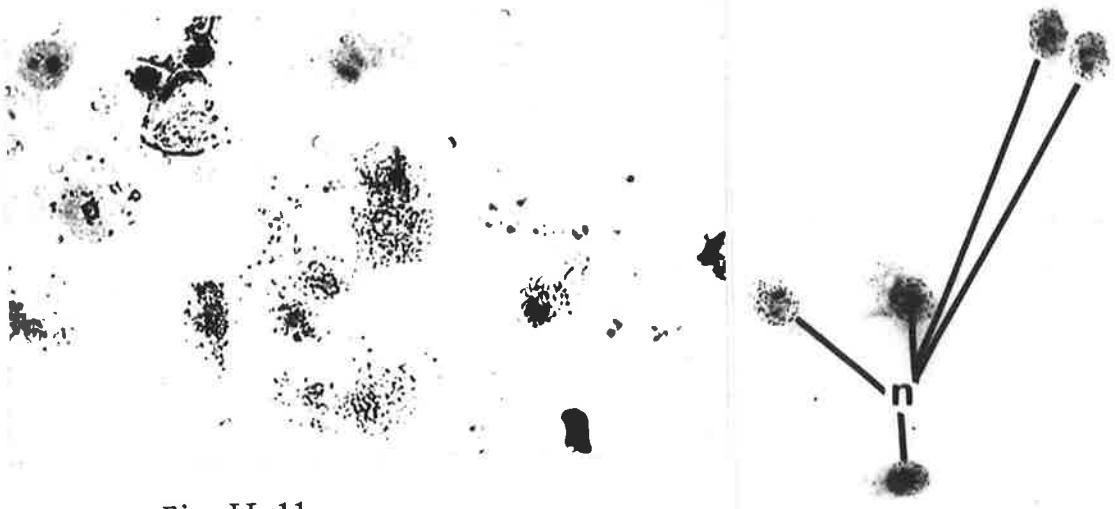


Fig.II-11

(a) Autoradiograph of (^{3}H)-acetate incorporation in prelabelled (5 min 'pulse') cells of amnion cultures after 120 min 'chase' incubation.
(b) The cells were washed in 0.63M $MgCl_2$ before coating with nuclear tracking emulsion. (Mag x1250) (n=nuclear label)

Table II-3 The occurrence of silver grains on auto-radiographs of adherent amnion cells

a. (^{35}S)-sulphate.

b. (^{3}H) -acetate.

All specimens were fixed in cetylpyridinium chloride and washed in one of three salt solutions of increasing electrolytic concentration. The three concentrations; 0.5M NaCl, 0.63M $MgCl_2$ and 1.25M $MgCl_2$; (represented critical electrolytic concentrations which dissociated the cetylpyridinium chloride/GAG complexes which corresponded to HA, ChS and Hep respectively, ie* 1,2,3, C = control slides).

s = observations on single cells.

a = observations on colonies of adjacent cells.

Minutes in ($^{35}SO_4^=$ medium ('pulse')	Minutes in 'chase' medium	Localization of silvergrains in autoradiographs					
		Nuclear *C 1 2 3	Peri Nuclear *C 1 2 3	Confined in cytoplasm *C 1 2 3	Periferal cytoplasm *C 1 2 3	Close extra cellular *C 1 2 3	Remote extra cellular *C 1 2 3
9	0 s		+	+			
	a		+	+			
5	2 s		+	+			
	a		+	+			
5	5 s		+	+ +			
	a		+	+ + +			
5	15 s						
	a						
5	30 s		+				
	a		+ +	+ + +			
5	45 s			+ + +			
	a			+ + +			
5	60 s			+ +	+ + +		
	a			+ +	+ + +		
15	90 s			+ + + +	+		
	a			+ + + +	+ + + +	+ +	
15	120 s			+ + + +	+ + + +	+ + +	+ + +
	a			+ + + +	+ + + +	+ + +	+ + +
15	150 s				+ + + +	+ + +	+ + +
	a				+ + + +	+ + +	+ + +

Minutes in (^{3}H)-AC medium ('pulse')	Minutes in 'chase' medium	Localization of silvergrains in autoradiographs											
		Nuclear				Peri Nuclear				Confined in cytoplasm			
		*C	1	2	3	*C	1	2	3	*C	1	2	3
9	0 s												
	a												
5	2 s												
	a												
5	5 s												
	a												
5	15 s												
	a												
5	30 s					+				+	+		
	a					+				+	+		
5	45 s									+	+	+	
	a									+	+	+	
5	60 s									+	+	+	
	a									+	+	+	
15	90 s									+	+	+	
	a									+	+	+	
										+	+	+	
										+	+	+	
15	120 s												
	a												
15	150 s	+											
	a	+											

Minutes in (^{3}H)-AC medium ('pulse')	Minutes in 'chase' medium	Localization of silvergrains in autoradiographs											
		Periferal cytoplasm				Close extra cellular				Remote extra cellular			
		*C	1	2	3	*C	1	2	3	*C	1	2	3
9	0 s												
	a												
5	2 s												
	a												
5	5 s												
	a												
5	15 s												
	a												
5	30 s												
	a												
5	45 s												
	a												
5	60 s	+	+	+									
	a	+	+	+									
15	90 s	+	+	+		+	+	+					
	a	+	+	+		+	+	+					
15	120 s	+				+	+	+					
	a	+				+	+	+					
15	150 s	+											
	a	+											

The observation of nuclear CPC precipitable (^{3}H)-acetate is in accord with this material being a PG; the evidence for such a location has been subsequently confirmed by a number of workers, BHAVANANDAN & DAVIDSON, 1975; FROME, BUDDECKE, von FIGURA & KRESSE, 1976. The apparent low levels of incorporation of (^{3}H)-acetate in this experimental regime would imply that there was a sufficiently large pool of acetylating moieties available prior to introduction of the tritiated precursor label, or that there was consistently insufficient levels of specific activity. Nevertheless the lowered labelling index did serendipidously reveal the existence of nuclear label which had been obscured by the greater levels of (^{35}S)-sulphate incorporation in those cell which had been incubated together with that isotope.

2.2. Comment on The "Halo".

At the commencement of this work (1968) the observation of the "halo" effect seemed novel and was thought to be fundamental.

More recently extracellular macromolecules, other than the CPC precipitable materials, have been investigated; notably the LETS (large external transformation sensitive) protein (CHEN, GUDOR, SUN CHEN & MOSESSON, 1977), which emphasize the importance of intimate extracellular molecules in the cells' microenvironment, and ultimately in their biological regulation.

The cell bodies of fully confluent cultures would themselves restrict the extent of spread of expansile macromolecules, as a consequence of the close proximity of one cell to another.

The biochemical data cited earlier indicated that the extracellular cell associated macromolecules around isolated cells represented a specialized chemical microenviroment. Furthermore in that state they would constitute an enviroment whose physicochemistry was different from that in the intercellular spaces between confluent cells or between cells in an organized tissue itself.

Indeed at the site of secretion on the cell surface, large extracellular macromolecules are in an inspissated form, possible from intracellular vacuoles (GODMAN & LANE, 1964). Export would result in hydration. In non confluent cultures, the "halo" may represent a concentration gradient of PG solution between the cell surface and the periferal extremities of the "halo" within the culture medium. This glycocalyx should be regarded as an integral part of the cell surface since it would characteristically effect a gradient of macromolecular exclusion properties. OGSTON & PHELPS (1961) have shown that the distribution coefficient - the ratio of the concentration of protein in, say an HA phase, to that in a buffer phase - was essentially independent of the the protein concentration. It varied with the HA concentration and with the type of protein studied. For example, they reported that HA excluded serum albumin to the extent of about 300ml/gm. Other workers have demonstrated the effect at lower values e.g. LAURENT (1964). The relevance to biological events, such as extracellular control mechanisms to these physical data is only now being more fully investigated. The organization of structures within multicomponent diffusion systems may explain the conformational requirements of extracellular materials during their interactive phases with cells. (PRESTON, LAURENT, COMPER & CHECKLEY, 1980). Intercellular PGs between adjacent cells of confluent cultures

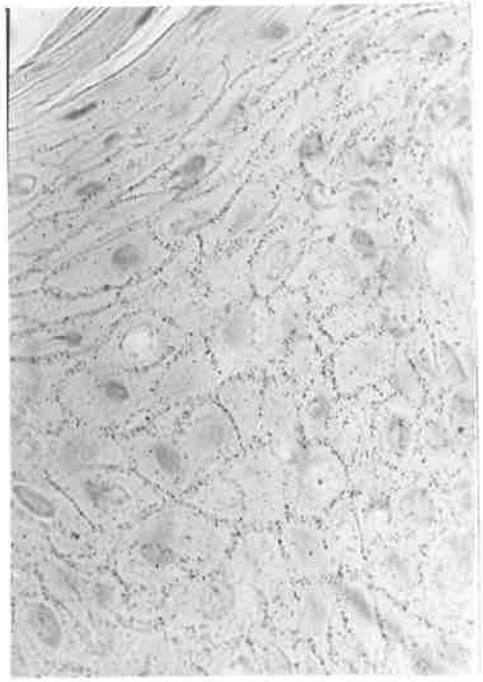
(and in tissues) would exert compartmentalizing effects on extraneous macromolecules different from those of the expanded extracellular glycocalyx of islolated cells. These molecules would be more vulnerable to dissolution, and/or degradation by enzymes, since more substrate cleavage site would be accessible eg. the susceptibility of extracellar matrix of non confluent cultures to testicular hyaluronidase.

3.2.3 Gingival epithelial culture.

Having estimated techniques for localizing the radioactive macromolecular products of amnion cell cultures the fate of biosynthetically radioactively labelled macromolecules in gingival epithelial tissue was followed. Advantages of autoradiography over histochemical staining are that fixation can be more chemically specific without further regard for the subsequent binding of an indicator dye (i.e. a specific histochemical stain) and that by using appropriate incubation times for incorporation of radiolabel, the biosynthesis and subsequent secretions by tissue pieces (or by cells) can be followed. The effects of various additives, such as HA and other extracellular GAGs in the incubation were also studied autoradiographically, vis à vis, Chapter IV, (WIEBKIN & THONARD 1982).

Histological sections of pieces of gingivae incubated for 15 min in Medium 199 containing (^{35}S)-sulphate or (^{3}H)-acetate followed by a 60 min "chase" in radioactive-free medium, showed autoradiographic labelling predominantly in the Stratum spinosum, (Fig.II-12). Despite some generalized labelling throughout the whole of the epithelial layer, little or no label was localized in the basement membrane. Almost all the epithelial label was confined to intercellular locations. The connective tissue was sparsely and diffusely labelled.

a)



b)

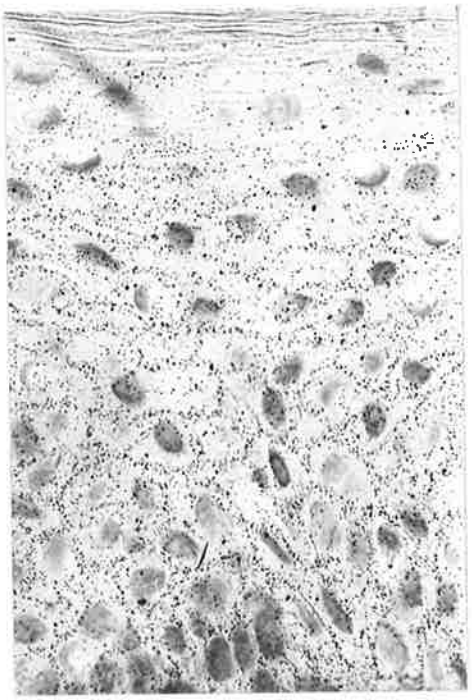


Fig.II-12 Autoradiograph of (a) (^{35}S)-sulphate (b) (^{3}H)-acetate incorporated by pre labelled (15 min 'pulse') gingival slices after 60 min 'chase' incubation. Sections fixed in 1% CPC as described in text. (Mag x1250)

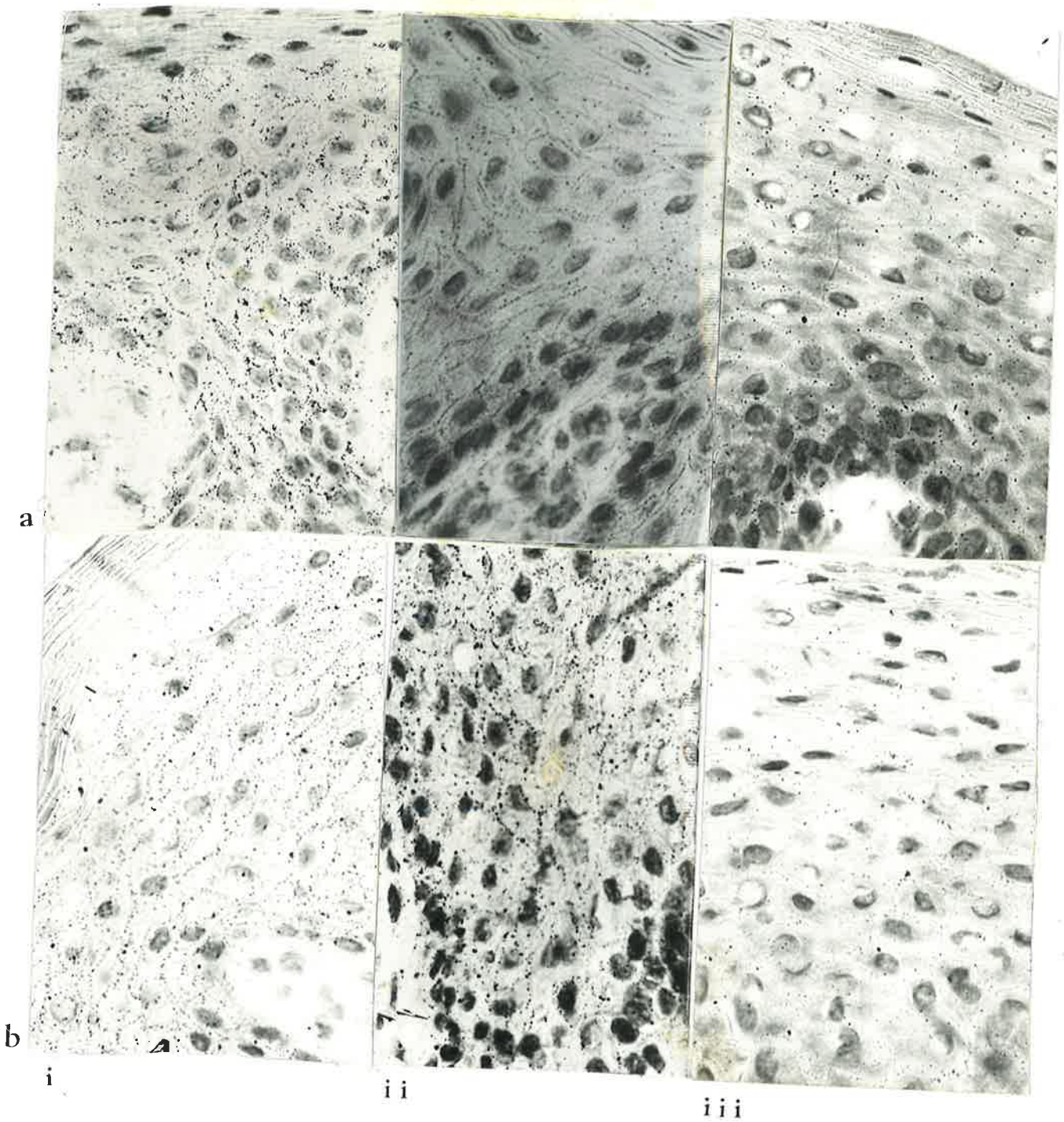


Fig.II-13

Autoradiograph of (a) (^{35}S)-sulphate, (b) (^{3}H)-acetate incorporated by gingival slices, fixed in cetylpyridinium chloride and eluted with:

(i) 0.5M NaCl,

(ii) 0.63M $MgCl_2$ &

(iii) 1.25M $MgCl_2$.

(Mag x500)

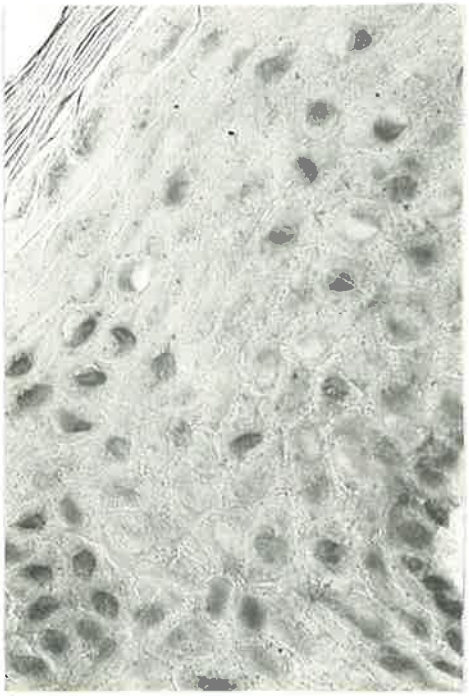
On sections fixed with CPC, autoradiographic silver grains corresponding to (^{3}H)-acetate incorporation were slightly diminished as compared with formal/ethanol fixation. In some sections intercellular architecture was less well pre - served, for example Fig.II-12a shows characteristic loss of nuclei. Nevertheless there appeared to be little or no difference between the distribution of (^{3}H)-acetate and (^{35}S)-sulphate label on respective slides.

Although most of either label was confined to intercellular locations there were discrete concentrations of label intracellularly. Elution of CPC fixed label with 0.5M NaCI did not reveal any difference between the distribution of (^{35}S)-sulphate and (^{3}H)-acetate. There was still a predominance of label in the intercellular location.

Almost all the intercellular label disappeared when the CPC fixed sections had been eluted with 0.63M $MgCl_2$ (This molarity of $MgCl_2$ was the lowest concentration of salt which removed 80% of ChS from a CPC impregnated filter paper) Control sections which were washed in 0.05% CPC in 0.05M NaCl for periods of time corresponding to the above elution regimes showed no marked diminution of label, (Fig.II-13).

Sections of tissue which had been fixed(killed)prior to incubation with radio label developed no autoradiographic pattern (Fig.II-14) indicating no isotope incorporation.

(a)



(b)

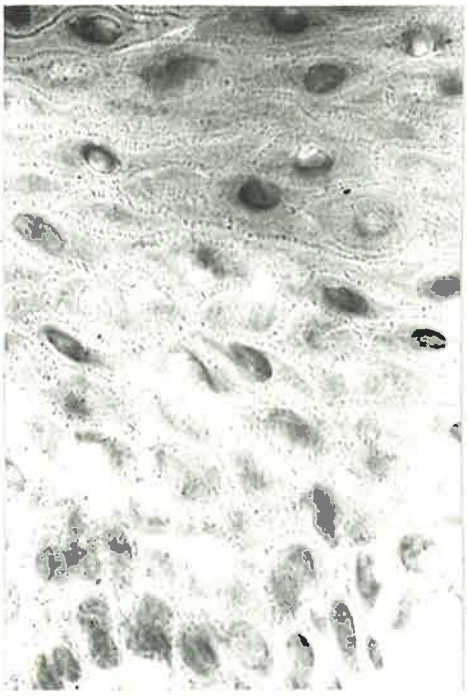
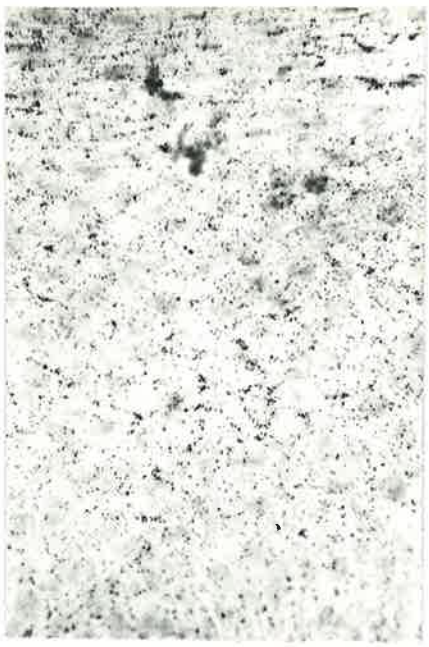


Fig.II-14

Autoradiograph of (a) (^{35}S)-sulphate (b) (^{3}H)-acetate incorporated by gingival slices, fixed (killed) prior to incubation with formal/ethanol. (Mag x1250)

(a)



(b)

Fig.II-15

Autoradiograph of (^{35}S)-sulphate incorporation into rat oral tissue, (a) buccal mucosa (b) tongue. (Mag x500)

In vivo radio isotope incorporation in rat buccal mucosa and tongue and subsequent autoradiography of sections of these tissues showed relatively high but indiscriminate labelling in the epithelium with equivocal concentrations in intercellular locations, (Fig.II-15). The middle layers of S.Spinosum showed the greatest metabolic activity. These results are in contrast to the relatively poor in vivo incorporation of (^{35}S)-sulphate into rat gingival tissue observed by MAGNUSSEN (1968), who used decalcified tissue. Both decalcification and fixation are important basic procedures.

3.2.4. SECTION SUMMARY.

The aforementioned studies have demonstrated that when amnion cell cultures were grown to high cell density, i.e. to confluence, the material surrounding the cell was condensed into an intercellular matrix which could be histochemically stained for polyanions or autoradiographically demonstrable following incorporation of radio actively labelled metabolic precursors.

By differential elimination of staining with appropriate enzymes the major polyanionic components of the intercellular substances appeared to have GAG properties as reported elsewhere by SISCA, LANGKAMP & THONARD, (1971) and THONARD & WIEBKIN (1973). Earlier studies using similar techniques applied to sections of human gingivae also revealed intercellular material which had GAG characteristics, (THONARD & SCHERP, 1962).

Criticisms of histological localization of intercellular material focus on whether dye substrates are on the inner or outer interface of cell membranes. In more recent studies using mild extraction methods under dissociative conditions the majority of PG can be shown to be extracellular (BARTOLD, WIEBKIN & THONARD, 1982) which confirms the early observations of DISCHE (1965) that the cytoplasmic sugars do contribute substantially to the total carbohydrate of epithelium. We will however see later that in culture at least a proportion of the newly synthesized polysaccharide is possibly never destined for export.

Furthermore, the in vivo incorporation of (^{35}S)-sulphate and (^{3}H)-acetate into oral epithelia (Fig.II-15) after 1h was predominantly localized (by autoradiography) in the intercellular sites of the S. spinosum. The known specificity of inorganic sulphate incorporation into GAGs (DZIEWAITKOWSKI, 1951, SCHILLER, MATHEWS, CIFONELLI & DORFMAN, 1956 BELANGER, 1954 and others), and the specific in situ CPC precipitation of extracellular macromolecules (fixation) followed by successive electrolyte elution is strong evidence for the GAG nature of the intercellular substances localised in this study. Indeed, it confirms the previous histochemical "identification" which strongly relied on cationic stains (THONARD & SCHERP, 1962).

The 'pulse-chase' regimes indicated that in the short term, in vitro GAG synthesis was relatively rapid (about 1-2h) since little intracellular label remained after 60 min. The basement membrane membrane incorporated little or no label, nor was there incorporation of (^{35}S)-sulphate and (^{3}H)-acetate into the mitotically active, S. germinativum. Indeed CASTOR & FRIES (1961) presented data which show an inverse relationship between cell generation time and AMPS synthesis in synovial cell cultures. Moreover, MPS

synthesis and DNA synthesis have been shown to be mutually antagonistic, (ABBOTT & HOLTZER, 1966).

The apparent similarity of incorporation of (^{3}H)-acetate and (^{35}S)-sulphate into intercellular material indicates that acetylation of those hexosamine moieties which will precipitate (fix) in tissue, can be used to follow both GAG synthesis and extra cellular matrix deposition. Furthermore, confluent epithelial cells synthesized about 60% of their total macromolecular uronic acid as HA (Fig.II-6). Unlike most of the other intercellular GAGs, HA is unsulphated. In the free state it is more easily eluted from CPC precipitated tissue extracts (0.5M-NaCl) than the more highly polyanionic sulphated GAGs (SCOTT, 1968). However, unlike the relative levels of radioactive incorporation demonstrated in amnion cell cultures, little or no difference was observed between the relative levels of (^{3}H) - acetate and (^{35}S)-sulphate deposition after 0.5M NaCl elution in gingival sections. SAVAGE & BERNARD, (1969), in an electron-microscopical investigation, reported that, intercellular MPS was observed throughout all layers of the gingival epithelium, including the keratinized layer. They commented that salivary sources may be contributory. Their observations seem at variance with the findings described above.

Furthermore, in the studies reported here, there was little evidence of radioactive labelling of intercellular material in the S. granulosum in the S. corneum.

Further refinement of the methodology is necessary to establish the quantitative proportions of GAGs at various localized sites.

However the autoradiographic techniques described appeared to be useful for the further study of intratissue biosynthetic responses to extraneous agents.

3.3 Biochemistry.

3.3.1 Amnion cell culture.

Biochemical analyses for uronic acid, sulphate and protein were performed on crude aqueous soluble preparations which could be precipitated with CPC from disrupted amnion cell cultures.

The sequential resolubilization of these precipitates by increasing the ionic concentration of the salt, provided some further purification of the amnion cell material. The adherent cells in monolayer cultures were analysed separately from non adherent cells ("floaters"). The CPC precipitable material secreted into the medium was also assayed. Due to the small amount of material available only the elctrophoretic mobilities of proteolytic digests of each fraction were attempted and these compared with available GAG standards. Recently published data on GAG content of gingival epithelium (BARTOLD, WIEBKIN & THONARD, 1981) provides increased specificity of analysis.
Since uronic acid is the ubiquitous monosaccharide of the polyanionic intercellular substance which is localized by histochemistry, and that its molar proportion within the GAG chain is common for all species the assay of this sugar has been quoted as the fundamental measure. Sulphation of GAG is known to be variable and it is therefore against uronic acid values that sulphate values are expressed. The comparison between uronic acid *per se*; as well as the degree of sulphation; of the CPC precipitable macromolecules derived from amnion cultures

of various levels of confluence has been described.

Cell associated material from monolayer.

The total macromolecular uronic acid content per cell of sparsely distributed adherent cultured amnion cells was less than half that of the cells from fully confluent culture (Table II-4).

Elution with 0.5M NaCl.

Only 32% of the uronic acid from the monodispersed cells (+ rating) was readily eluted from CPC precipitations by 0.5M NaCl compared with 71% from the fully confluent cells (++++ rating). Little or no sulphate was associated with readily elutable fractions (Table II-4). The CPC precipitable material, which could be solubilized in 0.5M NaCl, corresponded to the standard commercial HA when run electrophoretically on cellulose acetate strips. (Fig. II-16).

The protein content of the precipitable material (%) which was consistently solubilized in the low salt concentrations (0.5M NaCl) was greatest from fully confluent cells (Table II-4). In the first instance, this observation seemed to be anomalous since HA can be isolated from mammalian tissue essentially free of protein. Nevertheless the electrophoresic mobility corresponded to HA standards and might either imply very weak interaction with protein (such that the two components separated) or that any charge on the protein did not contribute to the mobility of the carbohydrate. The above findings may however lead to the speculation that the protein represents an associated "link" protein involved in macromolecular interactions.

Table II-4. Quantitation of cetylpyridinium chloride precipitable material associated with the amnion cells grown to various conditions of monolayer culture (see Fig. II-1).

Condition of monolayer culture: Ranking according to Fig. II-1	Cetylpyridinium chloride precipitable material			
	Total	solubilized in 0.5M NaCl	0.7M $MgCl_2$	1.25M $MgCl_2$
	uronic acid	uronic acid	uronic acid	uronic acid [1.]
+	0.068	0.022	0.029	0.017
++	0.064	0.013	0.021	0.03
+++	0.141	0.024	0.088	0.029
++++	0.128	0.091	0.031	0.006
	sulphate	sulphate	sulphate	sulphate [1.]
+	0.118	0	0.041	0.077
++	0.067	0	0.036	0.03
+++	0.0512	0.0015	0.038	0.0117
++++	0.0649	0.0	0.057	0.0079
	protein	protein	protein	protein [1.]
+	230.2	66.4	74.6	89.2
++	-	N.D.	71.3	8.1
+++	111.9	55.1	38.0	18.8
++++	65.1	45.0	17.1	3.0
	SO_4:UA	SO_4:UA	SO_4:UA	SO_4:UA [2.]
+		0.0	1.413	4.53
++		0.0	1.714	1.0
+++		0.0625	0.432	0.4
++++		0.0	1.83	1.32

1. umols/10^6 cells
see Fig. II-18 for range values.

2. molar ratios.

Such a hypothesis enjoys some credence now; ipso facto, proteins, glycoproteins and other PGs have all been reported to interact or stabilize aggregates of other intercellular matrix molecules with HA. Subsequent experimental evidence by this author, and by others will be discussed in Chapter V & VI.

Elution with 0.7M $MgCl_2$.

The uronic acid of adherent cells which was subsequently eluted from CPC precipitates by 0.7M $MgCl_2$ corresponded on electrophoresis to the ChS's and to ChS containing PG (Fig.II-16). The amount of uronic acid thus solubilized from adherent amnion cells was greatest in cultures just prior to fully constrained confluence (i.e. at +++ rating). The corresponding levels of sulphate values did not mimic the uronic acid values. However the amount of sulphate associated with cells, expressed both per cell or in terms of the uronic acid values rose when full confluence occurred. (Table II-4b & d).

The protein that was solubilized from CPC precipitates along with the macromolecular uronate in 0.7M $MgCl_2$ from cells of confluent cultures (+++ rating), was 25% of that assayed from + rated cultures, but remained between 26% and 32% of the total protein assayed.

This preliminary evidence was the first suggestion that the degree of sulphation, and the level of cell/cell contact may be regulatory.

ELECTROPHORETIC SEPARATION.

Fig.II-16.

Cellulose acetate strip electrophoresis of eluants of increasing salt concentration from cetylpyridinium chloride precipitates of an extract of human umbilical cord, and of commercial standards of AMPS. (This was representative of the separations achieved when the work was originally done. Highly quantitative electrophoretic separations have been published by the author and his colleagues, BARTOLD, WIEBKIN & THONARD,1982a).

Elution with 1.25M $MgCl_2$.

Cell associated sulphated macromolecules, solubilized from CPC precipitates by 1.25M $MgCl_2$ appeared to be heterogenous on electrophoresis, but corresponded in migration range to highly sulphated commercial Hep standards. Twenty five percent of the cell bound uronate associated with monodispersed cells (+ rating) had the high sulphate/uronic acid ratios consistent with the elevated sulphate ratios of the group of highly charged cell bound Hep-like-substances and Hep-S referred to by KRAEMER, 1971. Conversely, completely confluent cells contained only 5% total cell bound uronate resulting in a sulphate:uronic acid ratio of only 1.3.

In parallel with the decreasing sulphate and uronic acid values with increasing confluence, a quantitative decrease in the relative and absolute protein component was very marked, (Table II-4c).

In the light of the important data which have been variously reported from cell surface Hep-like-substances and Hep-S on several cell lines (KREAMER, 1971a,b,; KREAMER & SMITH, 1974; LINDAHL, 1976) the present data are strongly suggestive of such molecular species in amnion cultures. Moreover, the developmental consistency of this fraction of cell bound CPC precipitable material was also thought to imply a direct functional relationship to cell contact. KREAMER (1971 a,b) and KREAMER & SMITH (1974) have elegantly emphasized the importance of Hep-S as a cell surface component, and that it

Table II-5. Quantitation of cetylpyridinium chloride precipitable material associated with non-adherent amnion cells** separated from cultures having achieved various conditions of confluence. (see Fig. II-1).

Conditions of monolayer culture: Ranking according to Fig.II-1 "Floaters" **	Cetylpyridinium chloride precipitable material solubilized in 0.5M NaCl	1.25M $MgCl_2$	
	uronic acid	uronic acid	1.
+	0	0.014	
++	0	0.0042	
+++	0	0.024	
++++	0	0.0198	
	sulphate	sulphate	1.
+	0	0.0365	
++	0	0.003	
+++	0	0.0705	
++++	0	0	
	SO_4:UA	SO_4:UA	2.
+		2.607	
++		0.714	
+++		2 94	
++++		0	

Table II-6. Quantitation of cetylpyridinium chloride precipitable material in the medium of amnion cell cultures having achieved various conditions of confluence. (Fig. II-1).

Conditions of monolayer culture: Ranking according to Fig.II-1	Cetylpyridinium chloride precipitable material Total	solubilized in 0.5M NaCl	0.7M $MgCl_2$	1.25M $MgCl_2$	
	uronic acid	uronic acid	uronic acid	uronic acid	1.
+	0.0023	0.002	0.0003	None	
++	0.0054	0.005	0.0004	None	
+++	0.0002	None	0.0002	None	
++++	0.0005	None	None	0.0005	
	sulphate	sulphate	sulphate	sulphate	1.
+	0.0011	None	0.0011	None	
++	0.00054	None	0.00054	None	
+++	0.0007	None	0.0007	None	
++++	0.0004	None	None	0.0004	
	SO_4:UA	SO_4:UA	SO_4:UA	SO_4:UA	2.
+	0.47	-	3.66	-	
++	0.1	-	1.35	-	
+++	3.5	-	3.5	-	
++++	0.85	-	-	0.8	

1. umols/10^6cells.

2. molar ratios.

** cells that have sloughed off from the monolayer culture and separated from the medium before assaying the medium.

represented a "metabolically distinct compartment". Furthermore, it was derived directly from a small intracellular membrane associated pool which was trichloroacetic acid precipitable (i.e. associated with protein). Actual identification of N-sulphated moieties and N-acetylated hexosamine from epithelial cells has recently been described, (BARTOLD, WIEBKIN & THONARD, 1981).

Macromolecular uronate on non-adherent amnion cells sloughed off from monolayer cultures of various conditions of confluence (Floaters) ("Floaters").

Although the total amount of macromolecular uronic acid and corresponding sulphate was low in those non-adherent cells which had been centrifuged out of the medium from cultures at all rankings of confluence, the molar ratios ($SO_4^=$/UA) were high (Table II-5). In fact the only CPC precipitable material identified, was not solubilized at critical electrolyte concentrations below 1.25M $MgCl_2$. Microscopically these "floater" cells appeared to have recently undergone mitosis, showing dense nuclei. Those cells, from cultures which had barely achieved confluence (+++ rating) contained the greatest amount of uronic acid and sulphate; the relative amounts of the uronic acid values at confluences, + rating and +++ rating, correlated closely with the 1.25M $MgCl_2$ values for their adherent "parent" cells.

Macromolecular uronates secreted into the medium of amnion cell cultures.

Non confluent cells (+ and ++ratings) secreted small amounts of uronate over 24h into their respective nutrient media. Much of

this material (85-95%) was readily solubilized from CPC precipitates by 0.5M NaCl (Table II-6) the remaining sulphated material was solubilized by 0.7M $MgCl_2$. These molecules were highly sulphated. The small quantities of CPC precipitable material exported by fully confluent cells (++++ rating) were not readily solubilized at molarities less than 1.25M $MgCl_2$. However this quantitatively small fraction did not appear to represent the highly charged cell bound material described above, since its $SO_4^=$/uronic acid molar ratio did not exceed 0.8. These data, together with those for the non adherent cells further stress the cell bound nature of the highly sulphated material.

Sialic acid associated with amnion cells.

Although sialic acid was not previously thought to be an integral part of the PG molecule, its ubiquitous association with cell surfaces in the glycoproteins was considered to be sufficient reason to determine its presence in CPC precipitates of cell bound material. The small amounts of sialic acid which had precipitated with the uronate increased per cell as a function of culture development (Table II-7).

The macromolecular CPC precipitable uronate pool secreted by these cells into the medium did not contain significant amounts of sialic acid above control medium levels. The functional significance of this glycoprotein component are beyond the scope of this report but have been considered elsewhere (GREGORY, 1981)*.

Other chemical evaluations.

Paper chromatography of fully confluent cell cultures revealed

Table II-7 Sialic acid contents of amnion cells from monolayer cultures of varying confluence.

Degree of culture confluence	CPC* precipitable hydrolysed with H_2SO_4		CPC* soluble hydrolysed with H_2SO_4		CPC* soluble "free"	
	n mols/10^6cells		n mols/10^6cells		n mols/10^6cells	
+	0.15	(± 0.02)	0.63	(± 0.07)	0	
++	0		1.32	(± 0.12)	0.66	(± 0.07)
+++	0.45	(± 0.07)	0		0.33	(± 0.05)
++++	0.59	(± 0.07)	0		0.29	(± 0.03)

*CPC= cetylpyridimium chloride.

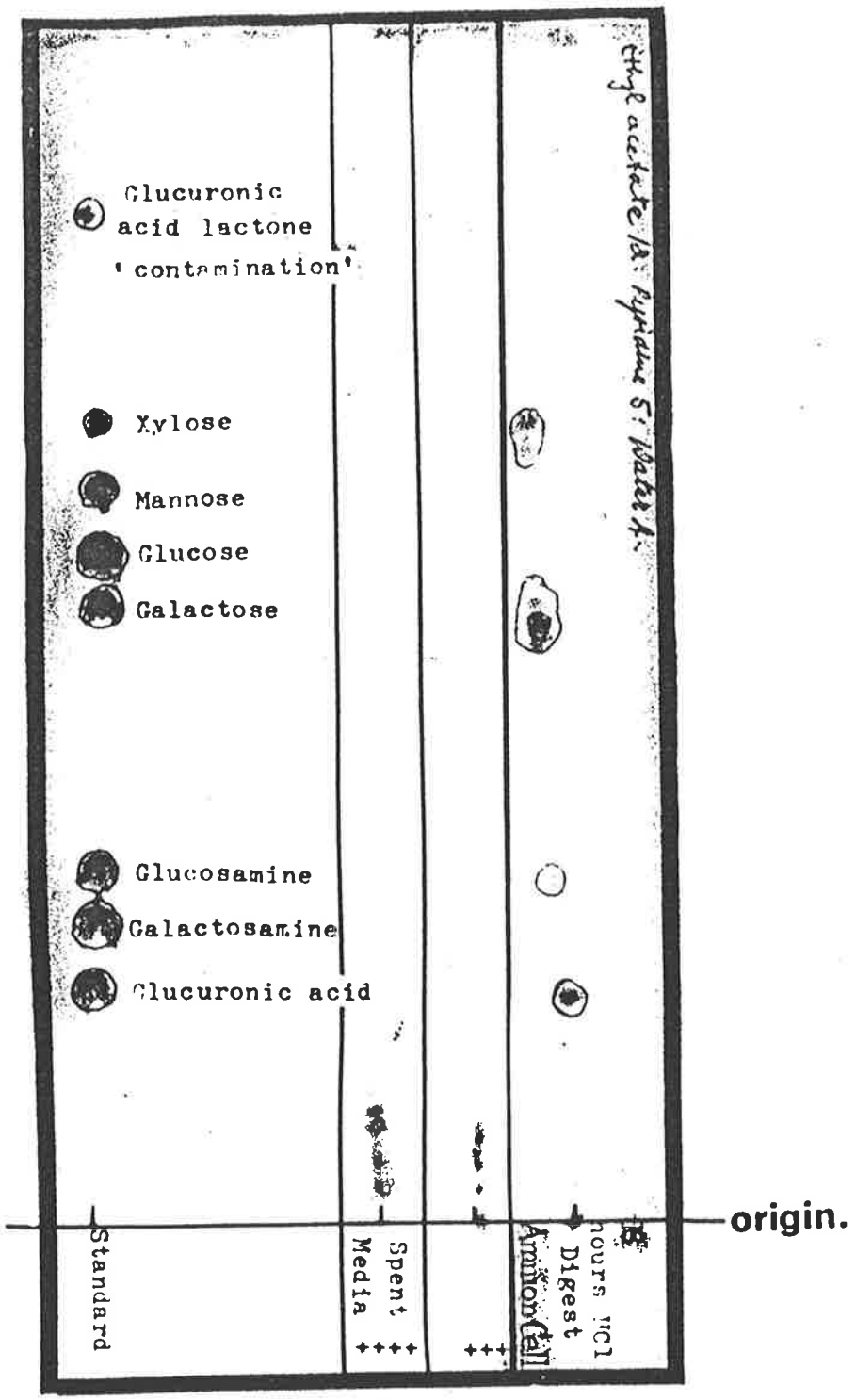


Fig. II-17. Paper chromatogram of a hydrolysate of a fully confluent amnion cell culture.
GlcUA, glucuronic acid; GalNAc, galactosamine; GlcNAc glucosamine; galactose and xylose.

glucuronic acid, glucosamine, uronic acid, some galactosamine, and xylose. (Fig. II-17). The variability of the hexosamine values as determined by the Elson-Morgan reaction appeared to be due to traces of $MgCl_2$ used in dissociating the AMPS from their CPC complexes since dialysis eliminated some of the depression of colour formation. ANTONOPOULOUS et al (1964) also comment on the depression of chromophore absorbancy in the Elson-Morgan assay, by traces of $MgCl_2$. Further, CHOTINER, SMITH & DAVIDSON (1968) note that combinations of amino acids together with hexosamines interfere with the assay. Since not all preparations were subjected to proteolytic digestion with papain/cysteine, hexosamine assays were found to be an impracticable source of routine data. Protein can also affect uronic acid colour* and data from non digested preparations have been omitted from the uronic acid results; but sulphate values were not shown to be affected by protein, amino acids, or salts (except thiocyanate used to precipitate excess CPC, which generated H_2S on hydrolysis).

It should be emphasized that the assayable amount, if any, of uronic acid, protein, hexosamine and sulphate was not previously known for ammion cultures at the outset of this study and comparable uronic/protein standards would have been helpful. This principle in which low levels of protein have been included in the assay systems, have been adopted for the recent continuation of this work.

The efficacy of the CPC and salt elution procedures described was tested by using small amounts of a human umbilical cord PG preparation (< 30ug AMPS). The cation affinities of ALDRICH

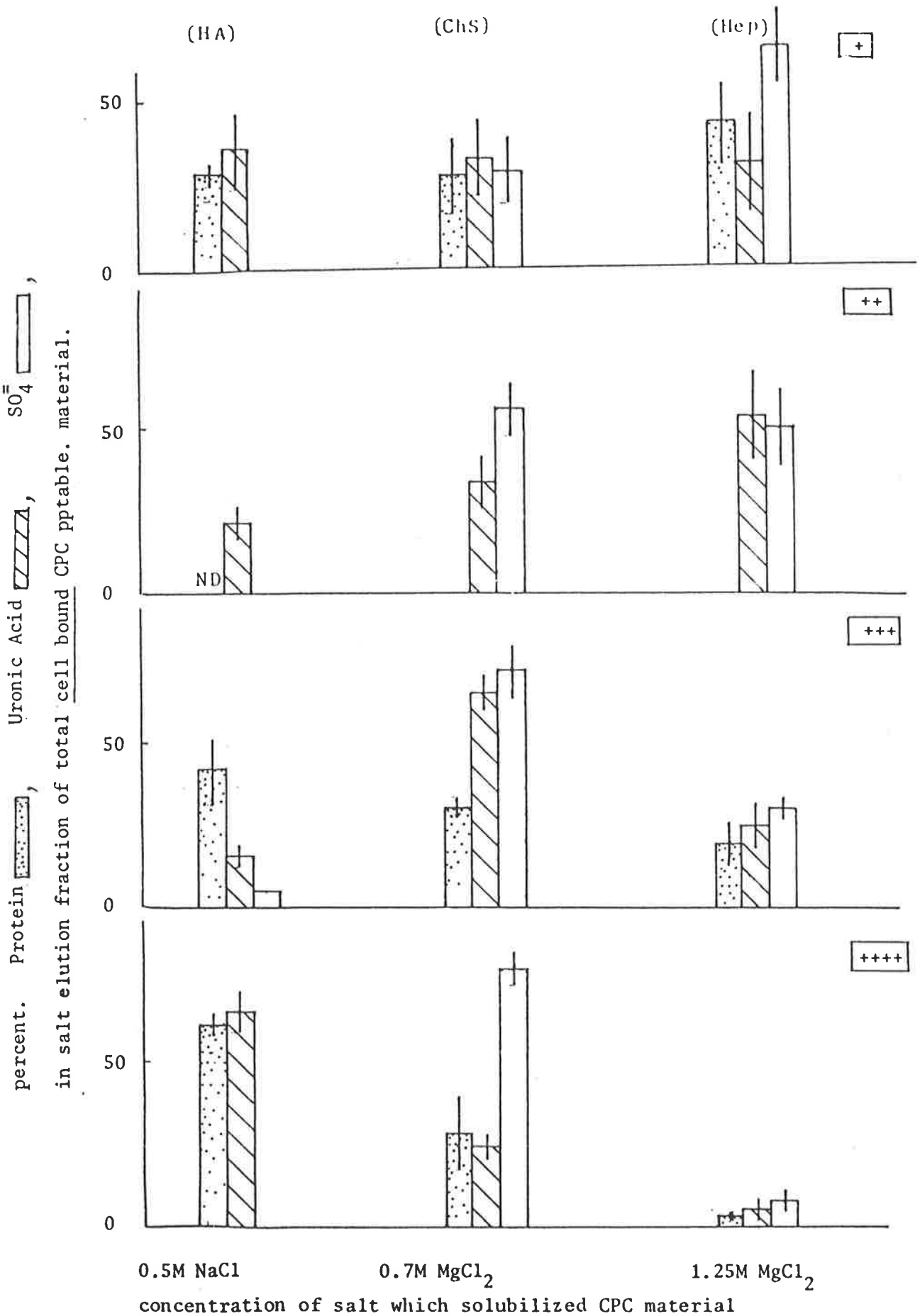


Fig.II-18

Proportion of macromolecular uronate , sulphate and associated protein in cells of the amnion cell line AV-2 at four stages of confluence (Cetylpyridinium chloride precipitable followed by critical electrolyte concentration elution). ND= not determined.

(1958), DUNSTONE(1962) and BUNGENBERG DE JONG (1949) and others were substantiated for ChS and Hep obtained from commercial sources. Furthermore, products that could be differentially eluted from CPC precipitates could be separated on starch gel (pH 3.2) (BROOKHART, 1965) or by cellulose acetate (pH 3.5) electrophoresis.

Despite the drawbacks in the practicability of chemically assaying very small amounts of material, the proportions of the different AMPS species which were bound to the cells varied characteristically. The amount of uronate in the fraction eluted from CPC complexes and representing ChS increased to a maximum as cell/cell interfaces developed to a condition in which the cells were just reaching confluence at +++ ratings (Fig.II-18). The increase in the intercellular interface surface area between cells from +++ rated and fully confluent (++++ rated) cultures, see Figs.II-1 & IV-17, was reflected in elevated levels of sulphation of the cell associated macromolecular uronates in the latter confluence condition (++++ rated).

3.3.2. Gingival cell culture.

Following the observation that the degree of confluence of amnion cell culture could be reflected in changes in the macromolecular uronate and in the sulphation of material associated with cells, assays were performed on gingival epithelial primary cultures. Uronic acid, and sulphate levels were determined on cells which had been disaggregated into a "fragmentary suspension culture"

a) according to culture age.

b) according to degree of cell/cell aggregation per se, (i.e. cell clump size) and

c) according to the development of the cellular aggregation in fragmentary suspension culture into the various filterable clumps (see Methods section, page 50.)

In vitro MPS synthesis related to culture age.

Primary fragmentary suspension cultures of gingival epithelial cells were grown for determined periods up to 21 days. These cultures had not been subjected to any prior fractionation which would separate variously sized cell clumps. The total cell-bound CPC precipitable uronic acid and sulphate per cell in such cultures reached a maximum at about day 6. However by day 9 these values decreased slightly remaining steady for several subsequent days. After 11 days and up to 21 days when the experiments were terminated, the level of uronic acid did not vary by more than 3% while the cultures remained healthy. There was always a substantial decrease in cell bound uronic acid and sulphate after the first 24h in culture but recovery invariably occurred by day 6. Dry weight values of cultures at day 6 were also consistently higher. (Table II-8).

Table II-8

Macromolecular uronic acid (a) and sulphate (b) content of gingival epithelial cells in "fragmentary suspension culture".

Time grown in fragmentary suspension culture (days)	Cetylpyridinium chloride precipitable material					Dry Mass of tissue ug per (10^6cells)
	(a)uronic acid			(b) sulphate umol per 10^6	Sulphate:uronic acid ratio	
	umol per gram tissue	umol per 10^6 cells; 0.5 M NaCl elution (HA.)	umol per 10^6 cells; 1.25 M $MgCl_2$ elution (ChS . Hep. etc.)			
1 (4)+	34.5 ± 4.3	0.0114 ± 0.0005	0.0031 ± 0.001	0.005 ± 0.005	1.6	420 ± 23
4 (5)+	2.4 ± 0.3	ND	0.0012 ± 0.0005	0.002 ± 0.003	1.65	500 ± 50
6 (4)+	33.6 ± 4.5	0.0072 ± 0.001	0.018 ± 0.0015	0.061 ± 0.005	3.5	750 ± 31
8 (3)+	30.5 ± 3.8	0.0065 ± 0.0015	0.013 ± 0.002	0.041 ± 0.007	3.1	635 ± 63
9 (5)+	30.0 ± 4.0	0.0062 ± 0.001	0.012 ± 0.001	0.038 ± 0.0005	2.9	640 ± 58
11 (1)+	-		0.007 ± 0.0015			

The suspension cultures consisted of disaggregated gingival epithelium grown in Medium 199; 15%BS. Culture Nos. ( )+

The uronic acid content of gingival epithelial cells in fragmentary culture remained about 0.3% of total dry weight. The value is both higher than that estimated by us for the uronic acid content of intact, separated human gingival epithelium (BARTOLD, WIEBKIN & THONARD, 1981) and for whole porcine gingivae (HIRAMATSU, ABE & MINAMI, 1979). These higher values probably represent an adaptation to the culture conditions, similar to those for cultured chondrocytes, (SANDY, BROWN & LOWTHER, 1979).

Assays of material eluted by 0.5M NaCl from the CPC precipitates, revealed that more than twice the uronic acid was synthesized by cells on the first day in culture than in the later days. The level of the sulphate values from the uronic acid containing material subsequently eluted with $MgCl_2$ of greater molarity (1.25M) was lower at day 1 than later in the cultures,(Table II-8).

In vitro MPS synthesis related to cell aggregation per se.

Since the purpose of this study was to investigate inter-cellular MPS, gingival cells in variously sized aggregations were studied in suspension cultures. Gingival cells were prepared and grown for 21 days in Medium 199 as described in the Materials and Methods section. These cultures were then fractionated according to constituent cell clump sizes. Cell preparations were thus divided into four categories depending upon their retention by the filter system.

Category I essentially consisted of the single cell suspension and Category IV consisted of large clumps of morphologically different cells, probably originating from the outer layers of the epithelium.

Results show that on a 'per cell' basis, cell bound macromolecular uronic acid values were highest in Category II (50μ filter) where cells were aggregated in small clumps (2-5 cells). Sulphate values were maximal in Category III (200μ filter) where cells were in larger clumps, (Table II-9).

In vitro MPS synthesis related to cell aggregate development.

As distinct from those experiments where both aggregated and single cells were permitted to remain in culture together throughout a determined experimental period, another experimental regime was adopted where cells were fractionated according to clump size at day 0 directly following the initial dissaggreation from the gingival tissue. Only the single cell suspensions were then cultured for 21 days and further fractionated. During this time, cells had self aggregated and in some cases appeared to have cloned.

The purpose of these experiments was to establish changes if any in the proportion of sulphate to uronic acid in cells during cellular aggregation development. Uronic acid values, per cell, of material eluted with 0.5M NaCl from CPC precipitates decreased as a function of increasing clump size. (Table II-10).

Table II-9

Macromolecular uronic acid (a) and sulphate (b) content of gingival epithelial cells fractionated according to cell clump size after 21 days in "fragmentary suspension culture". (Fig. II-3).

	Cetylpyridinium chloride precipitable material.						
	(a) uronic acid				(b) sulphate		
Cell Category	µmol per gram tissue	µmol per 10^6 cells; 0.5 M NaCl elution (HA.)	µmol per 10^6 cells; 0.7 M $MgCl_2$ elution (ChS)	µmol per 10^6 cells; 1.25 M $MgCl_2$ elution (Hep)	µmol per 10^6 cells; 0.7M $MgCl_2$ elution	µmol per 10^6 cells; 1.25 M $MgCl_2$ elution	$SO_4^=$: uronic acid ratio of sulphated material
Single cells I (6)+	28 ± 4.0	0.004 ± 0.0013	0.004 ± 0.0021	0.004 ± 0.0024	0.005 ± 0.0024	0.002 ± 0.001	0.8
50u mesh II (5)+	38 ± 4.5	0.006 ± 0.0004	0.005 ± 0.0015	0.008 ± 0.0034	0.008 ± 0.0011	0.013 ± 0.005	1.65
200u mesh III (6)+	26 ± 3.1	0.003 ± 0.0012	0.004 ± 0.0023	0.006 ± 0.0022	0.01 ± 0.0045	0.028 ± 0.006	3.8
Large clumps 500u mesh IV (4)+	10 ± 2.0	0	0.002 ± 0.0021	0.003 ± 0.0027	0.007 ± 0.0038	0.009 ± 0.0063	3.2

The suspension cultures consisted of epithelial cells of disaggregated gingivae N.B.*No sulphate was detected in 0.5M NaCl elution. Culture nos. ( )+ ± =range

Table II-10 Macromolecular uronic acid (a) and sulphate (b) content of gingival epithelial cells after 21 days in "fragmentary suspension culture".

	Cetylpyridinium chloride precipitable material.						
	(a) uronic acid				(b) sulphate		
ell Category (Fig. II-3) Cell Category	µmol per gram tissue	µmol per 10^6 cells; 0.5M NaCl	µmol per 10^6 cells; 0.7 M $MgCl_2$	µmol per 10^6 cells; 1.25 M $MgCl_2$	µmol per 10^6 cells; 0.7M $MgCl_2$	µmol per 10^6 cells; 1.25 M $MgCl_2$	$SO_4^=$: uronic acid molar ratio of sulphated material*
Single Cells I (5)+	28.5 ± 2.1	0.004 ±0.0021	0.004 ± 0.002	0.004 ±0.0017	0.004 ± 0.0025	0.006 ± 0.0005	1.25
50u mesh II (5)+	32.0 ± 4.2	0.005 ±0.0032	0.004 ± 0.00015	0.007 ±0.0028	0.006 ± 0.0037	0.016 ± 0.0052	2.0
00u mesh III (6)+	21.0 ± 2.7	0.004 ±0.0025	0.003 ±0.0024	0.004 ±0.0018	0.006 ± 0.0048	0.016 ± 0.0031	3.14
arge clumps 00u mesh IV (5)+	23.0 ± 2.5	0.001 ±0.001	0.004 ± 0.0013	0.0065±0.0007	0.011 ± 0.0055	0.02 ± 0.0038	2.95

These cells originated from single cell suspensions (I) obtained from initial gingival epithelial disaggregation (at day 0).

Cultures were grown in Medium 199, 15% BS. Culture nos. ( )+ No sulphate was detected in 0.5M NaCl elutions. ± = range

The uronic acid values of cell associated material eluted from CPC precipitates with 0.7M $MgCl_2$ were similar in each of the cell fractionation categories but in general they were lower than those assayed for material eluted with 1.25M $MgCl_2$.

Higher sulphate/uronic acid ratios were recorded from single cell fractions. Indeed, the trend for increased sulphate/uronic acid ratios was mirrored in the material eluted with the 0.7M $MgCl_2$. The total amounts of cell associated macromolecular uronic acid synthesized at day 21 was significantly different ($P \leq 0.05$) between the first two cell Categories (I & II) and together they were significantly higher than the amount synthesized by the larger clumps, Categories III and IV of cells. In all these cultures the ratio of macromolecular uronic acid eluted with 1.25M $MgCl_2$ to the total sulphated uronic acid values (0.7M $MgCl_2$ + 1.25M $MgCl_2$ eluents), was approximately 3:5. Uronic acid per dry weight of cells was increased in category II where it is tempting to regard the small aggregates as "young clones" and where they may be compared with the newly adapted cultures of day 6 in the previously described experiments. (Table II-9).

4.0. Interim Inference.

The larger clumps of cells in culture and those primary cultures which had been established for more than six days, demonstrated high sulphate to uronic acid ratios. Much of the high sulphate:uronic acid ratio can be attributed

to material eluted from CPC precipitates by 1.25M $MgCl_2$, a fraction which probably represents a tightly cell bound species of material. Such a fraction may be Hep or HepS or a Hep-like species of molecule, the functional roles of which continue to remain relatively obscure. Nevertheless, the control of biosynthesis of these highly sulphated macromolecules, which have been shown to be intimately associated with cell surfaces (KRAEMER, 1971), may be regulated by cell contact. On the other hand, the amount of non sulphated macromolecular material which was eluted from CPC complex with 0.5M NaCl, decreased as a function of clump size in all gingival epithelial cultures. This material, which corresponds to HA also decreased as the primary cultures developed. Several speculations could be advanced, among them; (i) Tissue integrity or cell/cell adhesiveness, is dependent upon highly sulphated polysaccharides of the cell surface. (ii) synthesis and secretion of the highly sulphated polysaccharides of cell surfaces is dependent upon cell contact and adhesion. (iii) Readily solubilized, non sulphated macromolecular polysaccharides, such as HA are important in regulating the synthesis and secretion of sulphated PG (cf. WIEBKIN & MUIR,1973a); in turn this latter synthesis is mutually dependent on the formation and development of cell/cell contact (cf. TOOLE, 1973).

The principal factor in (i) and (ii) above is the increase in GAG/sulphate as 'fragmented' gingival epithelium cells adapt to their in vitro enviroment, or as their cell contacts become established. The latter observation is indeed endorsed by a

similar interpretation, made earlier, with respect to the degrees of sulphation of macromolecular uronates during the development of cultured amnion cell monolayers, (Table II-4).

On the other hand the evidence supporting HA mediated control for the intra tissue maintenance of tissue integrity has subsequently appeared in the literature. However, characteristic tissue properties preclude a generalized principle. For example, the regulation of synthesis of cartilage PG by HA was thought to be unique since other connective tissue cells; skin fibroblasts and synovial cells did not demonstrate the relationship between HA and PG synthesis. Nevertheless an hypothesis, that HA was involved in the regulation of synthesis of intercellular PG by epithelial cells is inferred. Indeed, Chapter V describes the evidence verifying this early inference. Since epithelium shares with hyaline cartilage the properties that both tissues are avascular and that both tissues synthesize PG capable of interaction with HA, it may be coincidental that epithelial cells share with chondrocytes the mechanism which regulates PG synthesis in the presence of HA.

In summary, the two epithelial cell systems (amnion cells and fragmentary gingival cell suspensions) have demonstrated that the cells, in vitro are capable of synthesizing macromolecular uronate and their levels of sulphation reflect a state of cell/cell interaction ie confluence or clump size.
The limited amount of material and the lack of easily accessable healthy human gingival epithelium restricted the study to relatively crude preparative procedures to avoid loss of assayable

preparative procedures to avoid undue loss of assayable material. Nevertheless the determinations were reproducible and the electrophoretic separations which were consistent with standards have been confirmed by other systems,(eg vis à vis Chapter III and BARTOLD, WIEBKIN & THONARD 1981).

CHAPTER III

IN VITRO METABOLISM OF GLYCOSAMINOGLYCANS IN AN AMNION CELL LINE AND IN GINGIVAL EPITHELIUM.

1.0 INTRODUCTION.

In an attempt to understand biochemical events which may reflect the control mechanisms of synthesis and secretion of intercellular epithelial PG, cell cultures were incubated with radioactive precursors and the biosynthetic products were isolated. The relationships between the biosynthetic data so obtained and the extremes of confluence (+ rating and ++++ rating) of amnion cell cultures were studied. Similar preliminary biosynthetic investigations were also performed on the other epithelial cell model, the gingival cell "fragmentary suspension" culture.

2.0 MATERIALS & METHODS.

2.1 Amnion cell cultures.

Monolayer cell cultures were grown to four stages of confluence as described previously (Fig. II-1) in Medium 199 supplemented with 8% foetal calf serum (FCS).

2.2 Gingival epithelium - preparation of "fragmentary suspension" cultures.

Human gingival epithelium was obtained by micro dissection from gingivectomy specimens. A dissecting microscope (x 10) was used and the material appeared to be predominantly epithelial as evaluated by histology of representative preparations. The tissue was available for experimentation less than 45 min after surgery, during which time it had been stored in Medium 199 with antibiotics, as described previously. The separated epithelium was chopped with a sharp scalpel

and washed at 37°C with Medium 199 and 8% FCS on a 50μ nylon filter to remove single cells, damaged cell debris and extraneous cell products. The retained tissue was then incubated in Medium 199 and 3% F.C.S. containing radioactive isotope. Aliquots of the fragmentary cell suspensions were used to establish rough estimates of cell numbers. Haemocytometer counts were made of trypsin/EDTA disaggregated suspensions (see Chapter II).

All manipulations were carried out at 37°C.

2.3 Radioactive isotope incorporation.

Medium 199, 8% FCS containing one of either 50uCi/ml (^{35}S)-sulphate, 10uCi/ml (^{14}C) - UDP glucose or 10uCi/ml(^{3}H)- acetate was prepared.* Five ml of medium containing radioactive label and maintained at 37°C, was added to washed monolayer cultures ranked according to their degrees of confluence. Gingival epithelium at an approximate final concentration of 10^5 cells/ml was similarly incubated in radioactive medium. All cultures were pulsed with radio labelled precursors for 15 mins, after which the cells were quickly washed in pre-warmed radioactive isotope free medium. The washings were replaced by fresh medium, this procedure took 4 min. These 'chase' incubations continued for 2, 5, 10, 20, 35, 80 or 120 min.

2.4 Culture products.

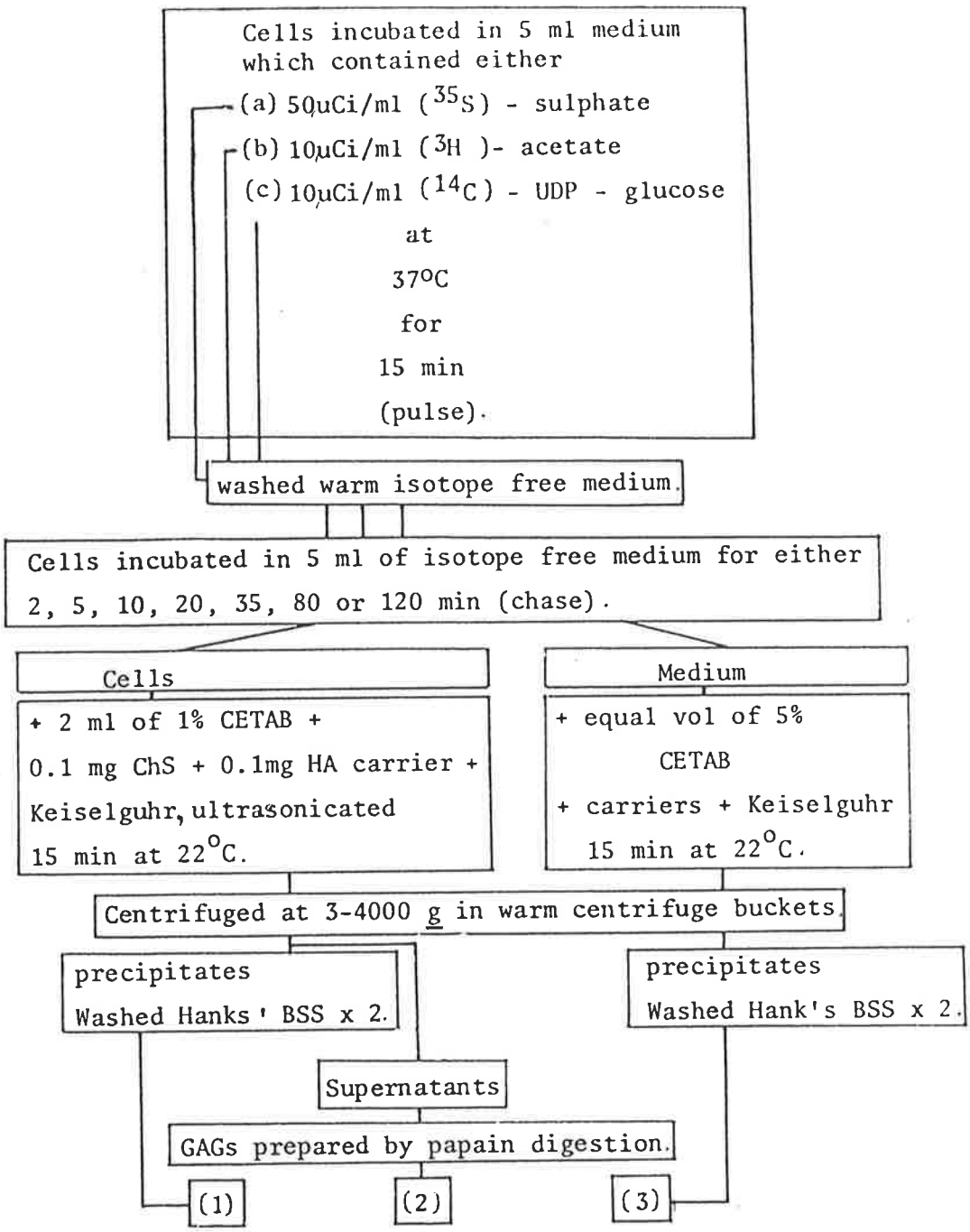
At the end of the appropriate incubation time intervals, the culture medium was removed from the cells, mixed with an equal volume of 5% cetyltrimethyl-ammonium bromide (CETAB) after which 0.1 mg of carrier ChS and of HA was added.

The cells were washed twice in 2 mls of cold medium at 4°C. These washings were then added to the detergent (CETAB) containing culture medium. The cells were ultrasonicated in 2 ml of 1% solution of CETAB at approximately 22°C and left at room temperature for 15 min. A small amount of Kieselguhr was added to each preparation. The Kieselguhr/CETAB precipitates were centrifuged at 3-4000$\underline{g}$ in warm centrifuge buckets. The supernatants were stored at 0°C and the precipitates were washed twice in Hanks' BSS. These detergent precipitable pellets were also stored at 0°C. Where the GAGs were to be identified, the resultant papain/cysteine digests were used. In those experiments assessing the rate of release of total macromolecular radioactive product from amnion cell incubations, the culture medium was completely removed at 5, 10, 20, 35 and 80 min after the radioactive 'pulse', and the macromolecular radioactivity measured. The culture was replenished with pre-warmed fresh medium. The total cell bound radioactivity was established at various time intervals by replacing the medium of some cultures with detergent and treating these and killed cells as described above.

The following fractions from amnion cell cultures of various confluences and from gingival epithelium were thus available for assay. (SchemeIII-1).

1. Detergent (CETAB) precipitated cell associated material.
2. Detergent (CETAB) soluble cell associated material.
3. Media containing secreted material.

The detergent precipitates were solubilized 3 x 1ml 1.25M-$MgCl_2$ and excess detergent was removed by precipitation with



Scheme III-1. Preparation of GAG in epithelial cells.

Flow chart of procedures for following the biosynthetic incorporation of radiolabelled precursors into detergent soluble and insoluble fractions by cultured epithelial cells.

saturated KCNS.

The fractions were individually dialysed against distilled water and concentrated in vacuo to 0.5 ml. Some cells associated precipitable material was not dialysed.

2.5 Standard preparations.

Standard GAG preparations were purchased from Sigma Chemical Co., St. Louis, MO., U.S.A. All radioactive isotopes were supplied by the Radiochemical Centre, Waterloo, N.S.W., Australia. 3'-phosphoadenosine 5' -phosphosulphate (PAP(^{35}S))* was prepared and purified by the method of ROBBINS (1962) using (^{35}S) -sulphate and brewer's yeast. UDP-N-acetyl galactosamine (^{35}S)-sulphate* was prepared as follows:-

A mixture of 5 ml butanol; 0.25 gm ammonium sulphate; 0.7 mCi $(Na)_2$ (^{35}S) 0_4 was cooled and 0.25 ml was added to 0.75 ml H_2SO_4 together with 0.1 gm UDP-N-acetylgalactosamine, stirred, allowed to reach room temperature and the precipitate centrifuged down, washed and brought into solution with 0.5 ml of 0.5% sodium acetate. This was spotted on Whatman for chromatography.

2.6 Electrophoresis.

Cellogel* 14x2.5 cm (Chemitron, Milan) and Sepraphore III 30 x2.5 cm (Gelman, Ann Arbor, Michigan) cellulose acetate strips were used for electrophoretic separations. The former were found to be superior for resolution, staining characteristics and for ease of handling.

The buffers were made up as follows:-

Acetic acid	- 100 ml
H_2O	- 895 ml
Pyridine	- 5 ml

pH 3.5

Known standards including HA, ChS (mixed isomers) and Hep were electrophoresed separately and as mixtures together with papain treated crude extracts of PGs from soft tissue homogenates. (FigII-16 and Fig.III-1.)

2.7 Chromatography (Paper).

Ascending paper chromatography was performed on Whatman No. 20 paper. Two different solvent systems were used and made up freshly as formulated below:-

System A (denoted in graphs ○—○ or ●—●)	ratio by vol.
Isobutyric acid	100
0.1M NH_3 (as NH_4OH)	60
0.1M Ethylenediamine tetra-acetic acid (sodium salt) EDTA.	1.6

System B (denoted in graphs △—△ or ▲—▲)	ratio by vol.
Isopropanol	60
0.4M Ammonium formate	40

2.8 High Voltage Electrophoresis.*

Either 0.05 ml or 0.01 ml samples were applied 1 cm from the bottom edge (cathode end) of carefully cut Whatman No. 3 MM filter paper (5x57 cm*) and electrophoresed in 0.05M citrate buffer (pH 5.1) at 1,500 volts (250 amps) for 60 min or 90 min. Standards included

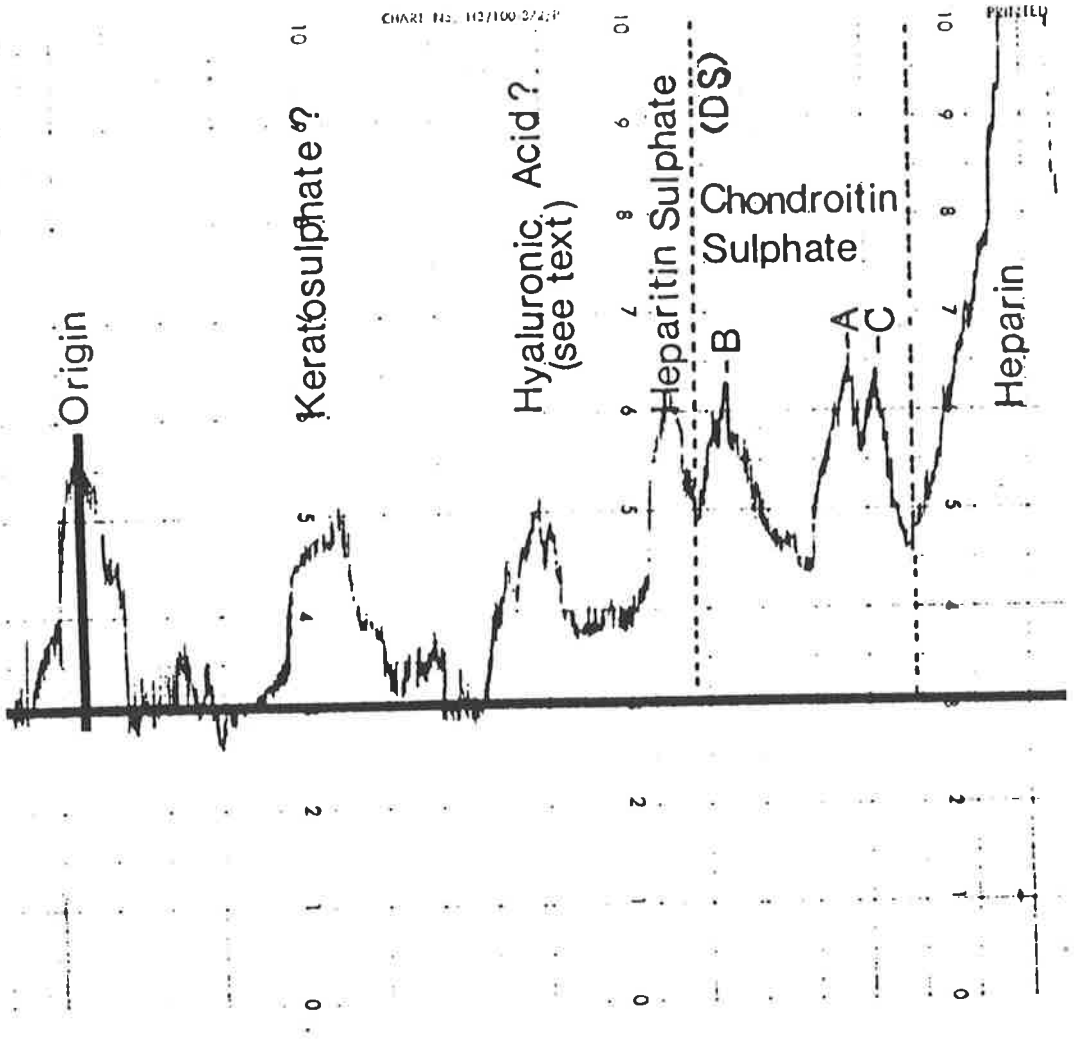


Fig III-1 A plot of a characteristic electrophoretic separation of(^{35}S) - sulphate labelled GAG on cellulose acetate. The separation was scanned for radioactivity. The peaks correspond to standard separations.

Mice were injected with(^{35}S)- sulphate (10uCi/gm) They were killed at 8-12hr, decapitated and eviscerated. The skin was removed and was homogenized in 1.25M $MgCl_2$. Four volumes of ethanol were added to cleared soluble extract. The precipitate formed over night at 4°C was resuspended in 0.1M Na acetate. The soluble material was reprecipitated twice in cold ethanol and the Na-acetate soluble material was subjected to alkaline hydrolysis and the GAGs were reprecipitated with ethanol. These precipitates were dissolved in buffer and electrophoretically separated.

ATP, AMP, PAP(^{35}S) AP(^{35}S) and $Na_2(^{35}S)O_4$. Electrophoretic mobilities of separated materials were identified as described below and were compared with control standards, (Fig.III-2). Care was taken to remove all Keiselguhr from samples prior to application to avoid charring of the paper during electrophoresis.

2.9 Detection of Separations.

Following papain digestion of CETAB precipitated extracts of epithelial cells, the GAGs were separated by cellulose acetate electrophoresis. The bands were visualized following staining with Alcian Blue (1% in 0.1% acetic acid) for 20 min. The strips were destained in 0.1% acetic acid for a further 20 mins.

Paper chromatograms and high voltage electrophoretic paper were dried and observed under an ultra violet lamp (3660^{o}_{A}) for fluorescing phosphoderivatives. An automatic recording Nuclear Chicago Actiscan III with slit width of 1.5mm and slow feed speed was used to detect radioactivity. The position of the radioactive peaks were recorded, the Rf calculated for chromotograms and the areas under the curves measured by differentially weighing the cutouts of tracings or with aid of a planimeter. The ratio of the intergrated peak value to the total radioactivity recorded was expressed as a percentage.

Some chromatograms and electrophoretograms were cut into pieces (1cm) across the direction of the separation and eluted into (5ml) scintillation fluid. This fluid contained 0.3% w/v.PPO; 0.03%w/v

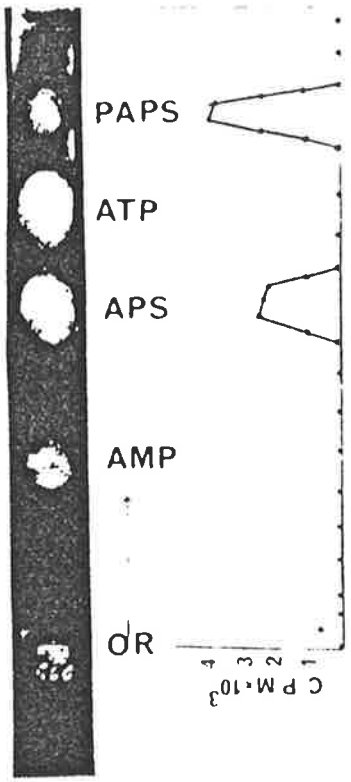


Fig.III-2.

High voltage electrophoresis of standard preparation of PAP^{35}S. (Negative print of an autoradiograph & radio active profile.) (see also Fig.III-13).

POPOP: in toluene. Radioactivity was measured in a Packard Tricarb liquid scintillation spectrophotometer. Counting rates were corrected for quenching by using a channels ratio method.

The migratory positions of the standards on the high voltage electrophoretograms were visualized by autoradiography*.

3.0 RESULTS AND DISCUSSION.

Previous data obtained from developing amnion cell cultures (as well as from aggregated clumps of gingival epithelium) indicated that there may be some relationship between cell contact and the overall macromolecular sulphate (see Chapter II). Moreover, within the limits of these chemical assays, there were some data which revealed that the distribution of GAG species within the total extracted from epithelial cultures varied according to the level of intercellular contact. Further, using radioactively labelled precursors the localization of *de novo* macromolecular sulphate appeared to be essentially intercellular.

This chapter describes a series of pulse-chase radio labelling experiments to determine whether the distribution of these *de novo* synthezised macromolecules were representative of the total GAG as assayed biochemically. The results indicate that during the *in vitro* development of monolayers of amnion cells there are indeed differences in the proportions of GAG ultimately secreted. However with only some minor differences, the sequential appearance of some of the major intermediate GAG precursors

was similar at the extreme degrees of confluence.

3.1 Amnion cell cultures.

The in vitro biosynthetic incorporation of either (^{3}H)-acetate or (^{35}S)-sulphate into macromolecular material by amnion cells was measured at various times after a 15 min pulse over 2h.

Cells were separated from their medium, ultrasonically disrupted and macromolecular material precipitated with CETAB (Fraction 1). Material resolubilized by 1.25M $MgCl_2$ from the CETAB precipitates was exhaustively digested with papain and then dialysed at 4^{o}C against water. The detergent soluble material was retained (Fraction 2).

The medium was separated from the cultures and was also stored for subsequent investigation at -4^{o}C (Fraction 3). Although no protease inhibitors were added, all preparations were kept frozen until needed.

Secretion of macromolecular (^{35}S)-sulphate by amnion cells into their media.

Following a 10 min 'chase' incubation after removal of the radio isotope, cells from fully confluent cultures (++++ ranking) contained about 90% of their original cell bound label. After 80 min of 'chase' incubation only 20-30% of the label was associated with the cells (Fig III-3). However, since less than 50% of the radioactively labelled material could be

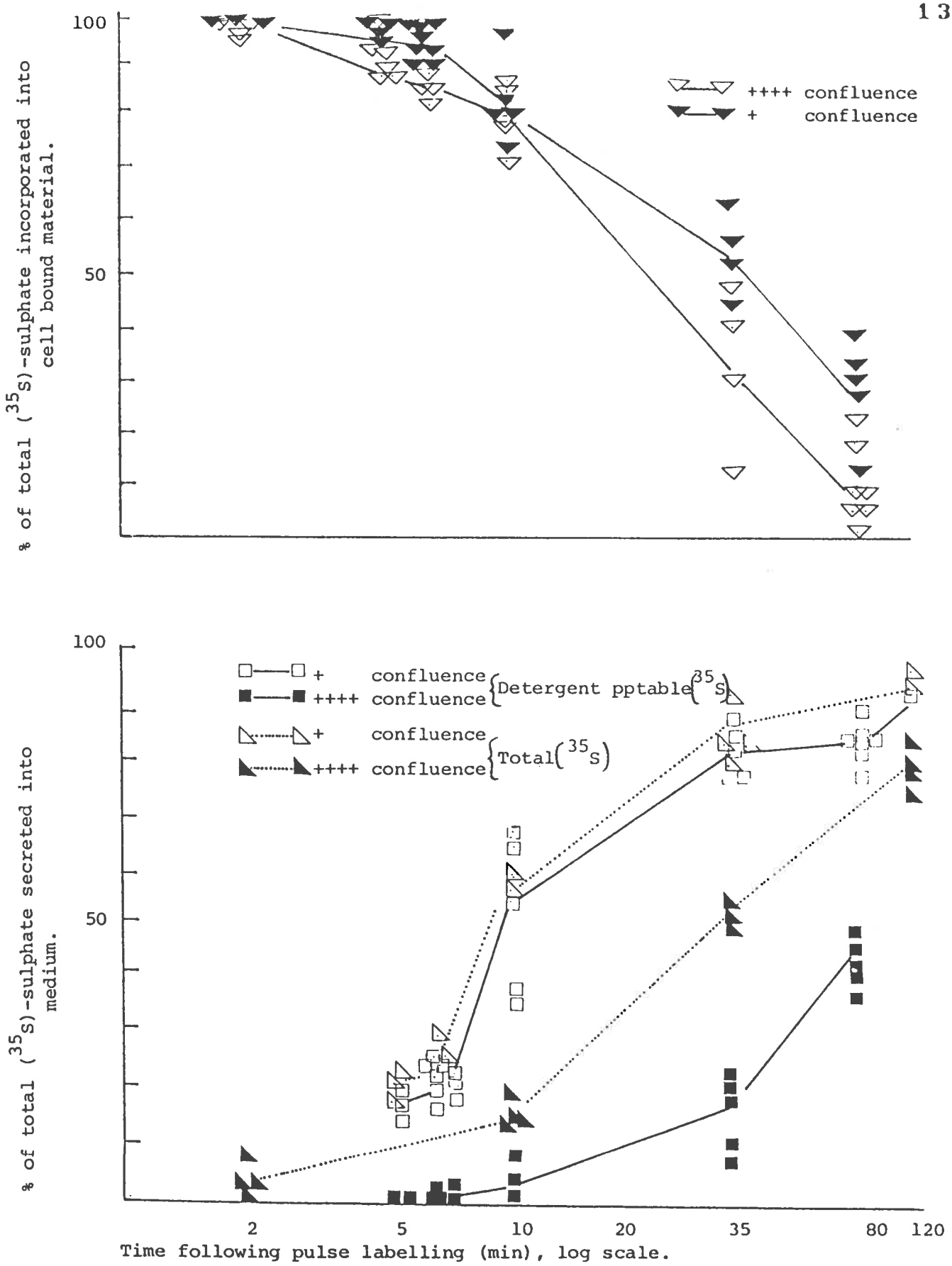


Fig III-3 Total (^{35}S)-sulphate incorporated by cultured amnion cells - the distribution between the cells and the medium.

precipitated by detergent from the medium, hydrolytic-enzymatic degradation may have occurred during the 80 min incubation. A 2h incubation of the cell free culture medium retrieved from these cultures resulted in a loss of 20% of detergent precipitable (^{35}S)-sulphate and 27% loss of total labelled material on dialysis at 4^{o}C. The rates of loss of macromolecules from low cell culture density monolayer culture (+ ranking) were more rapid; only 30% of the cell bound material remained after 35 min. Most of that macromolecular material representing loss from the cells, appeared as such in the medium, (70-80%). The observation that secreted macromolecular (^{35}S)-sulphate from non confluent cultures are not degraded to CPC soluble products probably reflects a limited enzymatic function consequent on the low cell numbers per culture (ie. + ranking).

Cell bound (^{35}S)-sulphated macromolecules - electrophoretic separations from cultured amnion cells.

The largest proportion of the total (^{35}S)-sulphated macromolecular material which was associated with the cells in fully confluent cultures at 2 min after removal of the radioactive 'pulse' was ChS (A&C)-about 50%,(Fig.III-4). The proportion of cell bound (^{35}S)-sulphate which corresponded electrophoretically to ChS-C was 30% of the total (^{35}S)-sulphate. This remained relatively constant throughout a 35 min of 'chase' incubation, while the material of slower mobility, which has been compared to the mobility of standard ChS-A, was detected in ++++ rating cultures only directly after removal of the (^{35}S)-sulphate.

The methods of SAITO, YAMAGATA & SUZUKI, (1968), for positive identification of these small amounts of material only became available as this project was ending. A positive identification of Hep, by electrophoresis alone, was also inconclusive since this GAG appears as a polydispersed band. However its susceptibility to prolonged incubation with Flavobacterium heparinase and the proportionately high sulphate to uronic acid ratios in macromolecular cell bound material in confluent cell cultures, is strongly suggestive of HepS or Hep (cf. BARTOLD, WIEBKIN & THONARD, 1981).

A "dumbelled" spot was observed on electrophoretograms of cell bound macromolecules at the position of DS & HepS (Fig. III-1). It was partially affected by heparinase but not by testicular hyaluronidase. By 35 min a more electrophoretically discrete spot was detected which was resistant to testicular hyaluronidase and migrated similarly and has been assumed to be DS.

Confluent cells also consistently synthesized a cell bound (^{35}S)-sulphate labelled product which had an electrophoretic mobility similar to HA. This was susceptible to testicular hyaluronidase and represented about 10% of the total cell bound label.

Together the above observation promoted speculation as to whether this moiety was partially sulphated HA or an incompletely sulphated chondroitin. Current evidence available from our laboratories which will be described in the final section strongly suggests that this macromolecule was HA with which a sulphated species of GAG, probably an aggregatable DS, had interacted.

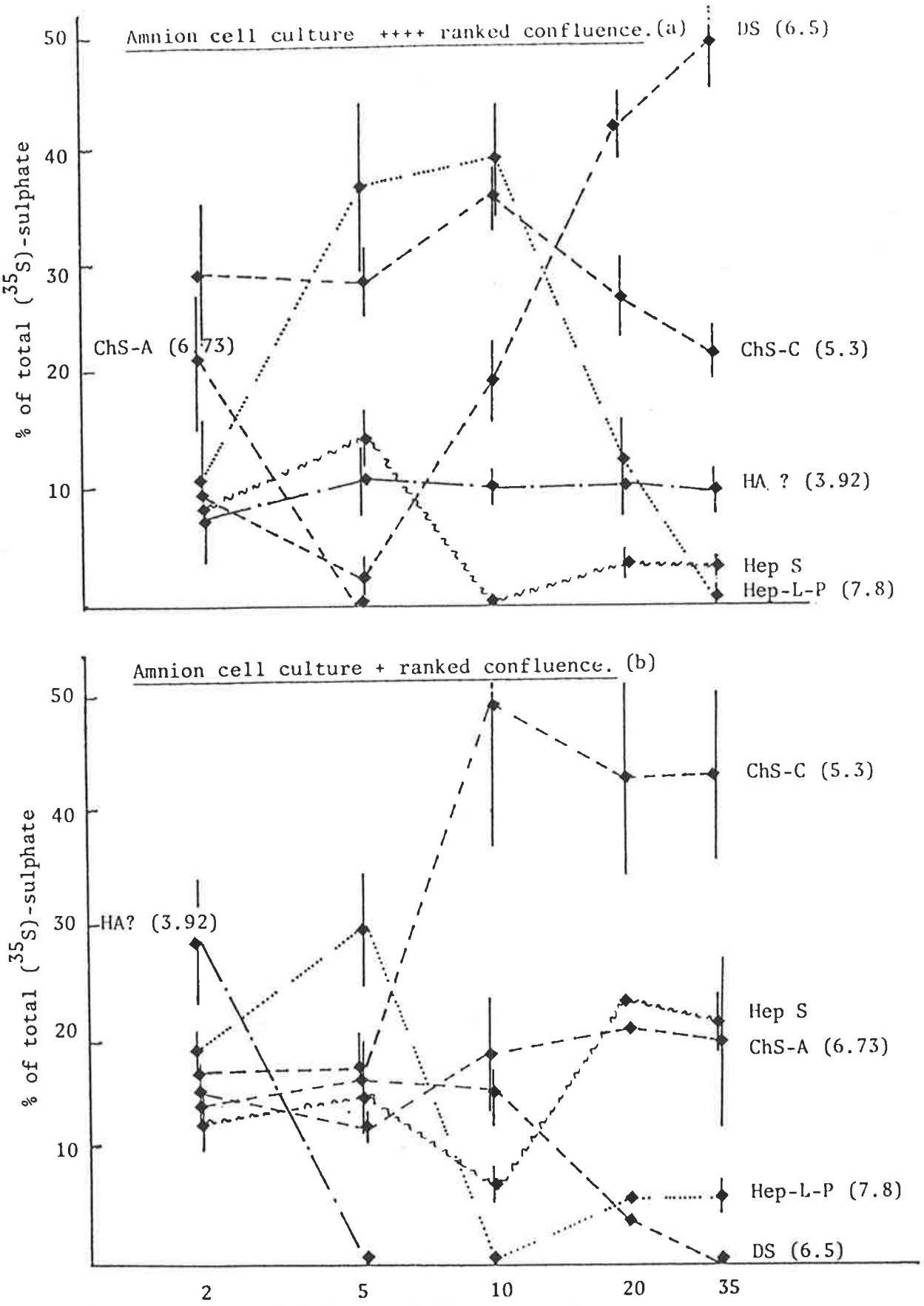


Fig. III-4.
Macromolecular (^{35}S)-sulphate contents of amnion cells. Non dialysable macromolecules were separated on cellulose acetate electrophoresis. (pH=3.5); numbers in brackets refer to electophoretic mobilities in cm/h/ma.; HA? ( ) represents ^{35}S- labelled material which migrated similarly to standard HA.

Furthermore, gingival epithelium has now been shown to synthesize PGs capable of interactions with HA and with each other, (WIEBKIN, BARTOLD & THONARD, 1979); these latter interactions are probably not similar to those in cartilage, but may be similar to those involving DS of specific iduronic acid/ uronic acid periodicity (CÖSTER & FRANSSON, 1977). None of this material was detectable in the non confluent cultures, (Fig. III-4b).

Non-confluent cultures (+ rating) appeared to achieve maximum incorporation of (^{35}S)-sulphate into material which corresponded to Hep after about 5 min of 'chase' incubation whereas a similar result was achieved earlier in the confluent cultures (within 5-10min). The former cultures contained a greater proportion of total sulphate in those Hep-like-polysaccharide bands, (Fig. III-4a) than did the non-confluent cultures.

In contrast to the confluent cultures, non-confluent, (+ rating) cells incorporated a greater proportion of their total (^{35}S)-sulphate into ChS-C than DS. Since the epimerization of uronic acid to iduronic acid in the final synthesis of DS occurs extracellularly, the larger number of cells of the ++++ rating cultures may provide higher levels of the epimerase in the medium. Materials with electrophoretic mobilities similar to both ChS-A and Hep S were identified in the non-confluent cells, whereas the confluent cells contained non-detectable amounts of these products after 35 min of 'chase'.

Cell bound (^{35}S)-sulphated macromolecular (Paper Chromatography).

Two chromatographic solvent systems were used for the separation of cell bound compounds contained in the detergent precipitable material. They were designated System A & System B (see Materials & Methods, Chapter III).

Dialysis was not performed after the resolubilization of the precipitated material. Since the chromatograms of the standard preparations described earlier corresponded closely to the Rf values quoted by PICARD (1964), the following data were duly considered to be representative.

When confluent and non-confluent cells were 'pulsed' for 15 min with (^{35}S)-sulphate and 'chased' for the time periods mentioned before, within 10-20 min of 'chase' incubation, the proportions of (^{35}S)-sulphate incorporated into cell bound AMPS by confluent cells was greater than by non-confluent cells. These values are in agreement with the net incorporation of (^{35}S)-sulphate into material corresponding to ChS-A and C which was identified by the cellulose acetate electrophoresis following papain digestion.

Within 20 mins the amount of (^{35}S)-sulphate appearing in the PAP (^{35}S) was 2-3 times greater in non-confluent cells than in confluent monolayers. Although there was no evidence of macromolecular sulphation occurring after 80 min in + rated cultures there was a decrease in radio labelled PAP (^{35}S), (Fig.III-5).

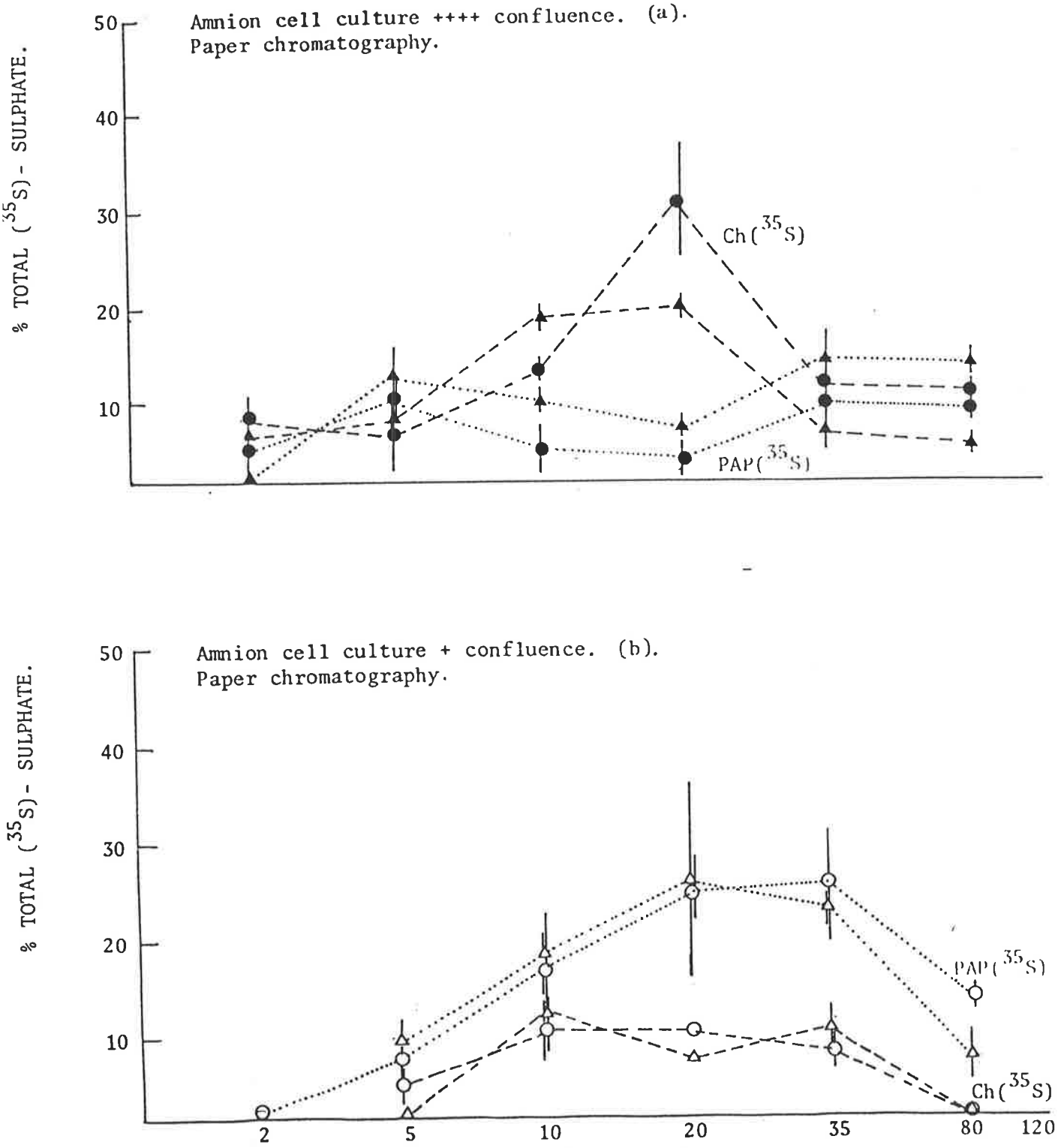


Fig.III-5 The content of chondroitin sulphate and PAP(^{35}S) in amnion cells.

(^{35}S)-sulphate labelled detergent precipitable components were separated by paper chromatography from an undialysed preparation of amnion cells by two solvent systems A (o & ●) & B(Δ &▲) (see p129 text).

However, a chromatographic spot was detected in the cell bound extracts from non-confluent cultures, which corresponded to the sulphate oligosaccharides reported by PICARD, (1964) (Fig.III-6). The PAP(^{35}S) could have acted as a sulphate donor for these products, the synthesis of which may be peculiar to tissue conditions.

Material containing (^{35}S)-sulphate which fluoresced under ultra violet light (3660Å) and coincided chromatographically with the standard of synthetic UDP-galactosamine sulphate was also detected in these cultures, (Fig.III-6). Since these sulphated components represented a significant pro -portion of the total (^{35}S)-sulphate incorporation (40-50%) at particular times in the 'chase' incubations their importance could not be dismissed. There is, however, no immediate explanation for this observation and its significance remains unknown.

Such amnion cell metabolites corresponding to UDP-hexosamine sulphate have not hitherto been shown to be utilized in GAG metabolism, although SUZUKI & STROMINGER,(1967) did positively identify this material in extracts of hen oviduct. Nevertheless they had concluded that sulphation followed polymerization. Further identification of "UDP- hexos -amine sulphate" separated in previously described experiments is required. Indeed it might be appropriate to comment that identification of all the products suffered from a lack of chemical or enzymatic analysis, e.g. iduronic acid in DS. However present work with enzyme elimination and with nitrous acid degradation of electrophoretic separations confirms some of the described data , (BARTOLD, WIEBKIN & THONARD, 1981).

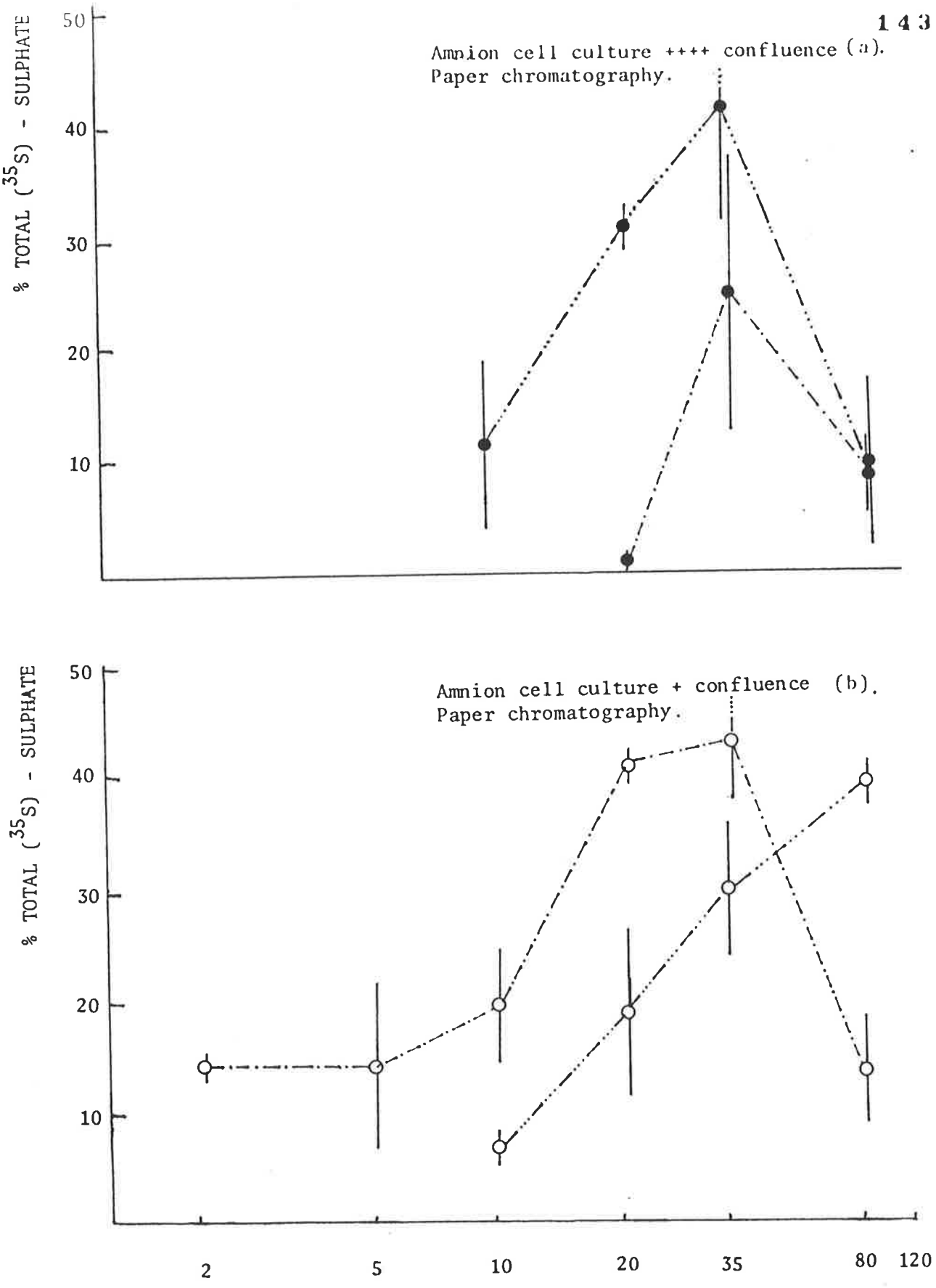


Fig.II-6 Relative proportion of two sulphated components of amnion cell cultures as detected on paper chromatography.

Corresponding to UDP -galactosamine sulphate (o-·-·-·) and sulphated oligosaccharides (o-··—··) Chromatography system A. (see p 129 text).

Paper chromatographic separation of amnion cell extracts revealed values corresponding to "free" sulphate and low levels of two nucleotides which were identified by ultra violet light.

The appearance of "free" (^{35}S)-sulphate peaks in material from 'pulsed' cultures, as late as between 10-20 min following the commencement of 'chase' incubations in ++++ rated cultures, would imply residual sulphatase activity. (Fig. III-7). A correspondingly smaller peak appeared between 20-30 min after the 'chase' incubation in non-confluent cells cultures. The variability of distribution of this degradable label through the different 'chase' incubations, suggests that biosynthetically labelled products differ in their susceptibility to enzyme action. Several references to such phenomena will be made in the final Chapter.

Sulphated material detected in the above mentioned chromatograms which corresponded to that described by PICARD (1964) as phospho-derivative nucleotides represented significant sulphate radio-activity. The distribution of these labels throughout amnion cultures are shown in Fig. III-8.

The levels of sulphated nucleotide appeared to rise from between 35 min and 80 min of 'chase' incubation in cells from both ++++ and + rated cultures. This material may be related to the afore mentioned nuclear PG.

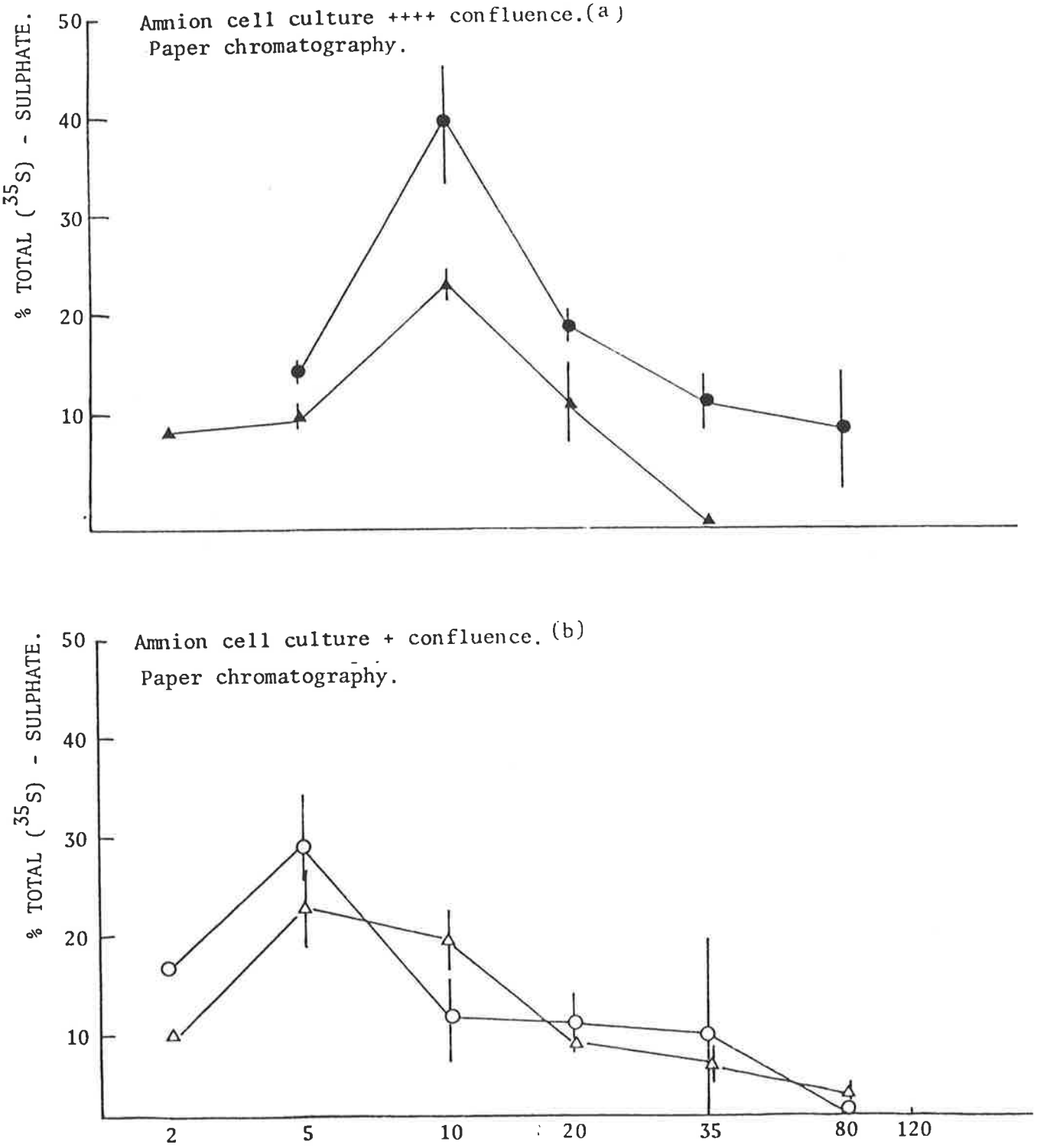


Fig.III-7
Relative proportions of (^{35}S)-sulphate which corresponded to "free" inorganic sulphate associated with cells of amnion cultures as detected on paper chromatography.

O–● Chromatographic system A; △–▲ Chromatographic system B, (see text).

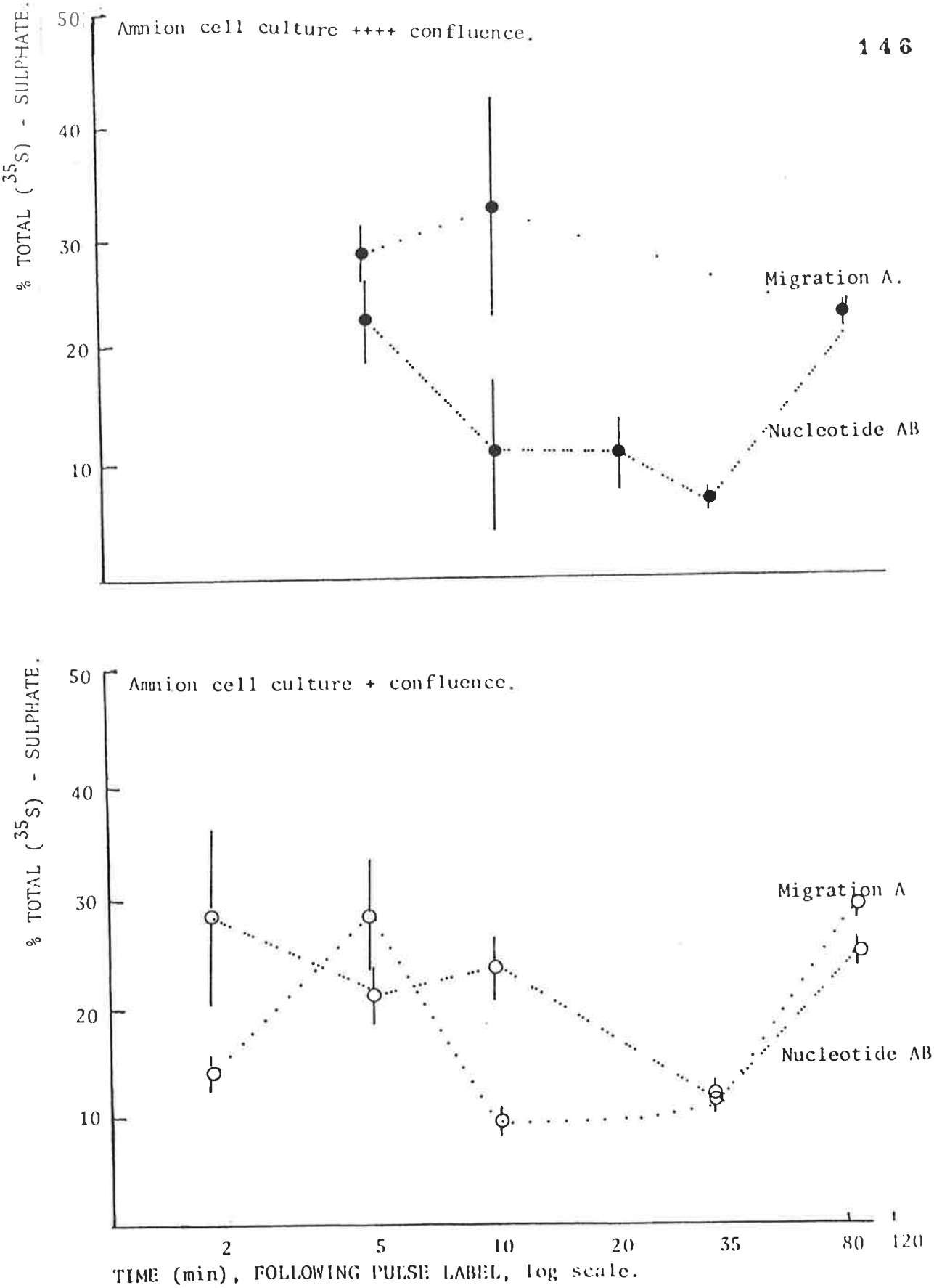


Fig. III-8.
Relative proportions of (^{35}S)-sulphate which corresponded to phosphoderivative nucleotides (according to Picard, 1964 and designated A, a fast migration o.....●; & AB, a slower migration o-----●, as detected on paper chromatography using chromatographic system A (see text).

Sulphated molecules in the medium of amnion cell cultures.(Paper chromatography).

The addition of the standard preparations, UDP-galactosamine sulphate, ChS and PAP (^{35}S) to Medium 199 + FCS (at concentrations up to 5 ug/ml) provided a control sample for paper chromatographic separations. Both chromatographic solvent systems were tested. Since resolution was not good for solvent system B, the following data are derived from separations solvent System A using (isobutanol, NH_4 and EDTA). Only those cultures which incorporated high specific activity radioactivity were used.

Material corresponding to AMPS began to appear in the medium after about 10min after 'chase' incubation of both fully and non-confluent cells, (Fig. III-9). However, the proportion of the total sulphate which was incorporated into AMPS by non-confluent cells was between 10 and 20% lower than the fully confluent cells.

Cells from +rated cultures did however continue to secrete AMPS over the ensuing 60-90 min of the 'chase' incubations. These cultures also continued to secrete material which corresponded to sulphated oligosaccharides, the proportion rising to 30% of the total sulphate counts.

(^{3}H)-acetate incorporation into macromolecules by cultured amnion cells. (Paper chromatography).

The appearance of AMPS in ++++ and + rated cell cultures to which (^{3}H)- acetate had been added, paralleled the (^{35}S)-sulphate data. The proportion of the tritium incorporated into AMPS in fully confluent cells was only 10-20% of the total (^{3}H)-acetate, but non confluent cells; + rating; (and the interim ++rated

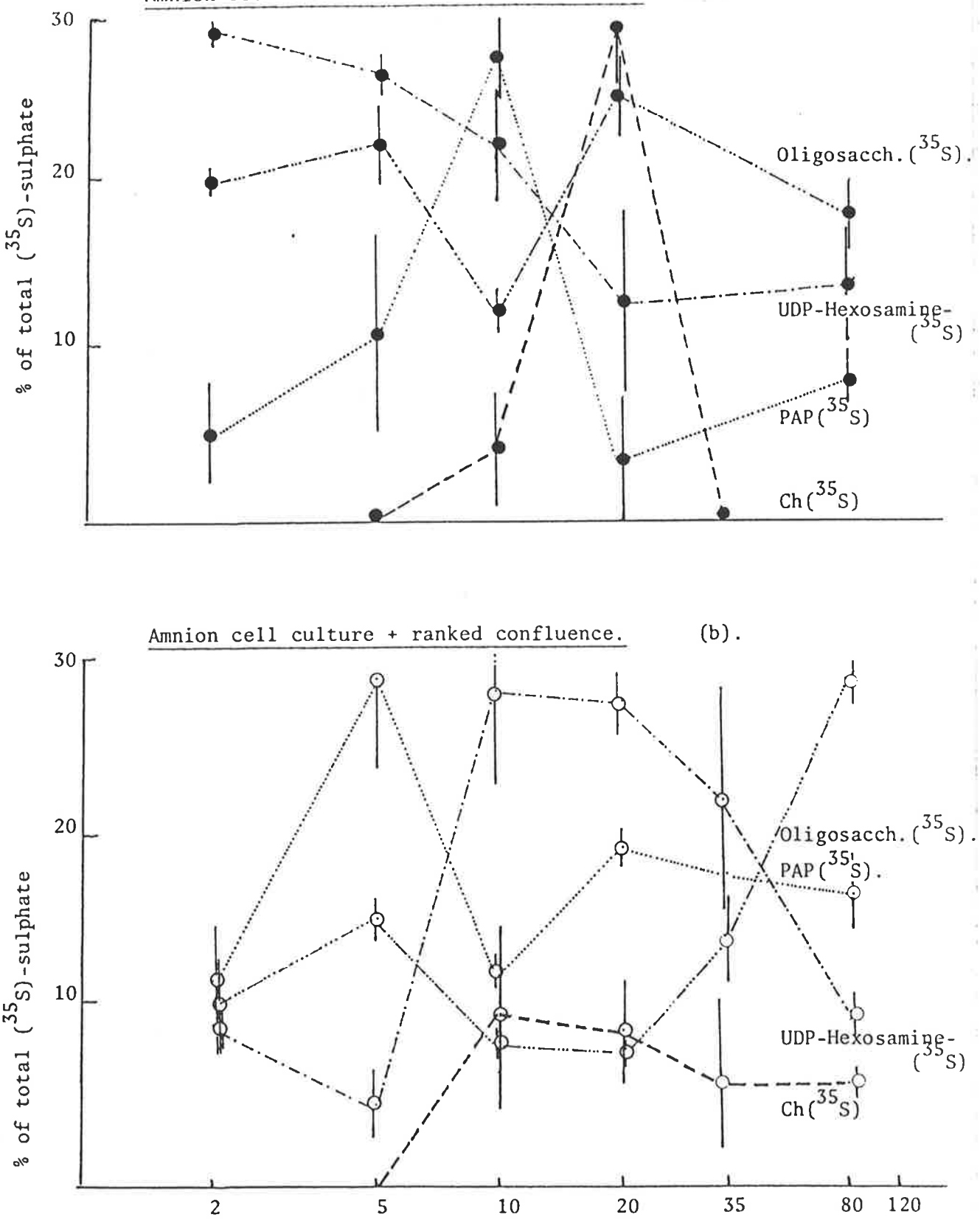


Fig. III-9.
Relative proportions of (^{35}S)-sulphate which corresponded to standards, UDP-hexosamine sulphate; oligosaccharide sulphates; PAPS; ChS, in the media of amnion cultures as detected on paper chromatography using solvent system A (see text).

cells) channelled up to 50% of the total incorporate (^{3}H)-acetate to the synthesis of macromolecular product corresponding to ChS, (Fig. III-10). (Some material conforming to the Rf value of UDP-galactosamine sulphate was observed in cultures in which there was cell/cell contact, i.e. ++ and ++++ rated confluence (cf Fig. III-6) but not in any + rated cultures).

(^{3}H)-acetate and (^{35}S)-sulphate incorporation into macromolecules secreted into culture media.

Despite the decreased level of tritium incorporation, the profiles representing the secretion of (^{3}H)-acetate labelled ChS into the media of fully and non confluent amnion cultures (Fig. III-11) were similar to those obtained from (^{35}S)- sulphate incorporation studies. However, no (^{3}H)-acetate had been incorporated into material with an Rf corresponding to sulphate oligosaccharides but material with an Rf coincident with that of UDP-hexosamine sulphate was evident in those media.

Interim rated (++) cultures incorporated (^{3}H)- acetate into material corresponding to ChS throughout a timecourse similarly to the + rated cultures, but a substantial amount of material with an Rf coincident with that of standard UDP-hexosamine sulphate was secreted into the media after only 2 min of 'chase'.

Soluble radio labelled macromolecular material from amnion cells following detergent precipitation (Paper chromatography).

The supernatants from amnion cells which had been ultrasonically disrupted and treated with detergent, contained high levels of (^{3}H)-acetate.

Chromatograms of System Λ of this soluble material revealed several discrete peaks (Fig. III-12), the identification of which would require further work. Nevertheless, a record of the various

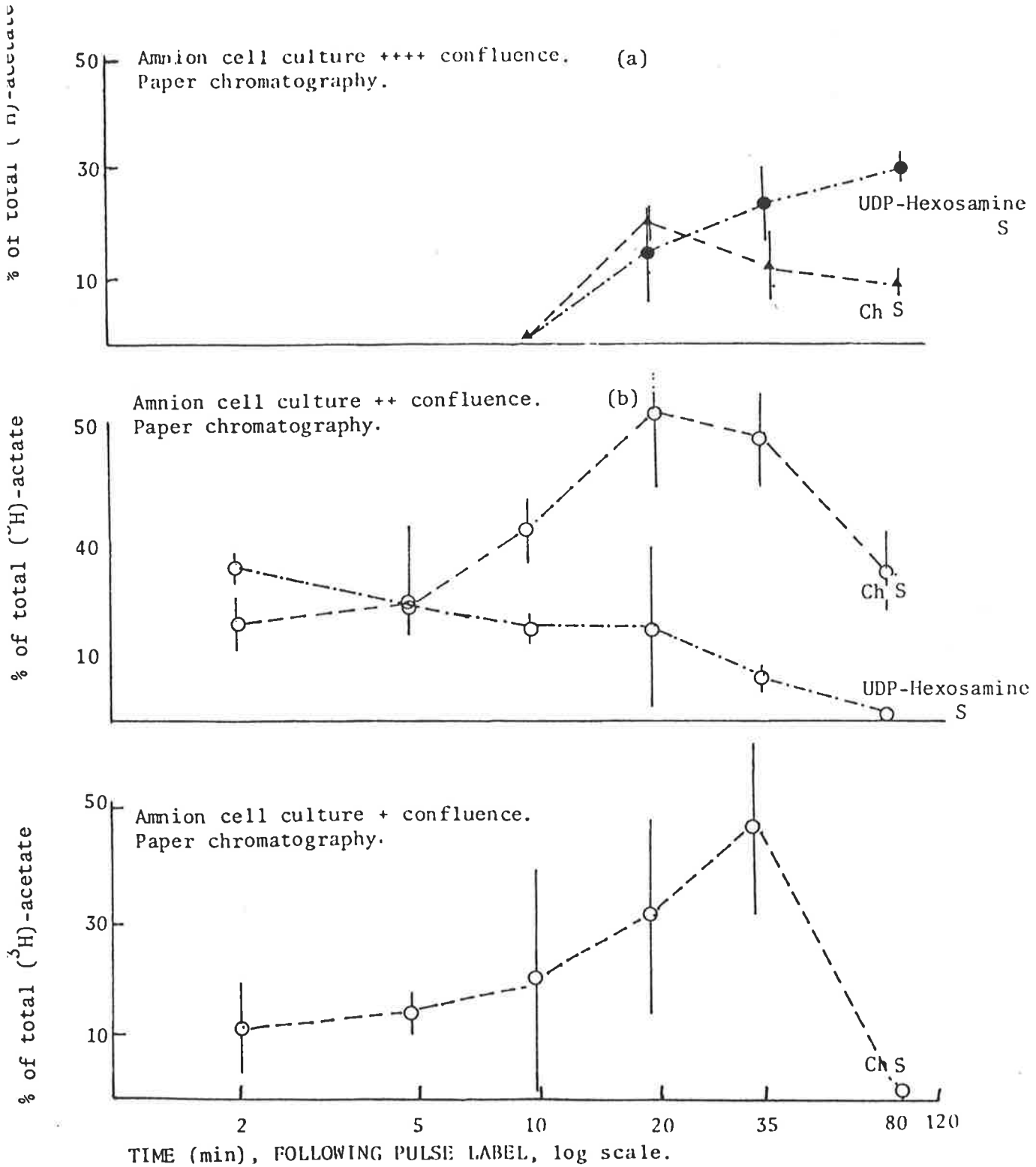


Fig.III-10.
Relative proportions of (^{3}H)-acetate which corresponded to standards, UDP-hexosamine sulphate; and ChS in cells of amnion cultures as detected on paper chromatography.
(o & o) chromatographic system A.
(▲ & ▲) chromatographic system B (see text).

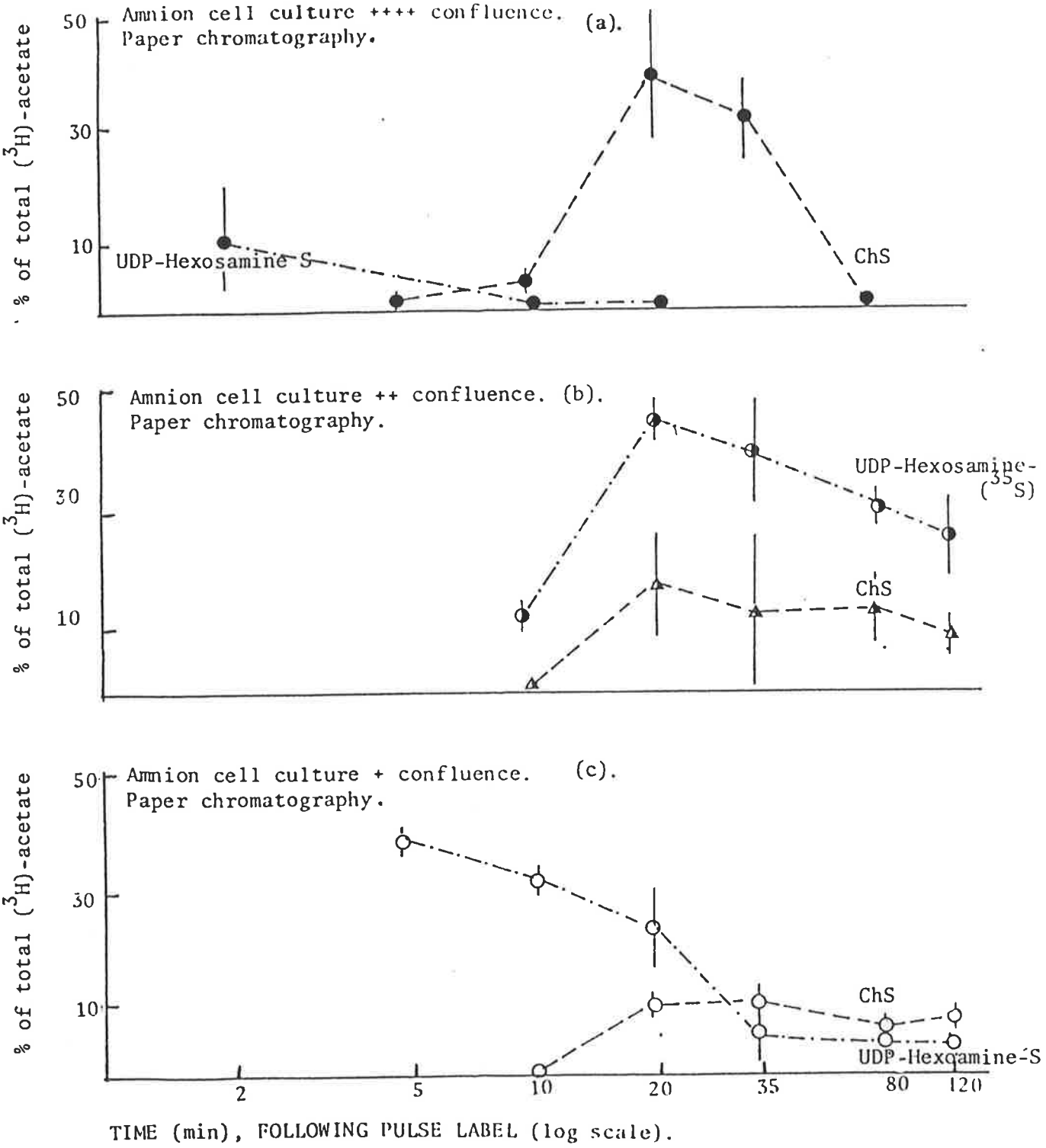


Fig. III-11.
Relative proportion of (^{3}H)-acetate which corresponded to standard UDP-hexosamine sulphate; and ChS in amnion cell culture medium as detected on paper chromatography using both chromatography systems A & B (see text).

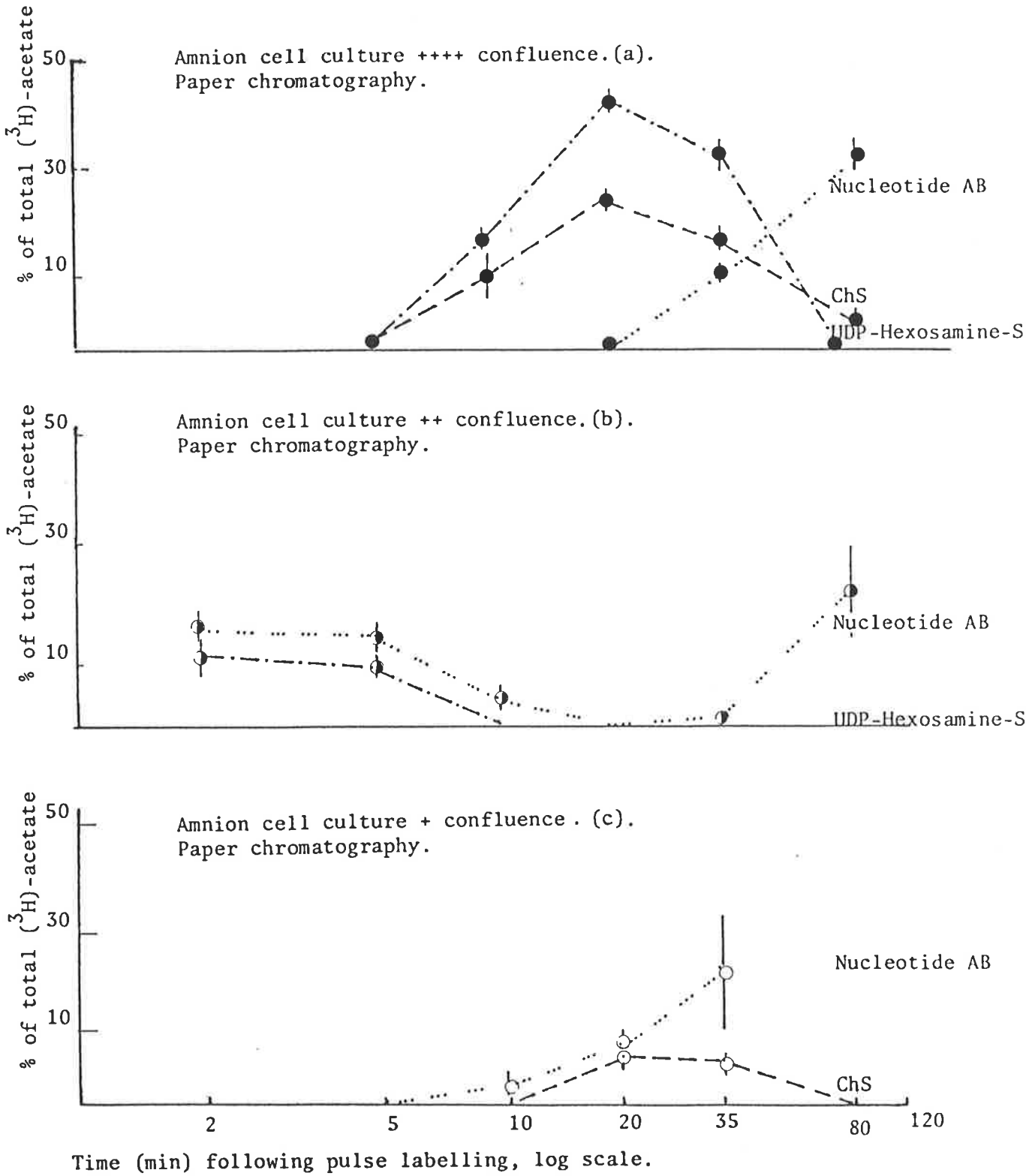


Fig.III-12.
The relative proportions of (^{3}H)-acetate which corresponded to standard UDP-hexosamine sulphate; nucleotide AB (Picard,1964); and ChS, in a detergent soluble fraction from cells of amnion cultures as detected on paper chromatography using solvent system A (see text).

proportions of these discrete fractions at various intervals of time during 'chase' incubation under different culture conditions is made here.

(^{3}H)-acetate was incorporated into a nucleotide which corresponded precisely in Rf to that referred to as AB by PICARD(1964). The initial appearance of this material was 10-20 min earlier in non confluent cultures than in cultures where the cell/cell contact was rated as ++++.

The UDP-hexosamine sulphate peak was evident in confluent cells at 20-30 min. Some (^{3}H)-acetylated AMPS appeared in ++++rated cultures as indeed it had done in the detergent precipitated fractions.

3.2 Fragmentary Gingival Epithelial Cell Cultures.

The incubations at 37^{o}C of fragmentary gingival epithelia in media containing (^{35}S)-sulphate precursors, provided preliminary data for an investigation into the sequence of events involved in epithelial PG synthesis and secretion as did the similar experiments described previously for amnion cultures.

Incorporation of (^{35}S)-sulphate into cellular components.

(High Voltage Electrophoresis).

High voltage electrophoresis of extracted material from incubations of gingival epithelial cells in parallel with standardized preparations of PAP(^{35}S) and AP(^{35}S)*(ROBBINS, 1962) revealed that some of the sulphated nucleotide donor from the cells, were not easily washed away from the detergent (CETAB) precipitable material, (Fig. III-13). Indeed the time dependent profiles of radiolabelled incorporation into CETAB soluble and CETAB insoluble donor nucleotides are similar (Fig. III-14).

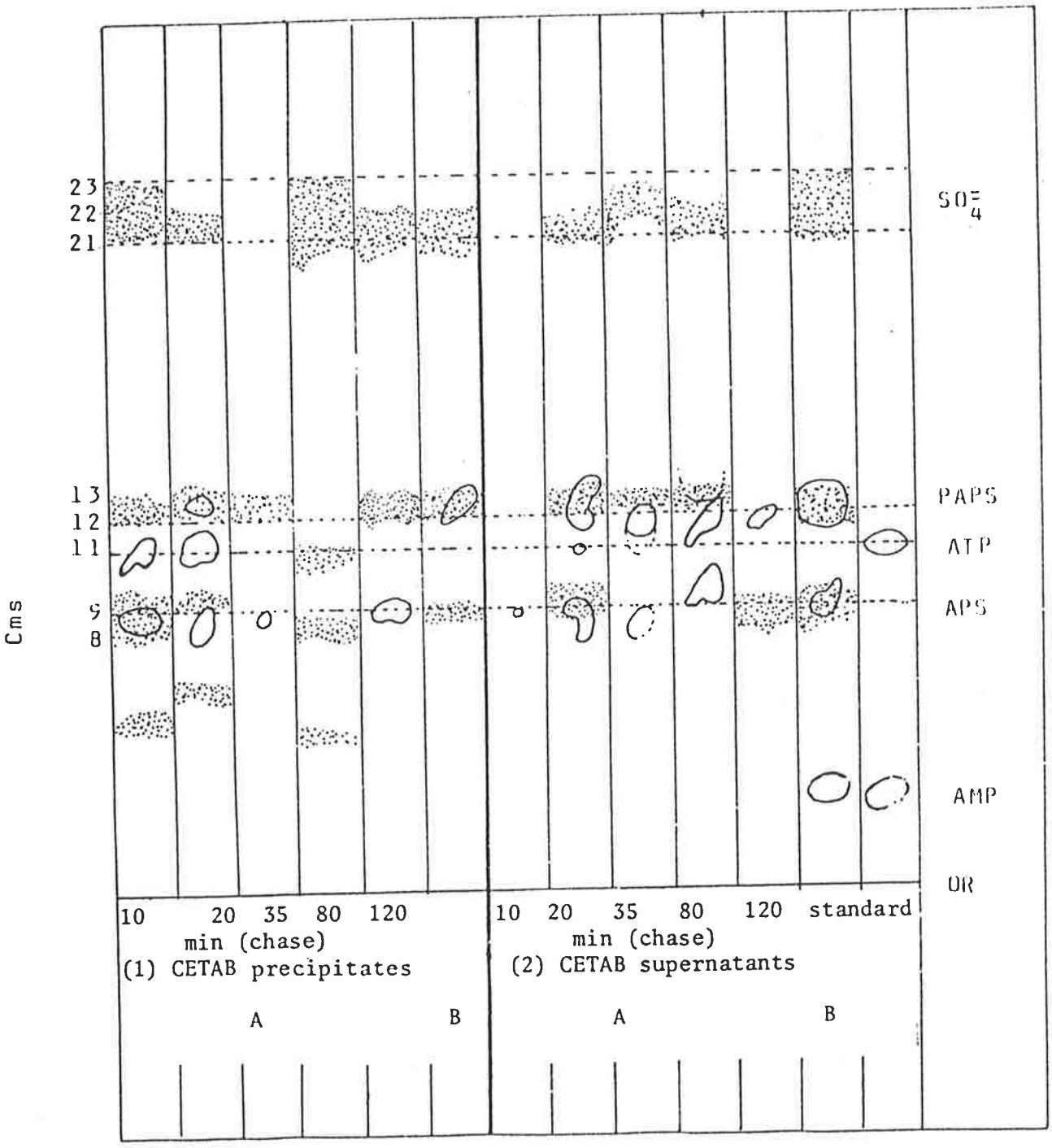


Fig. III-13.
Intracellular (^{35}S)-sulphate incorporated into gingival epithelial cells following 15min 'pulse' incubation.
High Voltage Electrophoresis of, A (1) (^{35}S)-sulphate in CETAB precipitable material (Scheme III-1(1) see p.127)
A (2) (^{35}S)-sulphate in CETAB soluble material (Scheme III-1(2) see p.127)
B standard preparations of PAP(^{35}S) & AP(^{35}S) (see text p.128)
Stippled areas represents autoradiographic demonstration of (^{35}S)-sulphate and U.V. sensitive areas are outlined.

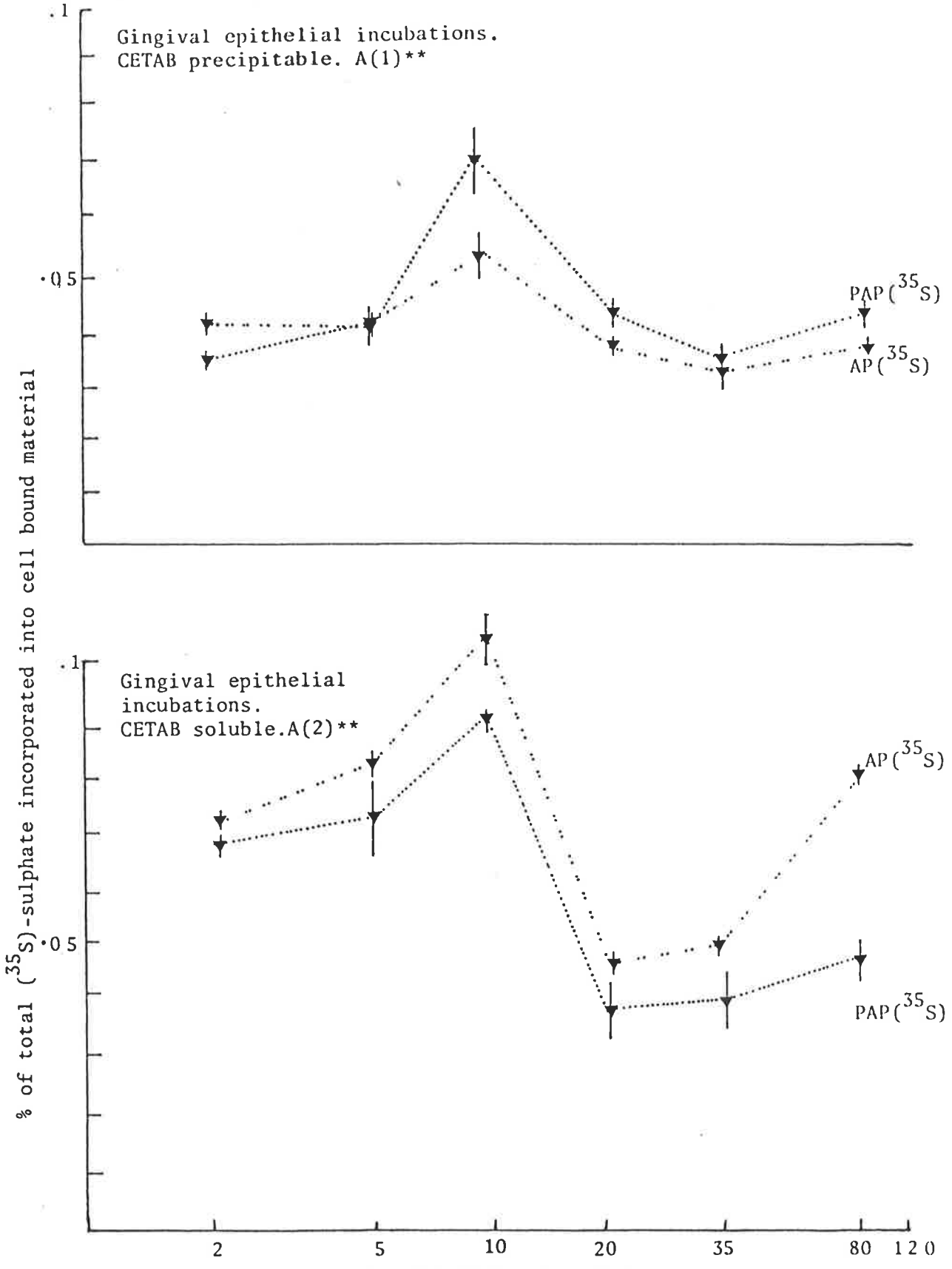


Fig. III-14.
Relative proportion of total (^{35}S)-sulphate which was incorporated into material which corresponded to standard PAPS and APS from extractions of gingival epithelial incubations. CETAB precipitable and CETAB soluble material. A(1)** & A(2)** see Scheme III-1 and page 126.

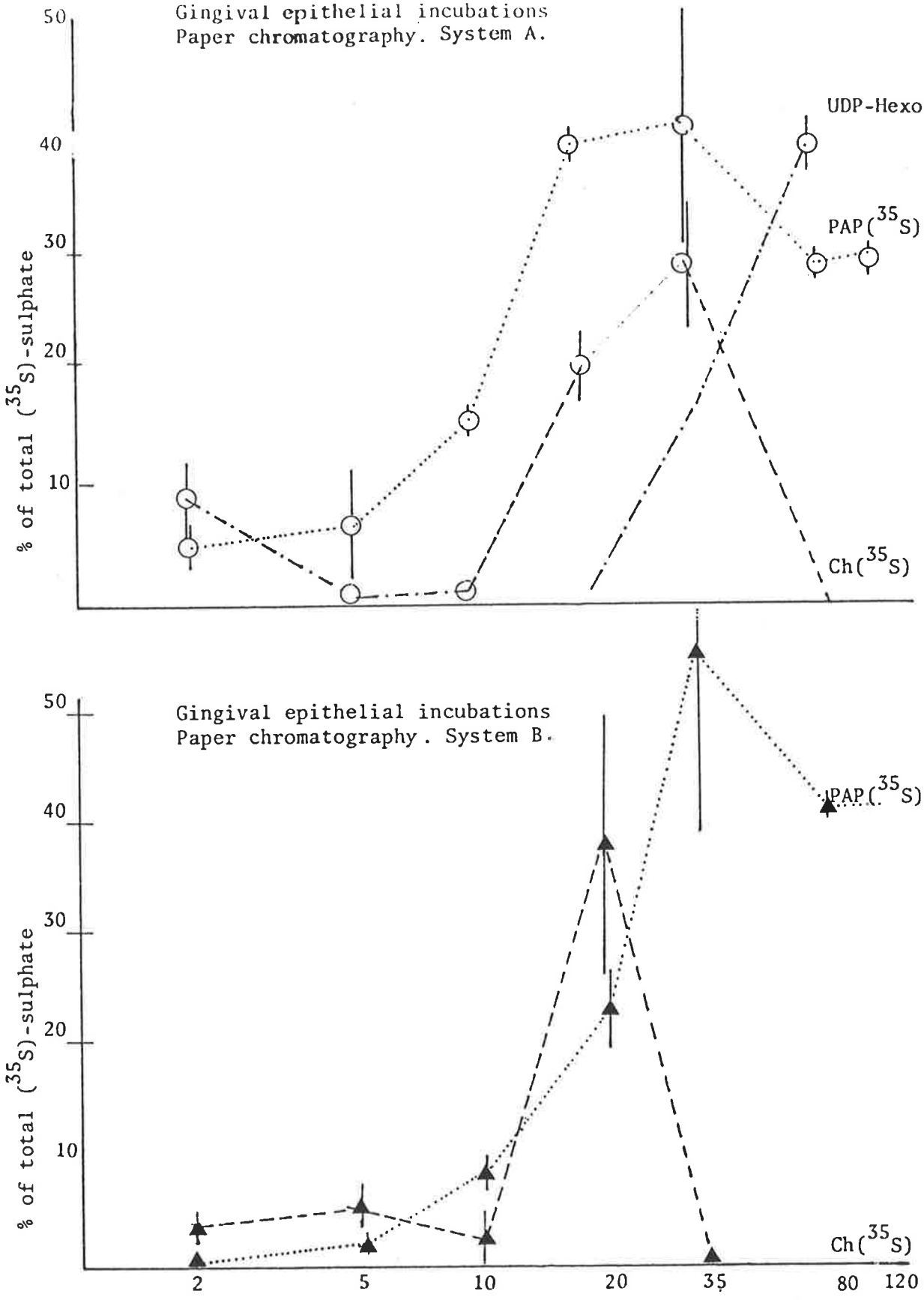
Of course the sulphated nucleotides represent a very small proportion of the total undialysed (^{35}S)-sulphate. The maximum (^{35}S)-sulphate incorporation into these sulphate donor nucleotides reached a peak after about 10 min 'chase' incubation.

Incorporation of (^{35}S)-sulphate into ChS by the gingival cells in vitro. (Paper Chromatography).

The solvent systems for paper chromatographic separations, described previously, were both used to identify ChS in detergent precipitable extracts of gingival epithelial cultures which had been 'pulse' labelled with (^{35}S)-sulphate (Fig. III-15). The label was incorporated into the cells within 20 min of 'chase' incubation and was secreted by 80 min. A radio-active spot corresponding to the Rf of UDP-hexosamine sulphate became significant after 35 min. The shape of the profile of PAP(^{35}S) was similar to that obtained from the high voltage techniques.

3.3 Comments on the Comparative Metabolic Patterns from Amnion and Gingival Epithelial Cultures.

Cell bound (^{35}S)-sulphated macromolecular material which corresponded to ChS appeared in both amnion cells and in fragmentary cultures of epithelial cells after about 10 min of 'chase' incubation. Most of the associated macromolecular label had been secreted by 80 min. Proportionately more (^{35}S)-sulphate incorporated into intermediate metabolites such as PAPS in non confluent amnion cells and gingival fragments than in fully confluent cultures of amnion cells. These intact cultures may be regarded as analogous to a tissue with complete integrity. The observations are in accord



Time (min) following pulse label, log scale.

Fig.III-15.
Relative proportions of (^{35}S)-sulphate which corresponded to UDP-hexosamine (^{35}S), Ch(^{35}S) and PAP(^{35}S) in fragmentary suspension cultures of gingival epithelial cells.
Paper chromatographic systems used A & B (see text).

with the view that the intercellular interactive status of of metabolizing cells is a function of the sulphation process of intercellular PG. Indeed the previously cited literature of LINDAHL and coworkers reminds us that sulphation plays an important role in the elaboration of the Hep-like polysaccharides, and KRAMER,(1971) has emphasized the intimate nature of these molecules with cell surfaces. Other circumstantial evidence for the hypothesis that sulphate levels in the intercellular material are regulated by the 'integrity' of the culture or tissue is available in the reports on the comparative levels of (in vitro) sulphate incorporation in monolayers and suspension cultures of skin fibroblasts of various passage and chronological age (MARSH, MAINI, WIEBKIN, SPENCER & DUMOND, 1978; MARSH,WIEBKIN, MUIR & MAINI, 1979)

Incorporation of inorganic (^{35}S)-sulphate into PAPS appeared to be one of the initial events in the sulphation of the GAG chain, appearing by 5 min of the chase incubation. Fully confluent cells appeared to contain smaller amounts of PAP(^{35}S) for a longer period of 'chase' incubation (Fig.III-5.).

In the described chromatogaphic separations the spots designated ChS were broader than the commercial standards, but have subsequently been identified by enzyme elimination, (BARTOLD, WIEBKIN & THONARD, 1981).

Data from studies on in vitro amnion cell cultures indicated that aneuploid epithelial like cells respond to their immediate environmental condition by synthesizing different proportions of AMPS fractions. SMULOW & GLICKMAN (1966) and SONNENSCHEIM & SMULOW (1966) established a triploid cell line from clinically normal

gingival epithelial mucosa. LANGKAMP,PLATT & THONARD,(1968) demonstrated AMPS in these cells. The diploid primary incubations in the presently described studies also synthesized AMPS. They showed their ability to respond to their immediate environment by synthesizing more cell bound AMPS as the clone sizes increased (vis à vis Chapter II). Proximal cellular relationships and cell/cell adhesion appear to regulate this. Enlarging de novo cell clumps demonstrated an increased sulphate value.

The concepts of contact inhibition, cell cycle inhibition (MACIEIRA -COELHO, 1967) and cell density dependent inhibition (STOKER & RUBIN 1967) may be explained biochemically with particular regard for the levels of sulphation of the intercellular PG and the proportion of HA. Although adhesion and rates of cell division (eg 3T3 fibroblasts, HOLLEY & KIERNAN, 1968) and the importance of membrane potential and interface properties have also received considerable attention (KANNO & MATSUI,1968) the intimate relationship of the intercellular polysaccharide was rarely discussed.

Since PG can be regarded as one of the major components of the epithelial cell microenvironment and of the cell surface in particular, the influences from cell systems cited in this thesis should prove worth of future consideration. The 1S1 epithelial like cell line which demonstrated contact inhibition may be an appropriate model. (CASTOR, 1968). In fact evidence for the inverse relationship between AMPS production (HA synthesis) and 'generation time' by synovial cell was published at least as early as 1961 (CASTOR & FRIES,1961), but the authors omitted to comment on these findings. But ABBOTT & HOLTZER, (1966) did demonstrate the mutual antagonism between AMPS and DNA synthesis in cell culture systems.

CHAPTER IV

THE EFFECT OF EXTRACELLULAR GLYCOSAMINOGLYCANS ON EPITHELIAL PROTEOGLYCAN BIOSYNTHESIS.

1.0 INTRODUCTION.

Epithelium is unique among soft tissues in respect to its avascularity. Postulates have been made and more recently substantiated, that macromolecules in the microenviroment of various cells (TOOLE 1972, NEVO & DORFMAN, 1972), in particular of epithelial cells, may exert some influence over synthetic activity and secretary functions (WIEBKIN, 1969, WIEBKIN & THONARD,1982). Enzymatic depletion of macromolecules from intercellular spaces of other tissues have been shown to cause rapid resynthesis of PGs etc., (BOSMANN, 1968; FITTON-JACKSON, 1970, HARDINGHAM, FITTON-JACKSON & MUIR, 1972). The following section describes experiments which demonstrate the effects of some macromolecules and enzymes on the regulation of epithelial PG biosynthesis and secretion. The biochemical implications from more recent investigations amplifies the importance of "extracellular matrix influences on gene expression", (SLAVKIN & GREULICH, 1975).

2.0 MATERIALS AND METHODS.

2.1 Cells and tissue.

Amnion cell cultures were grown on coverslips as described previously and short term whole gingival tissue incubations were used. These latter tissues consisted of small pieces of gingivae 2-3mm thick initially washed in Hanks' BSS and incubated

in Medium 199; 4% BS containing antibiotics at 37°C. (see Materials and Methods in Chapter II).

2.2 Media.

Test solutions of tissue culture Medium 199 prepared by the addition of one of the following;-

a) Hyaluronidase (Testicular, bovine) EC. 3.2.1.35
final concentrations, 30 TRU* /ml
15 TRU* /ml
2 TRU* /ml

b) Trypsin, EC 3.4.21.4
final concentrations, 0.5 µg/ml
0.3 µg/ml
0.15 µg/ml

c) Chondroitin sulphate (mixed isomers)
final concentrations, 0.3 µg/ml
0.15 µg/ml

d) Heparin,
final concentrations, 1000 u/ml
100 u/ml

e) Hyaluronic acid,
final concentrations, 0.3 µg/ml
0.15 µg/ml
0.05 µg/ml

f) No additives as controls.

Enzymes heated for 30 min at 100°C were used as controls and tissue specimens killed by formal/ethanol fixation before incubation were also used.

All additives except Hep were purchased from Sigma Chemical Co., and Hep was obtained from Burroughs Wellcome.

2.3 Incubations.

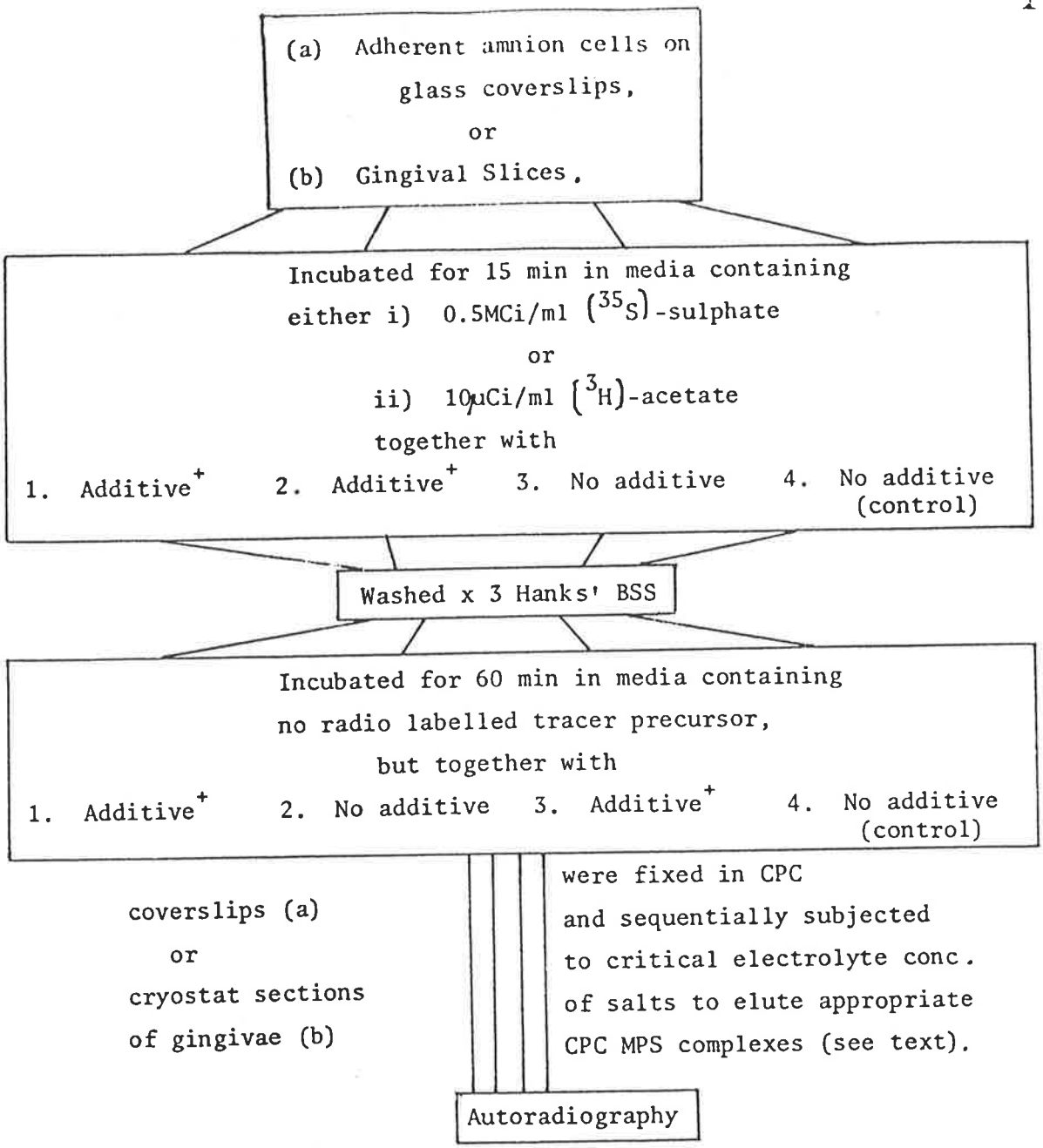
The incubations of coverslip cultures or the small pieces of gingival tissue were performed in Petri dishes using CO_2/ air to regulate the pH to 7.4. The experimental regimes for each test and control incubations were as follows.

Cells or tissues were initially incubated for 15 min in 0.5ml of medium which contained 0.5mCi/ml (^{35}S)-sulphate or 10uCi/ml (^{3}H)-acetate. They were washed X 3 in warm Hanks' BSS and transferred to medium containing no radioactive isotope for 60 min. The media containing the above mentioned additives were used either for the initial pulse incubation or for the subsequent 'chase' incubation. Some incubations included the additives during the entire 75 min of the experiment, (Scheme IV-1).

2.4 Autoradiography.

In the experiments where tissue pieces were used, cryostat histological sections (7μ) were cut, air dried and slide fixed with 1% CPC in 0.1M NaCl at 25°C for 4h. Similar fixation was used on the coverslip cultures of amnion cells.

Sequential histological sections of the gingival tissue from each test were subjected to "elution" in 0.05% CPC by increasing salt concentrations of 0.5M NaCl, 0.63M $MgCl_2$, 0.7M $MgCl_2$ or 1.25M $MgCl_2$, or in CPC alone at 37°C overnight. All the specimens were thoroughly rinsed in 0.05M Na_2SO_4 or 0.05M Na-acetate to displace inorganic radio isotope. They were coated with Ilford K2 nuclear tracking emulsion and subsequently developed as described previously.



\+ Hyaluronidase, Trypsin, ChS, Hep, HA.

Interference with metabolism in 2 indicates effect on synthesis.

Interference with metabolism in 3 indicates effect on secretion.

Scheme IV-1. Flow chart of procedures for the effect of extraneous GAGs on incorporation of (i) (^{35}S)-sulphate and (ii) (^{3}H)-acetate by

a) adherent cultured cells,

b) gingival pieces,

during short term incubation.

3.0 RESULTS AND DISCUSSION .

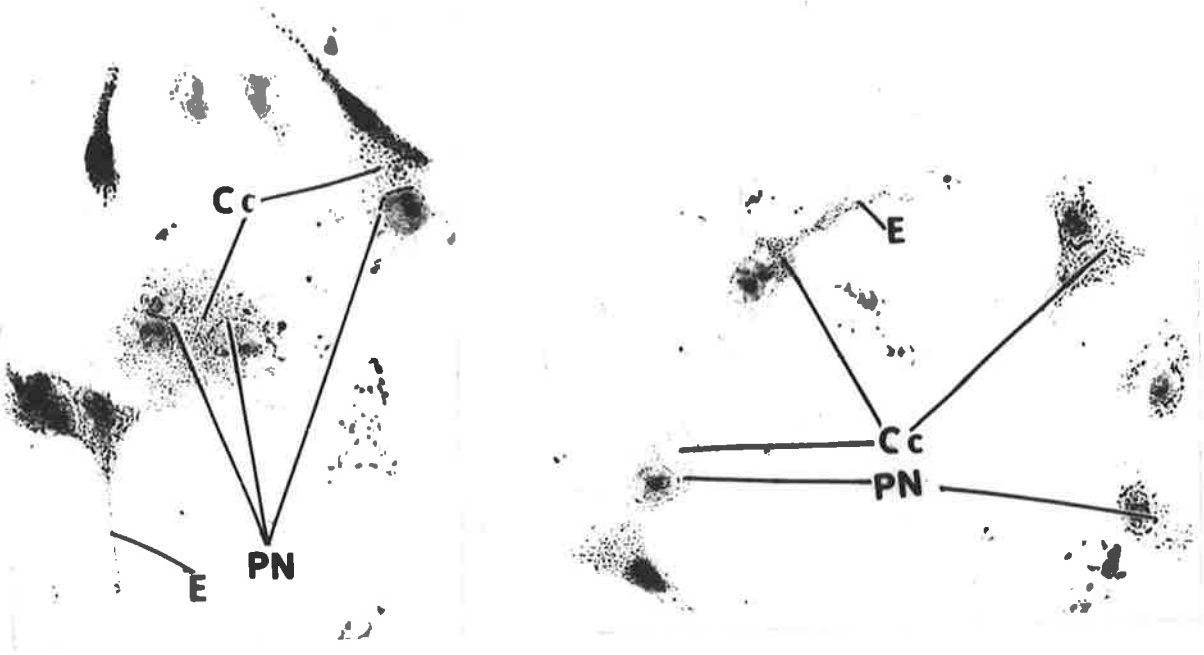
Amnion cells and gingival tissue pieces incubated with (^{3}H)-acetate and (^{35}S)-sulphate incorporated these radioactive precursors as described previously in Chapter II. The effects of various additives obtained from connective tissue or resembling them can now be described *.

3.1 Effect of chondroitin sulphate on amnion cells.

Tritiated acetate and (^{35}S)-sulphate labelled amnion cell cultures, incubated for periods between 45-120 min in medium containing ChS appeared to secrete less extracellular label than controls; the "halo" effect was absent in non confluent cultures, (Fig. IV-1). Good cytoplasmic label was observed, however, in all viable cultures. Further, the addition of ChS (1mg/ml) to the medium of (^{3}H)-acetate labelled amnion cells for the duration of the 1h 'chase' incubation abolished the intranuclear localization of the label described previously.

3.2 Effect of additives on gingival tissue pieces.

Previous results showed that human gingival pieces incubated at 37^{o}C for 15 min with (^{35}S)-sulphate or (^{3}H)-acetate and followed by 60 min in radioactive isotope free medium, incorporated label localized in the intercellular material of the epithelium, (Fig. IV-2), particularly in the Stratum spinosum (S). Elution from CPC fixed sections with increasing electrolytic concentrations sequentially removed label from the sections. Control washing without salt did not diminish the intensity of label. In sections treated with 1.25M $MgCl_2$ some intracellular label was retained (r), (Fig. IV-3) (cf. Fig.IV-2).



(a) (b)

Fig. IV -1.

Autoradiograph of
(a) ^{35}S - sulphate,
(b) ^{3}H - acetate,
by amnion cells incubated
in the presence of ChS. (Mag x1250)
(for details see text).

PN = perinuclear.

E = extension of cell.

Cc = Cytoplasmically confined.

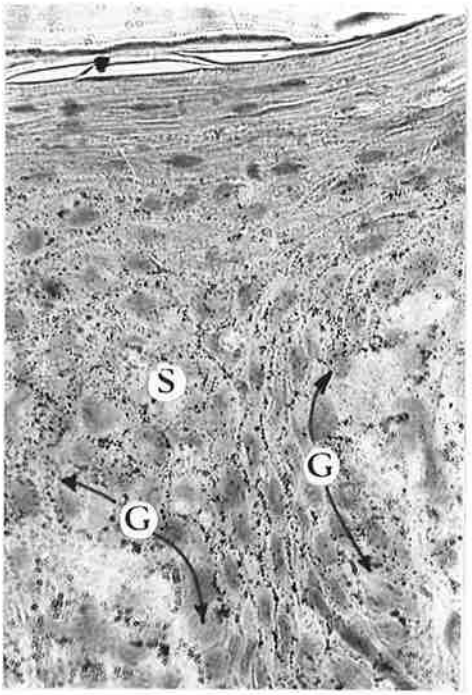


Fig. IV-2

Control autoradiograph of (^{3}H-acetate) incorporation into cetylpyridinium chloride precipitable material prelabelled (15 min 'pulse') gingival slices after 60 min 'chase' incubation (S=Stratum spinosum, G=Stratum germination). (Mag x1250)

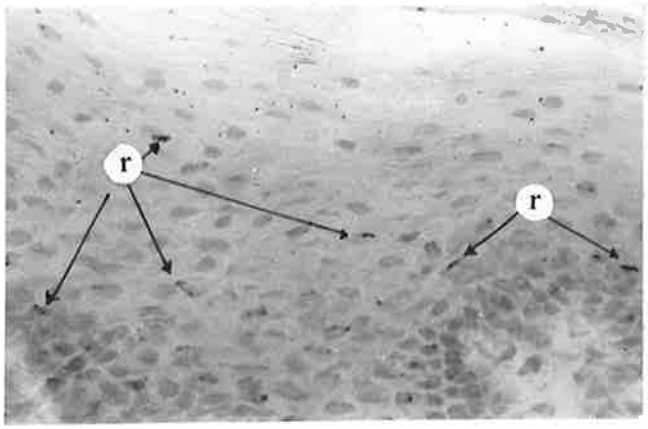


Fig. IV -3

Autoradiograph of(^{35}S)-sulphate incorporation into cetylpyridinium chloride precipitable material by prelabelled (15 min 'pulse') gingival slices after 60 min 'chase' incubation, histological sections were washed in 1.25M $MgCl_2$ prior to processing with nuclear tracking emulsion. (r = intracellular retention). (Mag x500)

3.2.1 The effect of hyaluronidase.

Gingival tissue was prelabelled with radio isotope for 15 min and then 'chase' incubated for 60 min in Medium 199 containing testicular hyaluronidase. Tissue integrity was disrupted at all concentrations of enzyme, but some intercellular localization of label was evident, particularly at the lower concentrations of enzyme, 15 & 2TRU (Fig. IV-4). Labelled material had diffused to most strata of the epithelium except in the S. lucidium, where there appeared to be some slight intracellular retention only (r). If the hyaluronidase was included throughout the whole of the 'pulse-chase' period, there was little or no localization of label, the intercellular label being confined to specimens incubated with the lower concentrations of enzyme (Fig. IV-4 b). When hyaluronidase was included in the 'pulse' alone, intracellular label was clearly evident both in S.spinosum (S) and S.granulosum (G). Heat inactivated hyaluronidase did not interfere with the pattern of incorporation of radio label demonstrated by controls.

3.2.2. The effect of trypsin.

Following an initial 15 min radioactive 'pulse' incubation, gingival slices were further incubated for 60 min in Medium 199 containing one of three concentrations of trypsin. The higher concentrations of trypsin (0.5 & 0.3 ug/ml) abolished intercellular localization of label. The lowest concentration (0.15 ug/ml) reduced the incorporation. Label was however observed intracellularly (r), (Fig. IV-5). Trypsin included

(a)

(b)

(c)

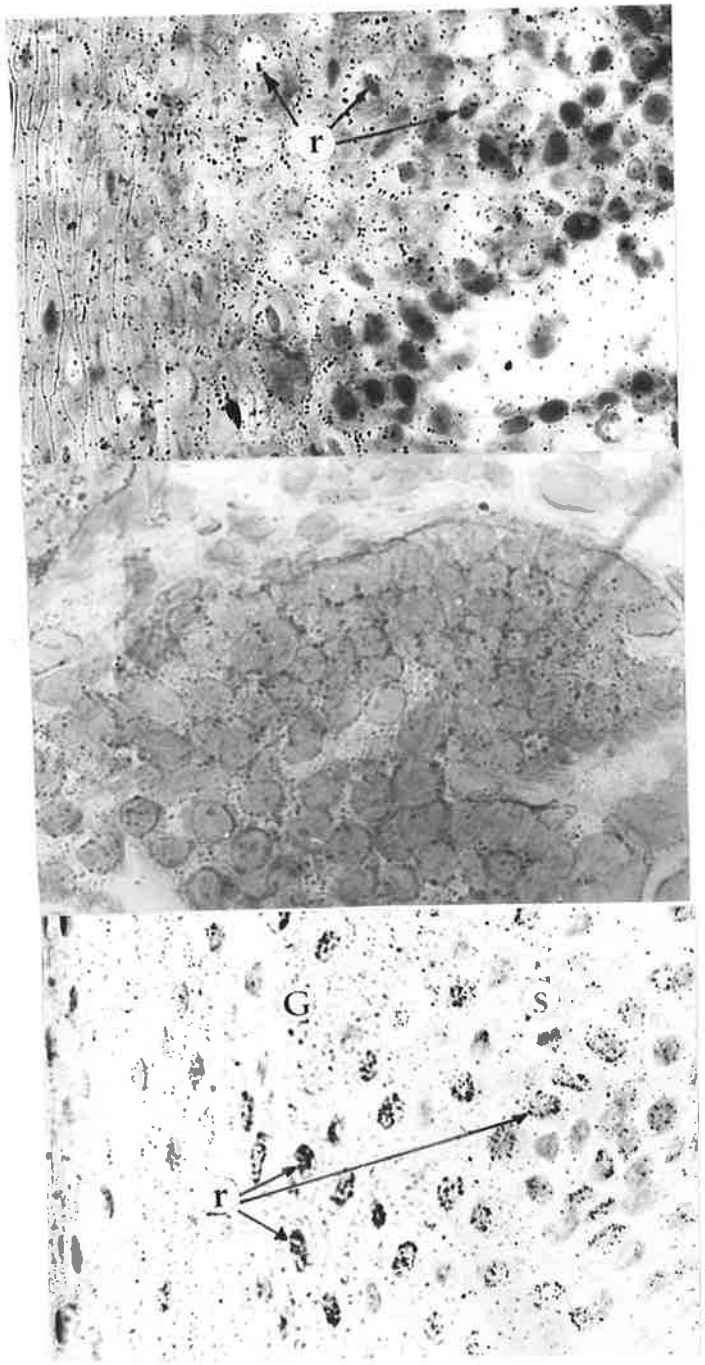


Fig.IV-4

Autoradiograph of (^{35}S)- sulphate incorporation by prelabelled (15 min 'pulse') gingival slices after;

a). 'chase' incubation (60min) in medium which contained 15 TRU/ml hyaluronidase.

b). 'chase' incubation (60min) 2TRU/ml of hyaluronidase was included throughout the 75min incubation (i.e. in 'pulse' and 'chase' incubations).

c). 'chase' incubation (60min). 2TRU/ml of hyaluronidase was only included in the 'pulse' (15min) incubation. (i.e. the 'chase' contained no additive). (Mag x1250) r = intracellular retention

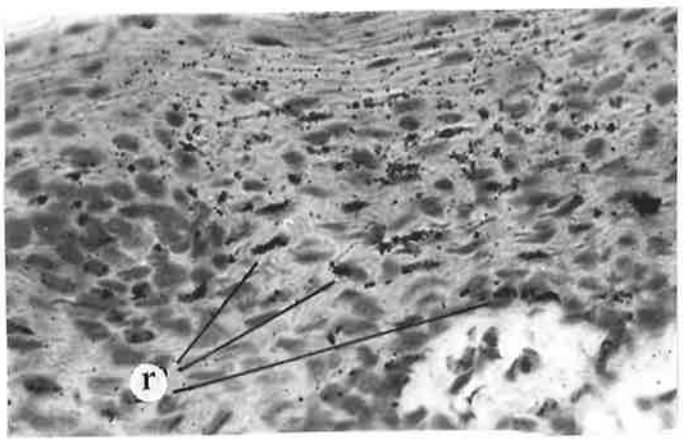


Fig. IV-5.
Autoradiograph of a histological section of a piece of human gingiva prelabelled with (^{35}S)-sulphate by 'pulse' incubation at 37^{o}C for 15min. A 'chase' incubation of 60min containing 0.3ug/ml of trypsin followed. Note complete absence of intercellular label and discrete intracellular retention, r (Mag x 500).

throughout the whole 'pulse-chase' incubation resulted in decreased intercellular localization with predominance of retention of the label intracellularly (r) (Fig. IV-6). However, in experiments in which the lowest concentration of enzyme had been used, and where intercellular localization was evident, the heavy confined discrete deposits of silver grains in the autoradiographs implied nidi of intense activity, (Fig. IV-7) . Low concentrations of trypsin included in 'pulse' incubations (first 15 min) appeared to have no effect on the localization of radio label but the highest concentration of trypsin prevented the uptake of the label, resulting in a decrease in the total number of autoradiographic silver grains.

Heat treated enzyme had no effect on uptake and localization of radio isotopes as compared with controls.

3.2.3. The effect of chondroitin sulphate.

When ChS was only included in the 'chase' incubation medium (0.3 to 0.15 µg/ml) of gingival slices prelabelled with radio-isotope, intercellular localization of label was inhibited and the intracellular retention (r) of label was diffuse, (Fig. IV-8). ChS did not appear to affect the initial incorp-oration of radioactive precursor. Autoradiographs of sections of tissue which were incubated with ChS which had been included with the 'pulse' alone were similar to the control incubations, however at the higher concentration of ChS (0.3 ug/ml), some label was observed in the outer S.spinosum where there were small pockets of intense activity, (Fig. IV-9).

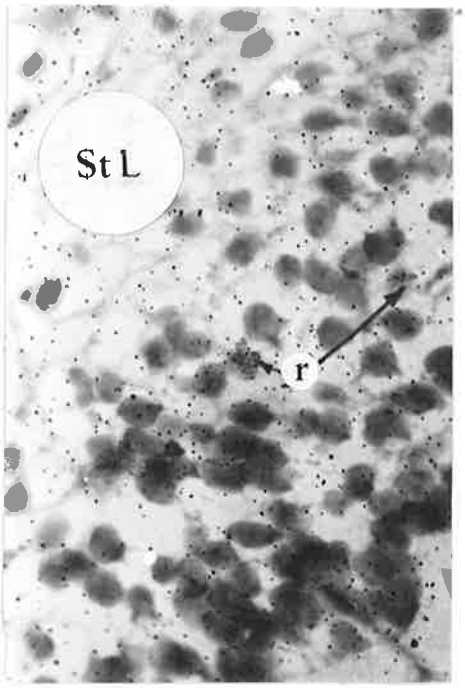


Fig.IV-6

Autoradiograph of (^{35}S)-sulphate incorporation by prelabelled (15 min 'pulse') gingival slices after 60 min 'chase' incubation; trypsin (0.5ug/ml) was included throughout the whole incubation 75 min. (Mag x500)
St L=Stratum Lucidium.
r= intracellular retention.

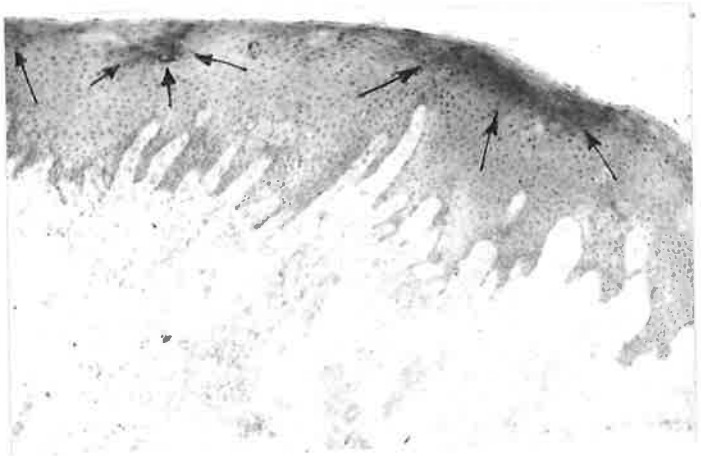


Fig.IV-7

Autoradiograph of (^{35}S)-sulphate incorporation by prelabelled (15min 'pulse') gingival slices after 60 min 'chase' incubation trypsin (0.15ug/ml) was included throughout the whole incubation (75 min). (Mag x125)

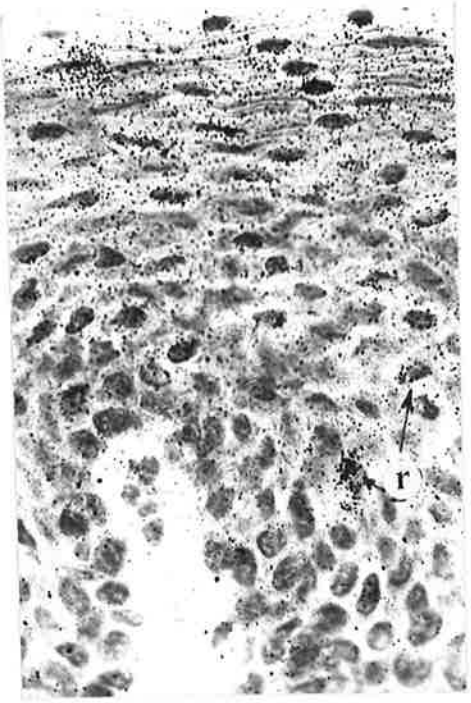


Fig. IV-8

Autoradiograph of (^{35}S)-sulphate incorporation by prelabelled (15 min 'pulse') gingival slices after 60 min 'chase' incubation in medium containing 0.3 ug/ml ChS. (Mag x500)

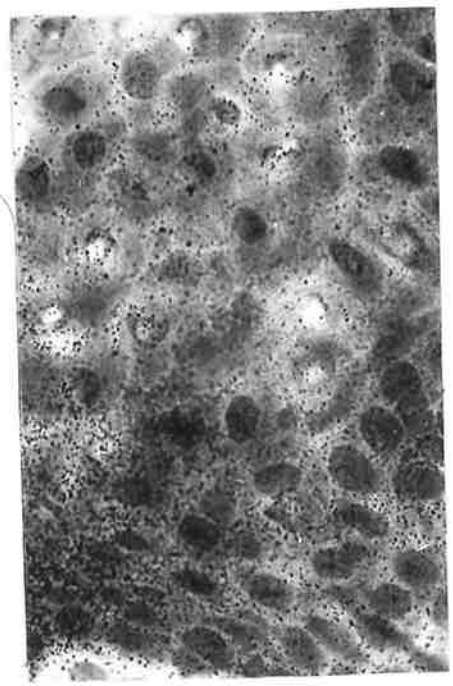


Fig. IV-9

Autoradiograph of (^{35}S) sulphate incorporation by gingival slices which were preincubated for 15 min 'pulse' in medium containing ChS (0.3 ug/ml) and were 'chase' incubated for a further 60 min. (Mag x500)

3.2.4. The effect of heparin.

When Hep was added to the incubations of gingival slices, the following concentrations were used; 100 and 1000u/ml. No intercellular localization of the labelled precursor was observed when the pre-labelled cells were incubated in radio isotope free medium containing the lower concentration of Hep. There was close perinuclear association of intracellular labelled material, (Fig. VI-10). The effect was less marked at the higher concentrations of Hep.

When Hep was included in the medium at 100u/ml throughout the whole of the 'pulse- chase' (75 min), the intercellular localization of the label was observed only at this lower concentration of Hep in this system. A similar effect was noted if the Hep was only included during the 'pulse" incubation; the higher concentration completely inhibited intracellular retention of the label, (Fig. IV-11).

3.2.5 The effect of hyaluronic acid.

After a 15 min 'pulse' labelling, gingival tissue was incubated in medium containing 0.3, 0.15 or 0.05ug/ml of HA. Under these conditions intercellular localization in gingival tissue was inhibited. In all experiments, the label appeared to be retained in a perinuclear location (r), (Fig. IV-12). This material could not be removed by previous treatment of CPC fixed sections with 0.5m NaCl nor with 1.25M $MgCl_2$. When HA was included for the whole incubation period; both in the medium containing the radio active label and in the 'chaser' medium; intracellular retention was noted at all concentrations, and only at the lowest concentration (0.05ug/ml) was there some evidence of intercellular localization (Fig. IV-13).

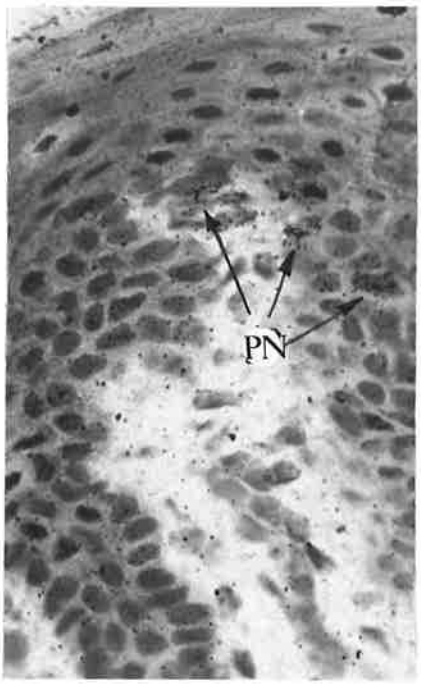


Fig. IV-10.
Autoradiograph of (^{35}S)-sulphate incorporation by preincubated (15 min 'pulse') gingival slices after 60 min 'chase' incubation in medium containing 100u/ml commecial heparin. (Mag x500) (PN=perinuclear label).

Fig. IV-11.
Autoradiograph of (^{35}S)-sulphate incorporation by gingival slices which were preincubated for 15 min 'pulse' in medium containing 1000u/ml heparin and were 'chase' incubated for a further 60 min. (Mag x500)

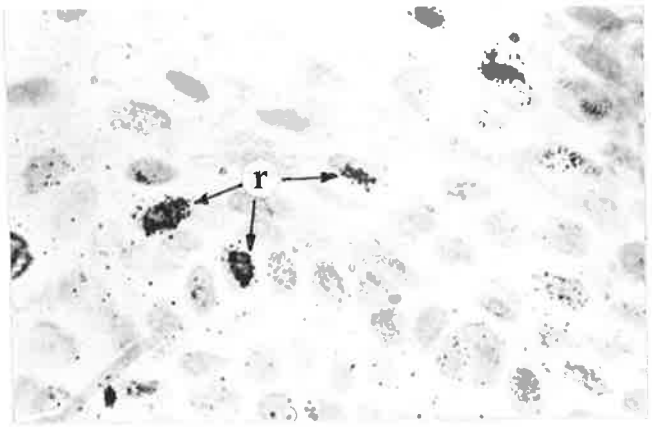


Fig. IV-12

Autoradiograph of (^{35}S)-sulphate incorporation by preincubated (15 min 'pulse') gingival slices after 60 min 'chase' incubation in medium containing 0.15 ug/ml hyaluronic acid. (r=intracellular retention of label. (Mag x1250)

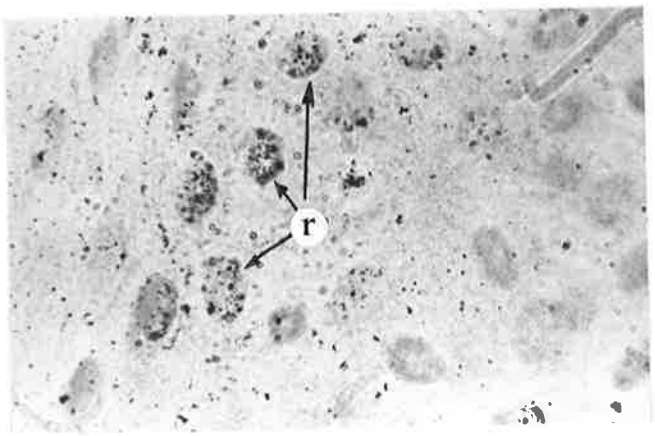


Fig. IV-13

Autoradiograph of (^{35}S)-sulphate incorporation by gingival slices 'pulse' labelled for 15 min and 'chase' incubated for 60 min. hyaluronic acid (0.05 ug/ml) was present throughout. (Mag x1250)

When HA was included with the radio active 'pulse' only, (initial 15 min, cells of the S.spinosum incorporated little label intracellularly, nor was there intercellular label, (Fig. IV-14).

4.0 SECTION SUMMARY WITH CONCLUSIONS AND SUPPLEMENTARY DATA.

Biochemical quantitation and microscopical localization of macromolecular uronates, synthesized both by an amnion cell line and by primary incubation of gingival epithelium and gingival epithelial cells confirm that epithelial cells are capable of synthesizing AMPS. Furthermore, successive quantitative assays throughout the development of the cultures were performed in order to investigate some of the qualitative and quantitative relationships, if any, between macromolecular uronates and cell contact. The localization of these species of molecules by classical histochemical means following substrate elimination with specific enzymes, together with the autoradiographic methods used, confirmed that macromolecular polysaccharide, designated AMPS, were deposited at intercellular interfaces of epithelial cells. Since most of these macromolecular uronates contained substantial sulphate, hexosamine, associated protein and some galactose and xylose (Chapter II), it was surmized that the macromolecules were PGs. Moreover, CPC precipitation with critical electrolyte elution (SCOTT,1960) as well as the relative specificity of biosynthetic incorporation of (^{35}S)-sulphate have provided stronger positive evidence (Chapter V; WIEBKIN, BARTOLD & THONARD,1979).

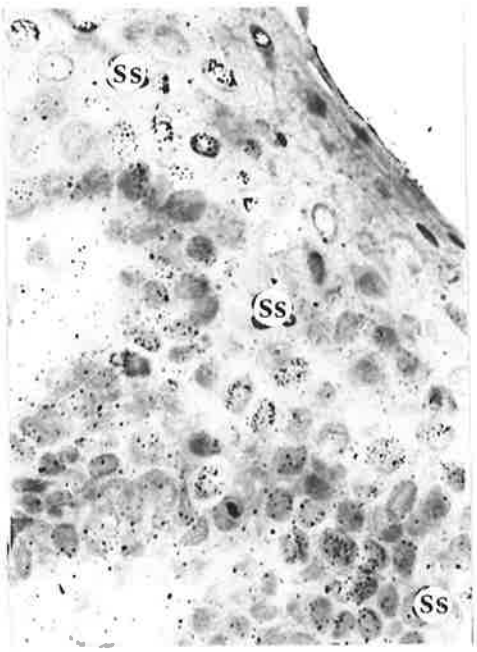


Fig. IV- 14.

Autoradiograph of (^{35}S)- sulphate incorporated by gingival slices which were preincubated for 15 min 'pulse' in medium containing 0.3 ug/ml hyaluronic acid and were 'chase' incubated for a further 60 min. (Mag x500)
(Ss=Stratum spinosum).

Amnion cell cultures - extrapolated function of cell-cell interface.

In the case of amnion cell cultures, as confluence increased, the amount, per cell, of macromolecular material with sulphated GAG properties increased until the cultures achieved +++rated confluence; namely that in the cells just experiencing extensive cellular contacts (Fig. IV-15).

ROLLET'S suggestion (1858) that mucin was an interfibrillar cement in the intercellular matrix must be considered heuristic even at that early date. On the cell surface of non adjacent cells there would be no need for "cementing material". On the other hand, the deposition of an intercellular cement between confluent cells could be an advantage in maintaining monolayer (or tissue) integrity. Furthermore at confluence, material between intercellular interfaces may be physically compartmentalized in a manner similar to the extracellular matrix of the in vivo tissue (cf. nodes of Bizzozaro, (Fig. IV-16)) and would thus be afforded some protection from ready dissolution into the culture medium. Indeed data derived from amnion cell culture indicate that the non confluent cells do secrete, into their medium, a greater proportion of their total synthesized PG than do confluent cells. However total PG synthesis per cell in non confluent cells is about half that of the confluent cells. The proportions of cell associated PG as compared with the proportions secreted into their media, of the total synthesized by +++rated and by fully confluent cultures (++++rating) were similar. However, the absolute total PG synthesized per cell was greater in the +++rated cultures than in ++++rated cells. If cell surface interface areas possess a regulating

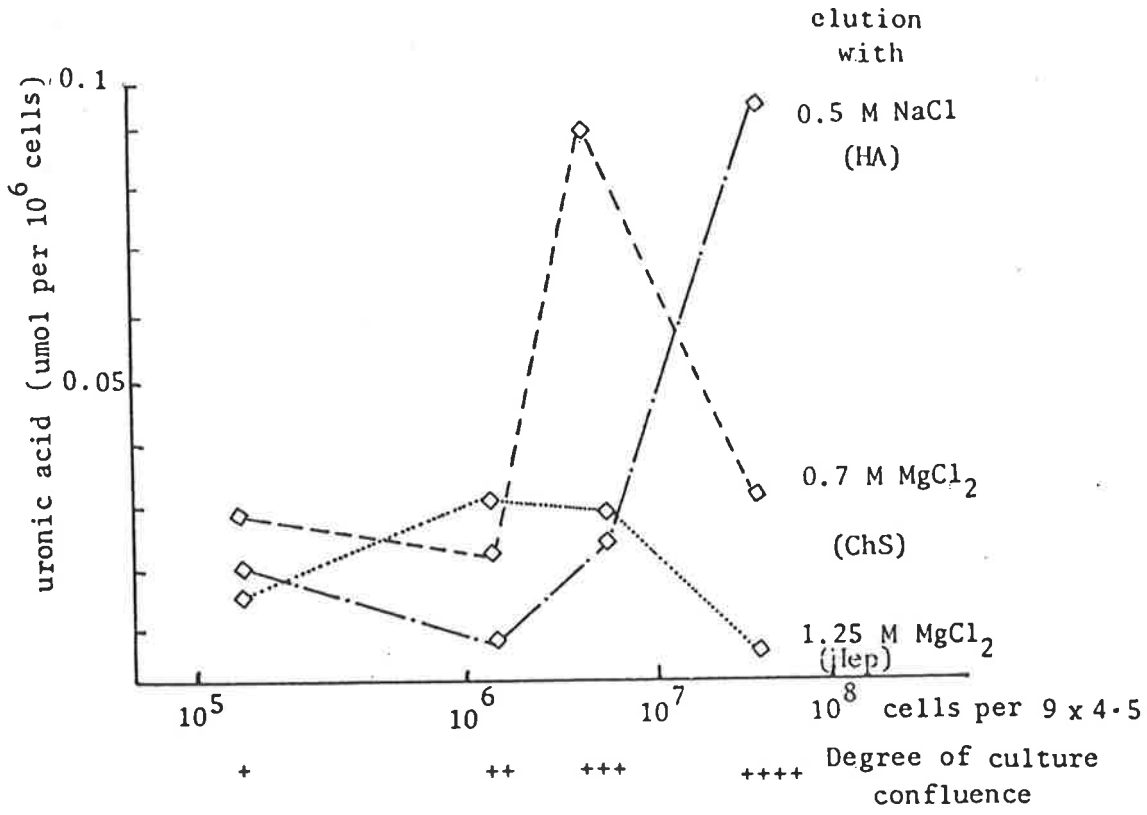


Fig. IV-15.

The distribution of macromolecular uronate associated with amnion cells in cultures of varying stages of developing confluence. The curves represent the data tabulated on page 102 (Table II-4). The material was sequentially eluted from CPC precipitates with 0.5M NaCl; 0.7M $MgCl_2$; 1.25M $MgCl_2$ to yield fractions which behaved in electrophoresis similarly to HA; ChS; Hep-L-P, respectively. (see page 102, Table II-4).

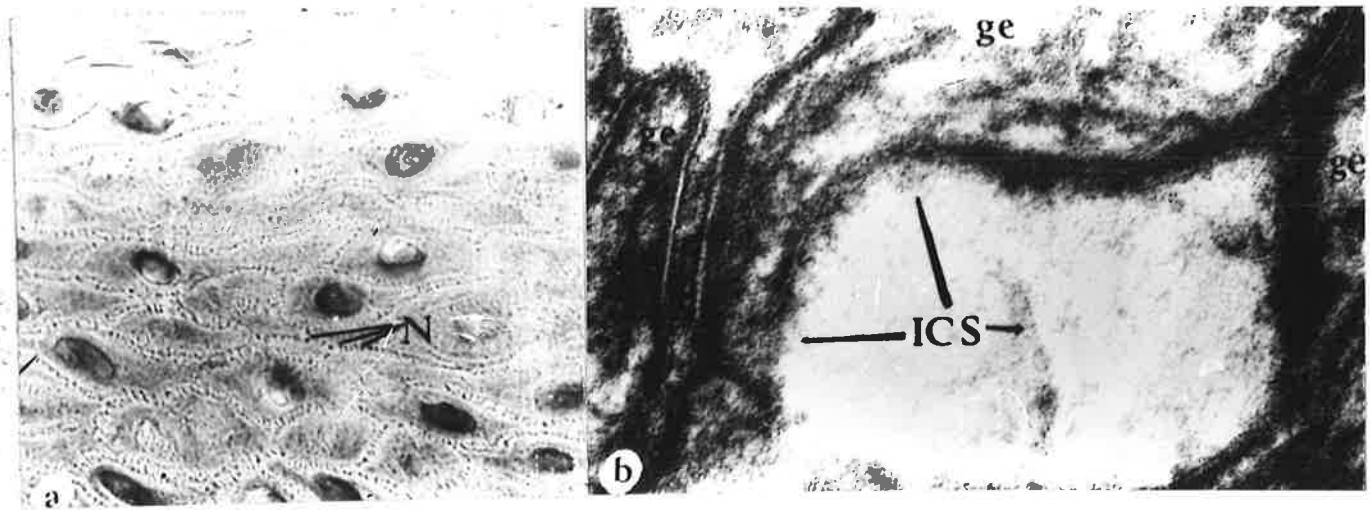


Fig. IV-16.

a. Nodes of Bizzozaro between cells of gingival epithelium (N). (Mag x1250)

b. Electronmicrograph of the interface between two cells of gingival epithelium (ge) showing large intercellular 'spaces' which contain macromolecular sulphated uronate (ICS), (WIEBKIN, BARTOLD & THONARD, 1979).

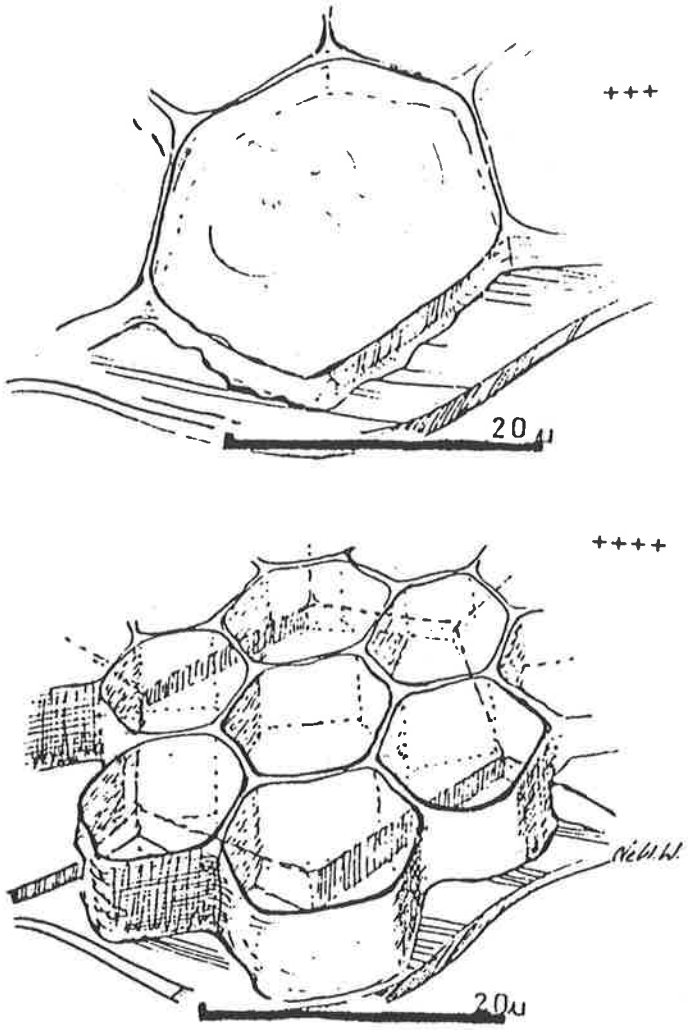


Fig.IV-17.

Schematized comparative model of the dimension of adherent cultured amnion cells at two stages of confluence (+++ and ++++ ratings).

function then interface geometry may further help to explain the consistent differences in assay values of +++rated and ++++rated cultures. Figure IV- 17 illustrates the possible relationship between the areas of cell surface interfaces per cell in two model cultures. The average cell volumes of detached amnion cells from +++rated and ++++rated cultures were similar. Microscopical vertical focus measurements demonstrate that tightly packed fully confluent (++++rated) cultures consisted of taller narrower columnar cells than the flatter spreadout cells of the +++rated cultures.

Given that the cell volumes of +++rated and ++++rated cultures remained similar throughout cultural development the cell surface interfaces of constituent cells from each of these cultures would vary per cell by a ratio of $\ell/\sqrt{\ell}$* where ℓ is the increase in height of the cells from the +++rated to ++++rated condition. Since a ++++rated culture contained about 40.0×10^6 cells per standard 9x4.5cm flask, whilst +++rated cultures contained about 7.5×10^6 cells a single cell in a ++++rated culture would spread over and adhere to only $\frac{1}{5}$ of the culture flask surface as compared to that covered by a cell from a +++rated culture. Thus their respective heights should be increased by a factor of 5. The cell surface interface areas per cell would increase then by a factor of $\frac{5}{\sqrt{5}} = 2.236$. There are however 5 times as many cells. The total cell: cell surface area per culture would therefore be increased by 11.18. Such an index could be used to compute the relationship of cell surface PG per unit of cell surface

interface as cultures developed from the +++rated condition to the fully confluent cells (++++rating).

Representative extrapolations have been tabulated for uronic acid (Table IV-1) and for sulphate (Table IV-2) as a measure of cell associated (intercellular) macromolecular component. These extrapolated values for intercellular components per "unit-cell-interface" (as opposed to 'per "basic cell numbers" ') clearly show that the amount of cell surface associated sulphated PG which appeared to contain ChS are predominant in the cultures which had just achieved cell contact (+++rating). However, the level of sulphation ($SO_4^{=}$:uronic acid ratio) increased when the culture reached full confluence (+++rating =0.43 vs ++++rating =1.81).

The amount of the less easily solubilized sulphated material (Hep-like polysaccharides) which eluted at 1.25M $MgCl_2$, appeared to attain constancy following initial cell contact (+++rating) but the level of sulphation decreased as ++++rating was achieved. Since the Hep-like-polysaccharides have been shown to be integral components of the cell surface itself (KRAMER, 1971), it is not surprising that such uronic acid values reflect total cell surface area (interface + free surfaces) rather than interface matrix material per se.

The amount of HA (0.5M NaCl eluted material) associated with cell interfaces in fully confluent cultures (++++rating) as calculated per "unit-cell-surface-interface", is of particular interest since HA has been shown to be an integral moiety in

Table IV-1. Relative amounts of macromolecular uronate calculated on the basis of 'umols per unit of cell surface area' (contrast with umol per 10^6 cells, Table II-4.p.102).

Conditions of monolayer culture: Ranking according to Fig.II-1	Cetylpyridinium chloride precipitable material solubilized in 0.5M NaCl uronic acid	0.7M $MgCl_2$ uronic acid	1.25M $MgCl_2$ uronic acid 1.
+++	0.18	0.66	0.217
++++	0.32	0.11	0.0214

Keynote: 1. umols/'unit of cell surface interface area', where a unit equals unity when the culture just attains confluence, ie. at +++ rating.

Table IV-2. Relative amounts of macromolecular sulphate calculated on the basis of 'umols per unit of cell surface area' (contrast with umol per 10^6 cells, Table II-4.p.102).

Conditions of monolayer culture: Ranking according to Fig.II-1	Cetylpyridnium chloride precipitable material solubilized in 0.5M NaCl sulphate	0.7M $MgCl_2$ sulphate	1.25M $MgCl_2$ sulphate 1.
+++	0.0112	0.285	0.087
++++	0	0.2039	0.028

Keynote: 1. umol/'unit of cell surface interface area' where a unit equals unity when the culture just attains confluence, ie. at +++ rating.

certain types of sulphated PG aggregates, (HARDINGHAM & MUIR, 1972). WIEBKIN & MUIR,(1975) have postulated that these aggregates are on the cell surfaces and can be involved in the regulation of PG biosynthesis itself (WIEBKIN & MUIR, 1973). As yet we are not certain that amnion cells respond to HA regulation with respect to PG synthesis. If they do, the concept of interface mediated function may prove to be enlightening in interpreting the role of HA at various stages of development.

Gingival epithelial cultures.

Suspension cultures of gingival epithelial cells derived from single cell preparations and which had been fractionated into clumps of cells (Categories II, III & IV) contained macromolecular material with markedly increased $SO_4^{=}$:uronic acid ratios. The relative constancy of the levels of uronic acid (0.7M $MgCl_2$) per cell in these cultures implies that some regulation of total PG synthesis was inherent in the culture as a whole. The dominant regulatory function appeared to be associated with the condition of the culture following excision and disaggregation processes of the gingival tissue. Indeed developmental biologists stress the importance of the interactions between approximating cells (induction). A method has recently been published for studying epithelial-mesenchymal interactions in human oral mucosal lesions (MACKENZIE, DABELSTEEN & ROED-PETERSEN, 1979) and they discuss the effects of keratinization. The effects of epithelial cells on fibroblasts and the converse, and in respect of this thesis, the role of PGs has also been briefly reported (MERRILEES & SCOTT, 1979). An exciting extension of the

work reported in this thesis would be to investigate the role of adherent cells on the synthesis of PG by non adherent cells, and also the effect of fibroblast cultures on the gingival epithelial culture. Indeed the gingival culture experiments described thus far have not eliminated any of the putative induction effects of clumped cells on PG synthesis and secretion by the single cells of the fragmentary suspension cultures, or vice versa . Nor have experiments been concerned with the role of homologous serum. Therefore the following preliminary studies were carried out to establish any regulatory role of homologous human serum on the biosynthesis of PG by fragmented gingival suspension cultures.

Two independent experimental regimes were adopted. Under each experimental protocol the gingival epithelial cells were cultured in medium containing their respective homologous serum (details as described earlier) at a concentration of 15%. They were compared with equivalent cultures supplemented with bovine serum. The first set of experiments were performed directly with unfractionated cells from fragmented gingival epithelium following 21 days of suspension culture. The second study focused on cells which had been fractionated into a single cell suspension (Category I) which formed the initial inoculum for the subsequent 21 day incubation, ie. their cultural development did not include their pre-exsisting clumps.

At 21 days of culture cells from each experiment were fractionated into Categories I,II,III,IV and uronic acid and sulphate values were measured.

When fragmented gingival epithelium was incubated unfractionated for 21 days in medium containing homologous human serum, small

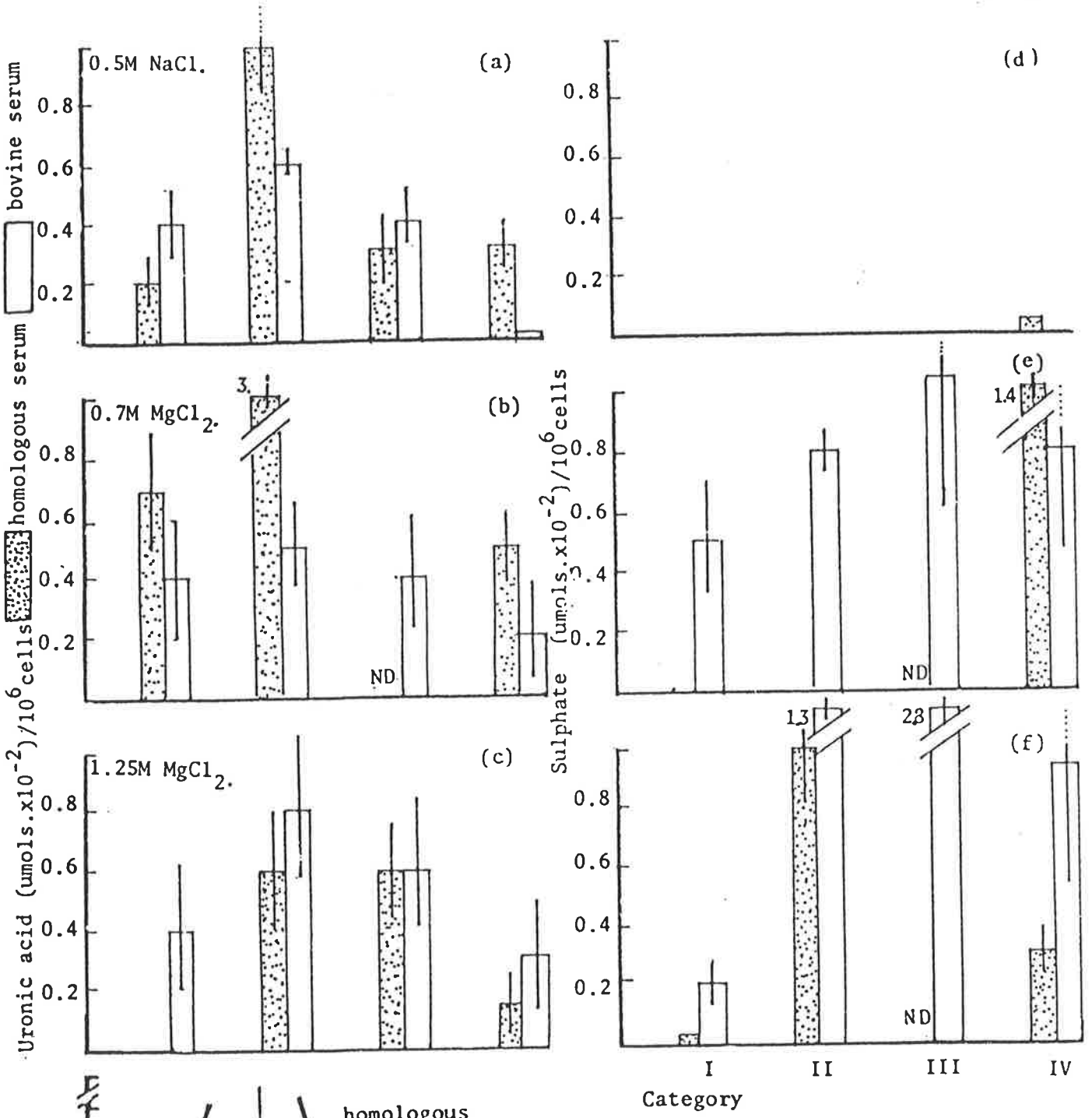
clumps of cells (Category II) synthesized relatively more polyuronate which corresponded to HA and ChS while they synthesized relatively less material corresponding to Hep-like- polysaccharides.

Generally homologous serum induced less sulphation.

Cultures originating from single cell inoculums showed a greater tendency for increased sulphation of the ChS and Hep- like- polysaccharides in single cells and Category II clumps of cells. A speculative interpretation based on these very preliminary data may imply that homologous serum in a culture containing an initial inoculum of a spectrum of clump sizes, ie.an elementary gingival epithelial fragmentation is either i) insufficient stimulus to induce the necessary sulphation of ChS to elaborate intercellular adhesion, or 2) the attempt of a single cell inoculum to achieve cohesiveness ie. to form clumps is reflected in the sulphation of the ChS synthesized. Even the single cells at 21 days were elaborating appropriately sulphated Hep-like -polysaccharides ie. a ratio of 3:1 for $SO_4^{=}$: uronate, (Fig.IV-20b.).

The Category II small cellular clumps contain more uronic acid per dry weight than the larger cell assemblages and homologous serum has an equally stimulatory effect on both experimental cell culture preparations.

Since a developmental stage of both these primary cultures may be represented by the metabolically active small cell clump Category II, perhaps it will be significant to focus attention on the significantly stimulated degree of sulphation of Category II cells in cultures originating from single cells, as opposed to the apparent inhibitory effect induced by the existence of a distribution of clump sizes in unfractionated cultures.



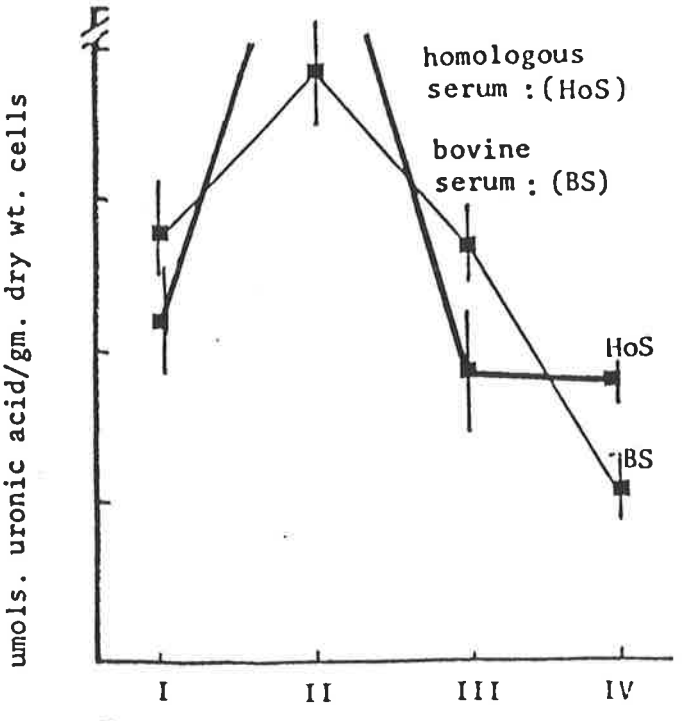


Fig. IV-18

The distribution of macromolecular uronic acid (a,b,c,) and sulphate (c,d,e,) associated with the cells cultured from fragmented gingival epithelium the total uronic acid content per gm. of cells. Unfractionated fragmentary gingival epithelium was cultured for 21 d in medium containing (i) 15% homo-logous human serum, (ii) 15% BSA. These suspension cultures were then fractionated according to cell clump size (Fig. II-3) into four Categories I,II,III,IV. representing single cells, small and larger clumps resp. Assays were performed on each of these Categories.

ND: not determined.

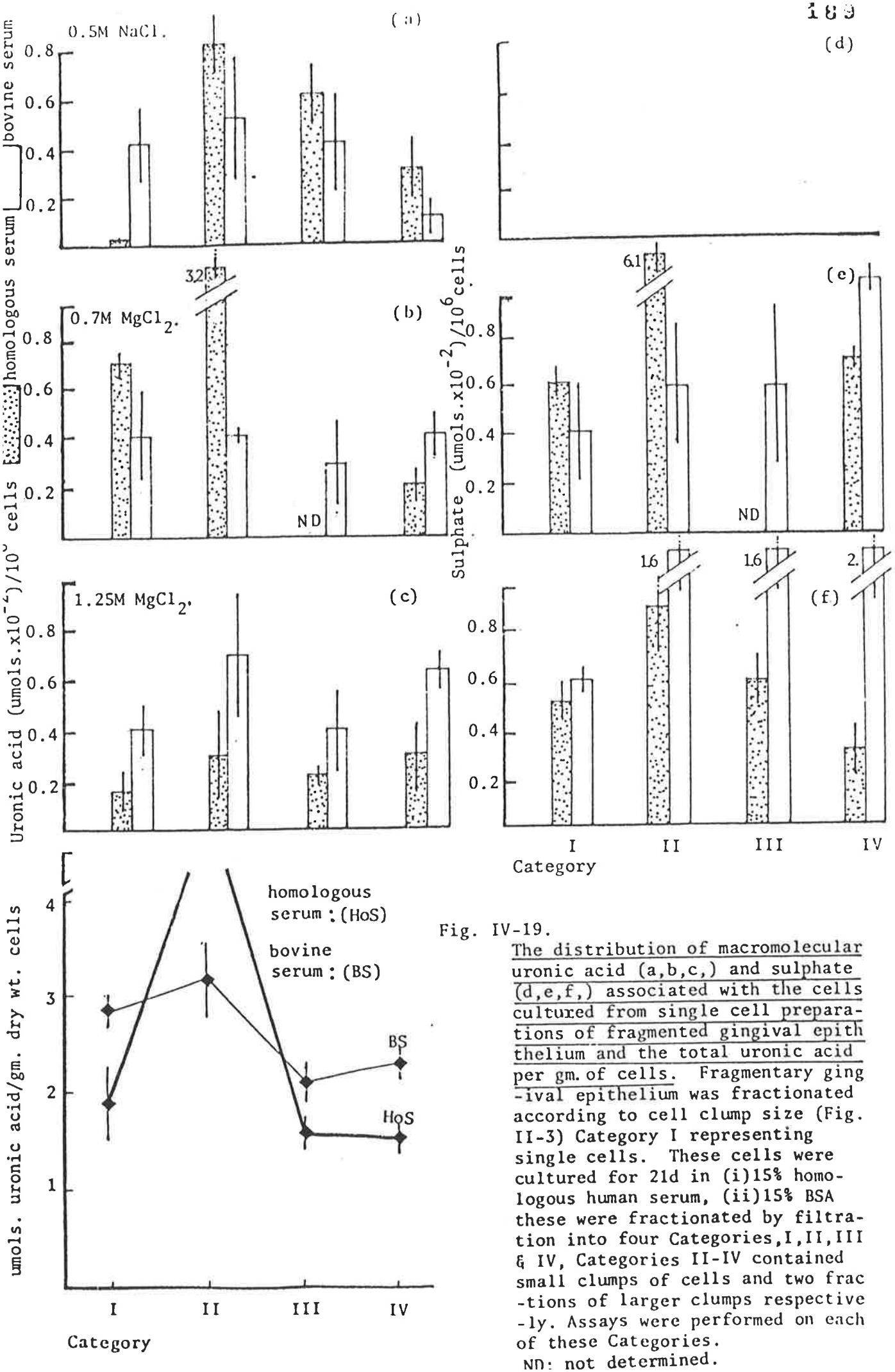


Fig. IV-19.

The distribution of macromolecular uronic acid (a,b,c,) and sulphate (d,e,f,) associated with the cells cultured from single cell preparations of fragmented gingival epithelium and the total uronic acid per gm. of cells. Fragmentary gingival epithelium was fractionated according to cell clump size (Fig. II-3) Category I representing single cells. These cells were cultured for 21d in (i)15% homologous human serum, (ii)15% BSA these were fractionated by filtration into four Categories, I, II, III & IV, Categories II-IV contained small clumps of cells and two fractions of larger clumps respectively. Assays were performed on each of these Categories.

ND: not determined.

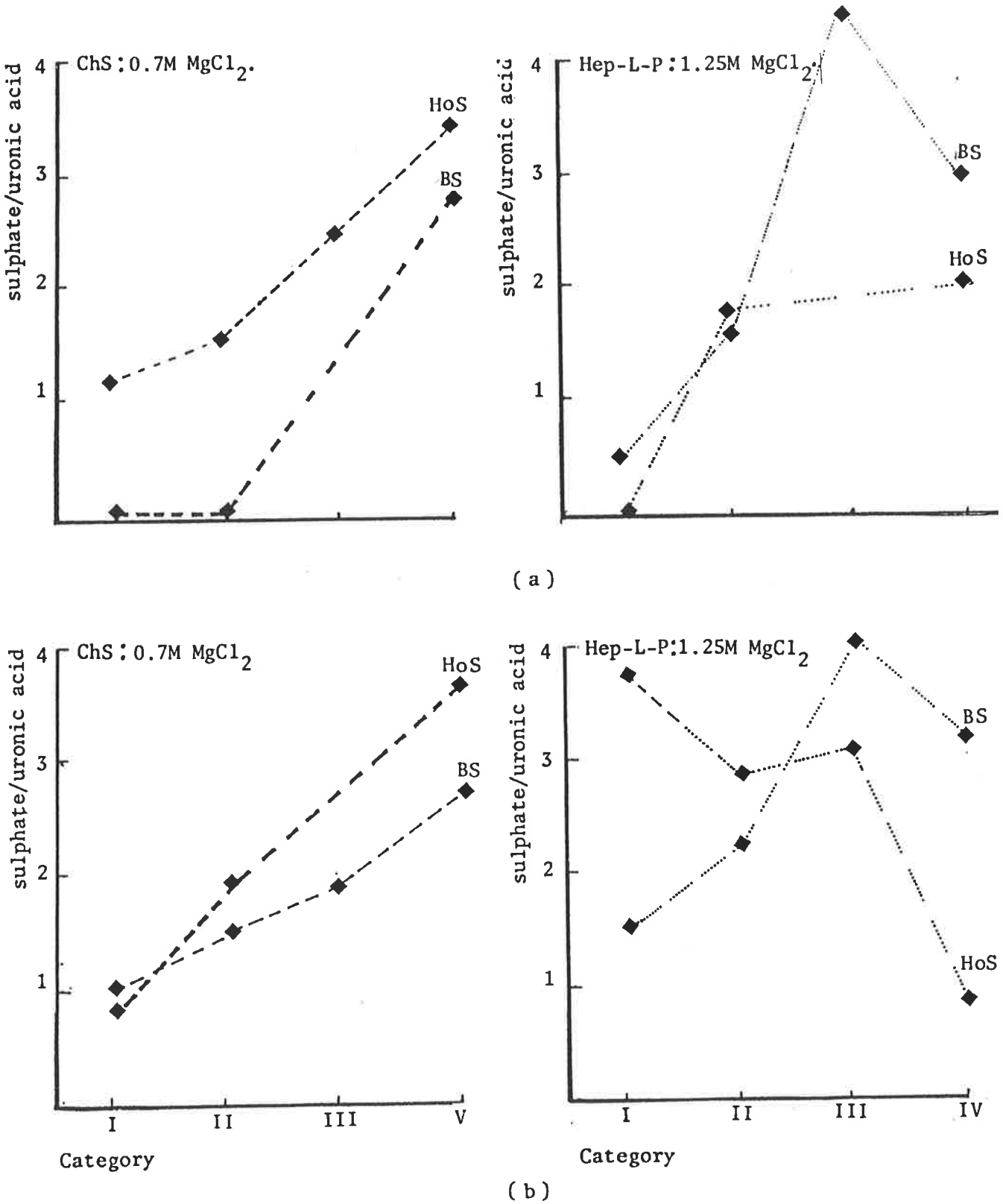


Fig. IV-20.
The sulphate to uronic acid ratio of fragmented gingival epithelium following culture for 21 days in (a) medium supplemented by 15% homologous serum or 15% BSA. Unfractionated fragmentary gingival epithelium was cultured for 21 days in medium . These suspension cultures were then fractionated according to cell clump size (Fig. II-3) into four Categories I,II,III & IV. representing single cells, small and larger clumps resp. (b) Fragmentary gingival epithelium was fractionated according to cell clump size (Fig. II-3). Cells from Category I representing single cells were cultured for 21 d and then fractionated by filtration into four Categories I,II,III,IV as above.

Homologous human serum, ◆——◆ HoS; Bovine serum ◆- - - -◆ BS.

The dynamic role of GAGs in gene expression- examples and inter-related biological mechanisms of regulation.

The experiments highlight the influence that extraneous intercellular materials might have on the further synthesis of intercellular macromolecules. The addition of nutrients and growth supplements ie. serum or vitamins etc. would probably serve to reinforce modulation of those influences. By analogy in another extracellular matrix function, GOLHABER (1965) had demonstrated that in vitro bone resorption was enhanced when Hep was added to a system containing sub-optimal concentrations of vitamin A & D_2 or parathyroid extract. However, following the addition of sulphated HA or dextran sulphate to his experimental system, bone resorption was not promoted.

Another example of the qualitative relationship of PG species to biosynthetic regulation is the blocking of thyroid hormone synthesis with propylthiouracil; this resulted in a decrease in sulphated PG and a concomitant increase in HA (SCHILLER, SLOVER & DORFMAN, 1964). The effect was reversible with thyroxin. The association between the microenvironmental status of the intercellular matrix and the constituent tissue cells was also implied in the work of BAZIN & DELAUNY, (1968) who demonstrated changes in the amount of AMPS and related substances secreted into inflammatory sites resulting from Arthus reactions. Such changes included the liberation of Hep from mast cells under trauma. In addition to synthetic control the depolymerization of high molecular weight Hep by reducing agents such as ascorbate and the depolymerization of other GAGs, especially those that are non sulphated such as HA, by a number of reducing agents (L-cysteine,

L-ascorbic acid, thiols, metal ions and hydroquinones eg. SUNBLAD & BALAZS, 1966; SCOTT, TIGWELL & SADJERA, 1972) and the presence of molecular oxygen or oxygen derived free radicals in tissues (McCORD 1974; McNEIL, WIEBKIN, BETTS & CLELAND, 1982), amplifies the complexity of the dynamic role which a wide range of interactive species plays in maintaining a) integrity of intercellular matrices, and b) the consequent regulation of genotypic expression by the extracellular matrix.

Thus, loss of tissue integrity may be related at least in part, to one or a combination of the following factors:

-disturbances in PG polymerization during synthesis;

-depolymerization after secretion;

-enzyme degradation;

-feedback control influenced by microenvironmental quality or quantity.

In repect of this last factor, the experiments performed for this thesis provided autoradiographic evidence for the effectiveness of three GAGs; ChS, Hep and HA; on the incorporation of (^{35}S)-sulphate by epithelial cells and by epithelium.

Some effects of ChS on biosynthesis of extracellular macromolecules.

Excess ChS did not appear to interrupt incorporation of radio-active precursor substance but it did limit the deposition of intercellular material as a function of the concentration of ChS in epithelium. Various quantitative studies on other cell types have shown to have various effects on PG synthesis. WIEBKIN & MUIR,(1973) using isolated adult chondrocytes have subsequently shown that relatively high (6000ug/ml) and relatively low concentrations (40ug/ml) of Ch-4-S had slightly stimulatory

effects on the incorporation of (^{35}S)-sulphate and at the high doses, 90% of the material synthesized in a 2h period was retained by the cell and not secreted into the medium. Interim concentrations (80-600ug/ml) showed no effect on synthesis, but secretion of (^{35}S)-sulphate labelled PG was inhibited. The same concentrations interfered with the secretion of (^{35}S)-sulphate PG by epithelial cells (amnion, Fig.IV -1 , and gingival cells, Fig.IV- 8,9).

We can only speculate about the modes of action of these GAG mediated effects. The polyionic nature of ChS in solution may cause it to occupy those sites within the intimately cell bound extracellular matrix normally vacant for attachment of de novo synthesized PG. The interactions of DS and ChS have been described (FRANSSON & CÖSTER, 1979) and the importance of fixed ion concentrations within the intercellular polysaccharide network upon K^+,Na^+ and Ca^{++} fluxes MAROUDAS,1974) are also likely to be mediating factors in macromolecular interactions. For example, induced changes in the local net charges near or on cell surface macromolecules have been demonstrated to affect the synthesis and secretion of different GAGs by tendons. Such net charge changes were induced by physical tension, or with synthetic polyamino acids in vitro. The switches from the synthesis of one GAG,eg. ChS, to another eg.DS, appeared to be reversible, (GILLARD, MERRILEES, BELL-BOOTH, REILLY & FLINT, 1977; GILLARD, BIRNBAUM, REILLY, MERRILEES & FLINT, 1979).

Some consequences of Hep-like-polysaccharides on biosynthetic events.

The effects of Hep on the incorporation of (^{3}H)-acetate and (^{35}S)-sulphate were curious. Hep has been shown to be involved in many biosynthetic and biochemical interactions. However, the molecular basis of the biological functions attributed to Hep such as haemostasis

remain largely obscure (SIMON, 1977). Although the chemistry of Hep, Hep S and Hep-like-polysaccharides confines them to a specific family of molecules, their biological functions are very varied. For example, mactin, the Hep from the clam Cyprinia islandica, has been shown to reversibly block cell division for 72h by binding to the cell surfaces without impairing cell growth (CIFONELLI & MATHEWS, 1972). The binding of Hep and other GAGs to the surfaces of many cell types appears to affect cell proliferation by influencing their selective ion binding function. While most studies on the effects of Hep added to cell or tissue cultures report reduced proliferation, others report no effect or stimulation (LIPPMANN & MATHEWS, 1977). There does not seem to be a simple correspondence between, say, the anti-coagulant capacity, molecular size and the other interactive reactions of Hep. LIPPMAN's comment (1977) is worth repeating:

> ".....that heparin may be the first case where a commercial drug should carry an FDA warning 'Suitable for Human Use Only; not for Investigational Use'....." !

Despite the confusion in the information available, at least the results described earlier in this section detailing the effect of Hep on in vitro (^{35}S)-sulphate incorporation by gingival epithelium lends support for the multifarious role of Hep in cell relationships.

For example when higher concentrations of commercial Hep were added to the 15 min 'pulse' of (^{35}S)-sulphate labelling period of gingival pieces, very little intracellular label was retained by the cells after 60 min 'chase' with or without Hep. Indeed good intercellular localization of radio label was observed. On the other hand at the lower concentrations of Hep, the labelled (^{35}S)-sulphate was observed within the cells. Since labelled macromolecular (^{35}S)-sulphate was eventually located

at the interfaces of cells when Hep was added to the initial radioactive 'pulse' incubation, it probably played no part in inhibiting uptake and initial incorporation of the inorganic sulphate.

Although Hep appeared to play no part in (^{35}S)-sulphate incorporation if included throughout the 'pulse/chase' incubation, Hep may have exerted an influence on the secretion or deposition of (^{35}S)-sulphated PG. Either endogenous or exogenous Hep may contribute some sulphate moieties, ie.N-suphate, to the metabolic pool (which contains the (^{35}S)-sulphate in these experiments) from which components for PG synthesis could be drawn. The likelihood of the radio labelled precursor preferentially achieving intercellular status would be thus lessened. Although evidence for cell surface binding, rapid endocytosis and lyzosomal degradation of GAGs by cells is good, (eg. PRINZ, SCHWERMAN, BUDDECKE & von FIGURA, 1978)*, direct re-utilization of degradation products may not be feasable in the short term experimental regime described in this thesis. On the other hand, an excess of exogenous Hep throughout the incubation may provide sufficient accessible sulphate to activate increased biosynthesis; thus increasing radio labelled incorporation. Indeed the rate of Hep and and Hep-like-polysaccharide syntheses themselves appear to be controlled in part by the process of sulphation (see Chapter I and LINDAHL, 1977).

Since (^{35}S)-sulphate incorporation into intercellular material is inhibited by the lower concentrations of Hep, it may nevertheless, be reasonable to suppose that the rate of inorganic (^{35}S)-sulphate incorporation into the synthetic pathway is slower than that of 'nascent' sulphate derived from degraded Hep, that is, sulphate derived from Hep could be more rapidly processed through the APS-PAPS activation pathway into

intercellular substance to the preclusion of inorganic radioactive sulphate; or that there is an inorganic sulphate-Hep equilibrium mechanism involved!

Other roles and modes of action of Hep are obscure; in lipoprotein interactivity; as biological ion exchangers; in local tissue detoxification in vivo and in anti-thrombin III interactions (DOUGHERTY & DOLOWITZ, 1964). The last examples are paradoxical, since during anaphylaxis, in most animals, the clotting time is not increased, despite the release of the metachromatic granules (containing Hep) from mast cells into tissue. RILEY (1959) suggested that Hep which will bind to protein, is rapidly metabolized by connective tissue cells. A 'mast cell cycle' was proposed in which histamine also released from the injured mast cells serves to condition more connective tissue cells than normally to receive the released Hep. As this Hep is probably electrostatically bound to proteins as a simple salt, reversible dissociation would occur according to mass law. Hep can then be metabolized by those cells which are stimulated to synthesize new and quantitatively distinct AMPS as a contribution to the formation of new intercellular matrix material. Finally, the proposal assumes subsequent enzymatic degradation of these AMPS, the components of which can be reutilized for the synthesis of new Hep. We are aware now that direct reutilization of breakdown products is not likely, however, KERBY & EADIE (1953) had shown that components of Hep were inhibitory to the degradative action of lysozyme, which is probably involved with PG biosynthetic control. (HUTTERER, 1966; TUDBALL, ARONSON & DAVIDSON, 1967). Subsequently KUETTNER, RAY, CROXEN & EISENSTEIN, (1967) and PITA, MULLER & HOWELL (1975) and others have demonstrated the importance of lysozyme in PG rich sites of cartilage, and its possible involvement of PG interactions. WIEBKIN & MUIR, (1973) described

slightly stimulatory effects of lysozyme on PG synthesis by cultured adult chondrocytes.

The role of HA in the regulation of secretion and synthesis of epithelial PGs.

Subsequent to the early experiments described in this thesis the influence of extracellular matrices on gene expression have now been more elegantly studied (eg.see SLAVKIN & GREULICH, 1975). Nonetheless, probably the most important observation from my early studies has proved to be the role of extraneous HA on PG secretion and synthesis by gingival cells; the implications of which will be discussed and emphasized in Chapters V & VI.

Specific tissue degradation; the effects of carbohydrase and of protease.

The biological consequences of the degradation of intercellular matrices varies according to the mode of depolymerization. For example depletion of 80% of the ChS from embryonic chick cartilage matrix by testicular hyaluronidase induced five times the normal rate of synthesis of PG by the chondrocytes *in vitro* (HARDINGHAM, FITTON-JACKSON & MUIR, 1972). DZIEWIATKOWSKI and his colleagues (1961, 1964 & 1966) have shown that epiphyseal cartilage lost polysaccharide prior to the deposition of mineral through the action of a proteinase. Destruction of the PG polypeptide core by cathepsin hydrolysis led to release of calcium in high local concentrations at the site of nucleation (WOODWARD & DAVIDSON, 1968). Even as recent as 1974 BARRETT (in discussion with DZIEWIATKOWSKI) comments on the sizes of PG pieces generated when intact cartilage PG subunits were degraded with purified cathepsin- indeed the physical dimensions of degradation products may be very important in determining the physical and survival characteristics of any particular tissue.

Although the extracellular degradation of intercellular matrix by tissue enzymes may result in non specific loss of mechanical and biophysical properties, per se, the mode of depolymerization of the intercellular polysaccharide network will result in specific degradation products which are themselves likely to effect specific biosynthetic consequences on the maintenance of tissue integrity. Indeed the characteristics of partially degraded polysaccharide network (by carbohydrase, or by protease) may have relevance to the proposal that the aetiology of degenerative diseases such as periodontal disease may involve controlled rates of diffusion of extracellular enzymes derived from oral microbiota and their subsequent action on specific substrate in the intercellular matrix of the gingivae. (SCHULTZ-HAUDT, 1958; THONARD & SCHERP, 1962; WIEBKIN, BARTOLD & THONARD, 1983).

As recorded earlier in this Chapter, the effects of a carbohydrase on the in vitro incubation of gingival slices as well as incubation with a protease, as represented by hyaluronidase and trypsin respectively, were observed.

High concentrations of testicular hyaluronidase (30TRU/ml) caused very great tissue disruption. Since some CPC precipitable radio labelled material was observed, despite its general dispersion only incomplete depolymerization of newly synthesized macromolecular intercellular substance can be supposed. At lower concentrations of hyaluronidase tissue disruption was less marked and label appeared to be more confined to intercellular locations. There was therefore a pool of macromolecular material which was less susceptible to degradation.

The reasons for investigating the effects of trypsin in the medium on (^{35}S)-sulphate incorporation into gingival slices was based

on the fact that the GAG components of the intercellular matrix would remain intact in the presence of trypsin whilst the spatial and stoicheiometric relationship of those GAGs to the cell surface receptors may be disrupted due to the loss of protein specific binding. Comparisons of the autoradiographs of gingival slices incubated with trypsin and with hyaluronidase revealed that the secretion and biosynthetic incorporation of the (^{35}S)-sulphate into intercellular macromolecules was influenced differently.

Low concentrations of trypsin appeared to have no marked effect on the initial (^{35}S)-sulphate uptake and incorporation, since the inclusion of the enzyme in the 'pulse' incubation resulted in autoradiographs similar to control incubations. Higher concentrations appeared to cause reduced radioactivity throughout the whole tissue. Label was noted intracellularly in prelabelled gingival slices which was 'chase' incubated in the presence of trypsin. Where low concentrations had been used, some nidi of intense metabolic activity, as evidenced by intercellular localization within the epithelium, were observed. This indicated a heterogeneity of the tissue structure in terms of either diffusive capacity of the enzyme or of cellular response to the action of the enzyme.

The effects of specific enzyme treatment on GAGs (or PG) in live gingival tissue pose at lease two fundamental questions;

I. What is the validity of 'specific' substrate elimination with 'specific' enzymes in histochemical studies (eg. THONARD & SCHERP,1962) using fixed non vital tissue?

II.What are the biological significances of the diffusion of exogenous enzymes in controlling cell metabolic products ?

Some responses might be;

I. Although the removal by enzymes of certain stainable substrates

from histological sections would strongly imply a localized identification of a specific substrate, the non susceptibility to enzyme attack of a substrate may indicate that:

a) inaccessibility of the enzyme active site or of the appropriate cleavage sites. Such stoicheiometric properties may be explained by the conformational changes which result from the 'active' diffusion characteristics of flexible linear macromolecules of the intercellular matrix in contrast to denatured 'fixed' molecules.

b) insufficient cleavage to permit elution of those 'fixed' substrate residues from the tissue before staining.

c) the requirement of complementary enzymes or activations to effect appropriate digestion; eg.lyzosomal enzymes, cathepsin etc...

d) non activation of the enzymes themselves.

e)the possible inactivation of the enzymes by tissue inhibitors; eg. cartilage protease inhibitor (KEUTTNER, 1977).

In the event of any of these events occuring, false positive staining will remain in the tissue.

II. The most apparent difference in the biological action of the two enzymes was that hyaluronidase caused morphological disruption of epithelial tissue whist trypsin caused metabolic and secretory disruption. Indeed the PG content of the intercellular matrix appears to represent a balance between the effects of biosynthesis and the effects of degradation. The ingress of an exogenous matrix specific enzyme will influence that balance by degrading the matrix, depositing degradation products and stimulating resynthesis by constituent cells. The depolymerized matrix and the newly secreted remodelled replacement polymer will exhibit their matrix specific diffusion characteristics

which may or may not further enhance the rate of diffusion of enzymes (or other solute) through the network. Thus, far from being a passive ground substance, the intercellular macromolecular polysaccharide matrix plays an important regulatory role in maintaining tissue integrity. It is important that the biosynthetic reactivity to the effects of extraneous enzymes is appropriate to stem further degradation. Perhaps the evidence quoted for the effect of a protease implies that tissue can recover from cleavage to the protein core better than from depolymerization of the carbohydrate. Indeed the characteristic effects of different GAGs on the synthesis and secretion of intercellular material in vivo would emphasize that any depolymerization of the GAG may be diagnostic of continued tissue degradation and contrary to restitution of tissue integrity.

CHAPTER V

CHARACTERIZATION OF PROTEOGLYCANS FROM GINGIVAL EPITHELIUM.

1.0 INTRODUCTION.

Biochemical analyses of digests of suspension cultures of gingival epithelium have revealed uronic acid-containing macromolecules some of which are sulphated to varying degrees (WIEBKIN & THONARD, 1968, 1969, 1970).

Quantitatively, the proteoglycans of epithelium appear to be the major species of macromolecules in the intercellular substance (SCHULTZ HAUDT, 1958). Since this intercellular substance plays an integral part in tissue integrity (THONARD & SCHERP, 1962; PAGE & SCHROEDER, 1976), further studies have been carried out which characterize some of the properties of these molecules. The results which follow show that epithelium dissected from human gingivae excised at operation and incubated in nutrient medium without serum is capable of synthesizing PG, some of which will interact with HA. In so doing, they become large enough to be excluded by gel exclusion chromatography of Sepharose 2B-CL.

2.0 MATERIALS & METHODS.

Powdered ingredients of Hanks' BSS, Leibovitz L-15 nutrient medium* and gentamycin were supplied by Flow Laboratories, Scotland, U.K. Isotopically labelled compounds were supplied by the Radiochemical Centre, Bucks., U.K. Sepharose 2B and Blue Dextran 200 were purchased from Pharmacia (South Seas) Pty. Ltd., North Ryde, New South Wales, Australia.

Umbilical cord HA was obtained from Sigma Chemical Co., Mo., U.S.A. Scintillation fluid (toluene: 2- methoxyethanol 3:2 v/v) contained per litre, 80g of napthalene and 4g of 2,5-bis-(5-t-butyl benzoxazol - 2-yl) thiophen. Trasylol was supplied by Bayer Pharmaceuticals, Haywards Heath, Sussex, U.K.

Leech hyaluronidase was supplied by Biotrics Inc., 24 Beck Road, Arlington, Mass., U.S.A.

Tissue Source.

Small pieces of human gingival tissue clinically free from inflammation, 2-3 mm thick were obtained from gingivectomy specimens. The tissue was dissected under a microscope (x10) into small pieces containing predominantly either epithelium or connective tissue. The pieces from these two tissue types were initially washed in Hanks' BSS and incubated at 37°C in Leibovitz L-15 nutrient medium without serum supplements but with 50 ug/ml of gentamycin for 2-3h. They were then transferred to nutrient medium containing both 20μCi of Na (^{35}S) O_4/ml and 10μCi of (^{3}H) acetate/ml, and incubated further. After 24h, the tissue pieces were separated from their incubation medium by low speed centrifugation (150g) and washed once. The tissue pieces were extracted in 4M guanidinium chloride in 0.5M sodium acetate, (pH 5.8) by gently stirring for 48h at 4°C. A mixture of protease inhibitors was added to this extraction step i.e. 1mg soya bean trypsin inhibitor and 0.1ml of Trasylol (1000 Kallikrein Inactivation Units) per litre of a solution of 0.01M EDTA/0.1M 6-amino hexanoic acid/ 0.005M benzamidine hydrochloride.

Extraction and Fractionation.

The macromolecular material thus extracted from gingival epithelium and separated on Sepharose 2B-CL in 0.5 sodium acetate, contained uronic acid, was sulphated and could be precipitated by 1% cetylpyridinium chloride in 0.05M NaCl. The guanidinium chloride extracts were then dialysed against two changes of 19 vols, of distilled water at 4°C. Where slight precipitation occurred, supernatants were separated by centrifugation at approximately 3000g. The supernatants were concentrated by vacuum dialysis and adjusted to 0.5M sodium acetate (pH 5,8) or were lyophilized. Solutions of these extracts in sodium acetate (0.2 ml containing up to 4500 d.p.m. of (^{35}S)-sulphate) were supplied to the tops of columns (30cm x 0.9cm) of Sepharose 2B-CL. The columns were eluted with 0.5M sodium acetate at flow rates of about 4ml/h. Fractions of not more than 0.2ml were collected. Aliquots of either 10ul or 40ul of each fraction were taken for the determination of radioactivities.

Columns were characterized by elutions of standard HA, Dextran Blue 2000, (^{35}S)-sulphate and by standard preparations of PC extracted from laryngeal cartilage and purified by density gradient centrifugation under associative and dissociative conditions (WIEBKIN, HARDINGHAM & MUIR, 1975).

The material represented by fractions at the void volume was pooled and concentrated by vacuum dialysis and adjusted to 4M guanidinium chloride. This was then applied to a column (30cm x 0.9cm) of Sepharose 2B-CL and eluted with 4M guanidinium chloride in acetate (pH 5.8) at a flow rate of about 4ml/h. Since much of the (^{35}S) - sulphate and a proportion of the (^{3}H) -acetate radioactive counts were quenched by 4M

guanidinium chloride, the determination of radioactively profiles relied on tritium values. Fractions representing partially retarded material were pooled, concentrated and readjusted to 0.5M sodium acetate. This was then applied to a column equilibrated with sodium acetate. The retarded material was collected, concentrated to about 0.25ml and mixed with 10µl of HA (2µg/ml). The mixture was further fractionated on the same Sepharose 2B-CL column with sodium acetate. The radioactivities of the fractions eluted were measured and the resultant profiles compared with those produced from the previous separation, i.e. without HA. In a further series of experiments, material extracted with 4M guanidinium chloride from four prelabelled human gingival epithelial specimens was pooled. Using the procedure cited earlier, the extract was dialysed against distilled water, concentrated and readjusted to 0.5M sodium acetate. This material was fractioned sequentially on Sepharose 2B-CL in 0.5M sodium acetate, and in 4M guanidinium hydrochloride as described above. The partially retarded material was concentrated to 1ml, divided into two aliquots of 0.25ml and one of 0.5ml. One of the smaller samples was mixed with 10 ml HA (2µg/ml) and fractioned as previously described. The largest sample (0.5ml) was applied to a Sepharose 2B-CL column equilibrated and eluted with 0.5M sodium acetate. The material representing the retarded fraction was vacuum dialysed, then dialysed against 0.5M sodium acetate and again applied to the same column. This procedure was repeated twice.

The remaining 0.25ml aliquot was treated with Leech hyaluronidase; 0.25ml of a solution of 0.7 µg/ml leech hyaluronidase was added to the sample, followed by 3 further additions at 30 min intervals to

replenish the enzymatic activity. The total mixture was then applied to a Sepharose 2B-CL column and eluted with 0.5M sodium acetate. Fractions (0.2ml) were collected and 10 ul of each tested for radioactivity. The retarded material was pooled, dialysed, concentrated to 0.25ml and finally chromatographed on the Sepharose 2B-CL column with 0.5M sodium acetate. The activity of the leech hyaluronidase was established by demonstrating an abolition of interaction between HA and cartilage PGs.

3.0. RESULTS AND DISCUSSION.

3.1. Intercellular Substances in Gingival Epithelium.

The intercellular substance of gingival epithelium consists predominantly of PGs and glycoproteins (SISCA et al., 1971; THONARD & WIEBKIN, 1973) there being little or no collagen. In the pathogenesis of periodontal disease there is a loss of connective tissue integrity and the disappearance of the intercellular substances of the crevicular epithelium (PAGE & SCHROEDER, 1976). The presence of oral bacterial or tissue enzymes acting on the substrate in the intercellular location has been suggested as a possible factor in the aetiology of periodontal disease (THONARD & SCHERP, 1962). Studies on suspension and monolayer cultures of either gingival or amniotic epithelium indicated that cell aggregation correlated positively with the degree of sulphation of intercellular PGs and that HA synthesis was limited in cultures where there was reduced cell/cell contact (WIEBKIN & THONARD, 1969).

Radioactively labelled macromolecular material extracted from small pieces of gingivae (predominantly epithelium) under dissociative conditions was chromatographed on Sepharose 2B-CL with 0.5M sodium acetate. The elution profile (Fig, V-1a) shows a distribution of labelled material in which about 17% of the total tritium counts and 18% of the (^{35}S) -sulphate counts were excluded from the gel. When the fractions containing this large molecular material (E) were pooled, concentrated and reseparated on Sepharose 2B-CL under dissociative conditions in 4M guanidinium chloride, 80% of its total radioactivity (tritium) was partially retarded (R_{1_D}) by the gel (Fig. V-1b). This material (R_{1_D}) behaved similarly when further eluted with 0.5M sodium acetate from the same Sepharose 2B-CL column (Fig. V-1c). However, a proportion of the radioactive label eluted at the void colume (Vo) (22%) and was not included in further characterization. When HA was mixed with partially retarded material (R_{1_A}), up to 26% of the tritium and 37% of the (^{35}S) -sulphate appeared at the void volume of an elution from Sepharose 2B-CL under associative conditions (Fig. V-1d). Omission of protease inhibitors in the extraction and elution procedures resulted in the appearance of radioactive label distributed throughout the retarded portions of the profiles.

High molecular weight material similarly extracted from the underlying connective tissue, and subjected to gel exclusion chromatography, was not dissociated by 4M guanidinium chloride

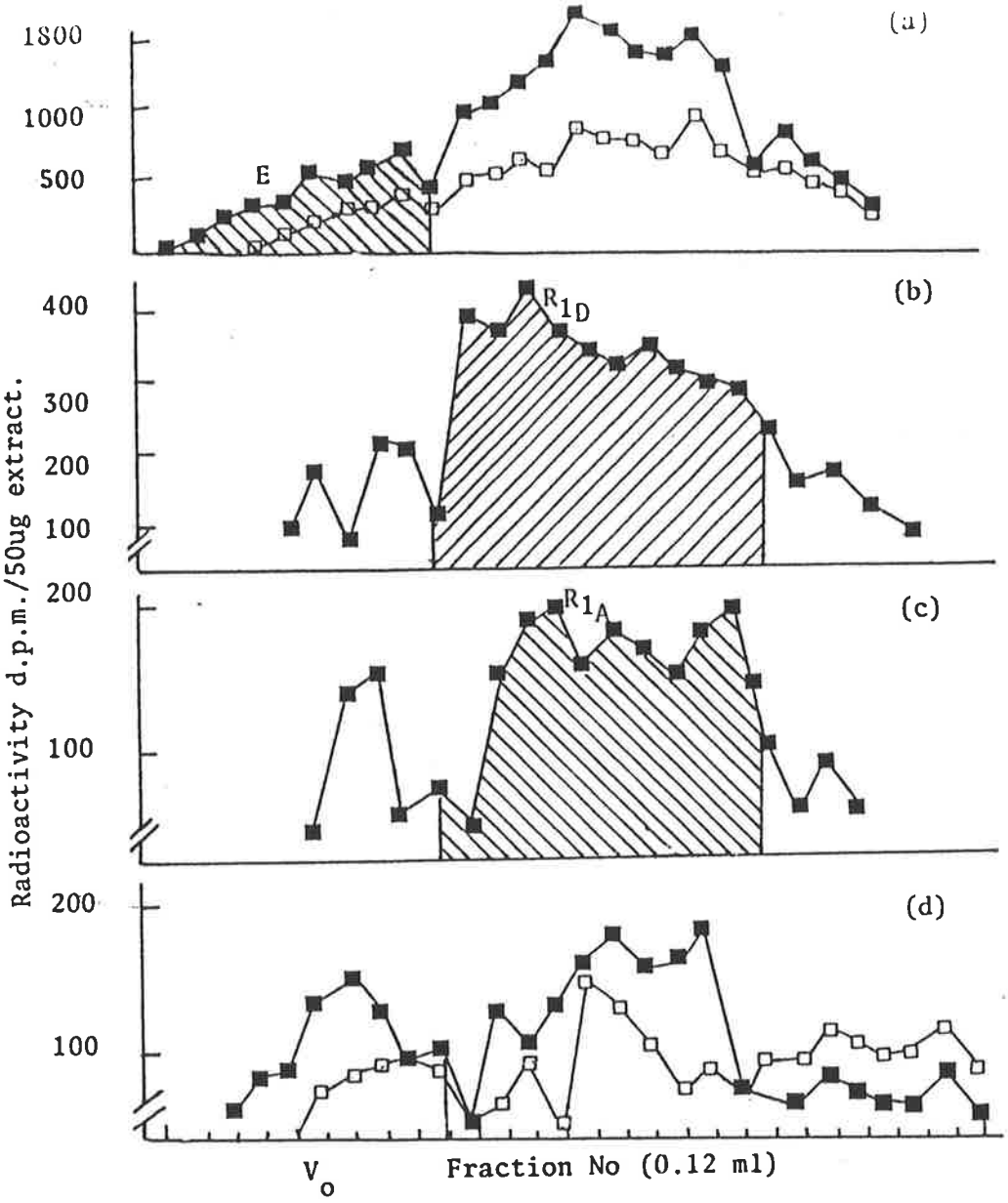


Fig. V-1

Gel chromatography of material extracted from pieces of gingival epithelium. Tissue was incubated for 24h in nutrient medium containing (^{3}H)- acetate and(^{35}S)0_4^{2-}. Macromolecular material extracted with 4M-guanidinium chloride was applied to a column of Sepharose 2B-CL and eluted with 0.5M sodium acetate at pH 6.8 (a). The excluded peak (e) was then fractionated under dissociative conditions in 4M-guanidinium chloride at pH 5.8 (b). The partially retarded material (R_{1D}) of (b) was further separated under associative conditions in 0.5M sodium acetate (pH 6.8) (c) and its retarded material (R_{1A}) was mixed with HA and finally fractionated under associative conditions (d).

(^{35}S)0_4^{2-} □——□ (^{3}H)-acetate ■——■

Column size (30cm x 0.9cm); fraction size 0.12ml.

and remained in the void volume in the absence of HA, (Fig. V-2). If the R_{1A} fraction was again concentrated by vacuum dialysis and further fractionated on Sepharose 2B-CL under associative conditions less than 5% of the radioactive counts appeared at the Vo. Subsequent chromatographic separations of the retarded material on Sepharose 2B (R_{1A}) revealed no labelled material at the Vo. The initial chromatographic elution (Fig. V-1) separated all the self-aggregated material from the totally aggregatable fraction which had been extracted with 4M guanidinium hydrochloride. Similar elution profiles were obtained from chromatographic separations of material from which HA had been specifically removed following exhaustive digestion with leech hyaluronidase.

The previous results reported herein and elsewhere indicated that the ratio of tissue HA to total PGs in cultures of gingival and amnion epithelial cells appeared to be governed by and correspond to the microscopic development of the culture, namely, the extent of cell/cell contact (WIEBKIN & THONARD, 1968, 1969, 1970). Control of synthesis and secretion of PG by epithelium may be regulated by a physico-chemical interaction with HA (HARDINGHAM & MUIR, 1972, 1973), similar, but not necessarily identical to that described by WIEBKIN & MUIR (1975) and WIEBKIN, HARDINGHAM & MUIR (1975) for cartilage cells.

3.2. Aggregatable Proteoglycan.

The present results indicate that associated proteoglycans of large molecular size ($\leqslant$ 500,000) (Fig. V-1a) can be dissociated (Fig.V-1b) and that not more than 20% (as determined by tritium incorporation) of them readily self-aggregate (Fig.V-1c). Enzymatic degradation

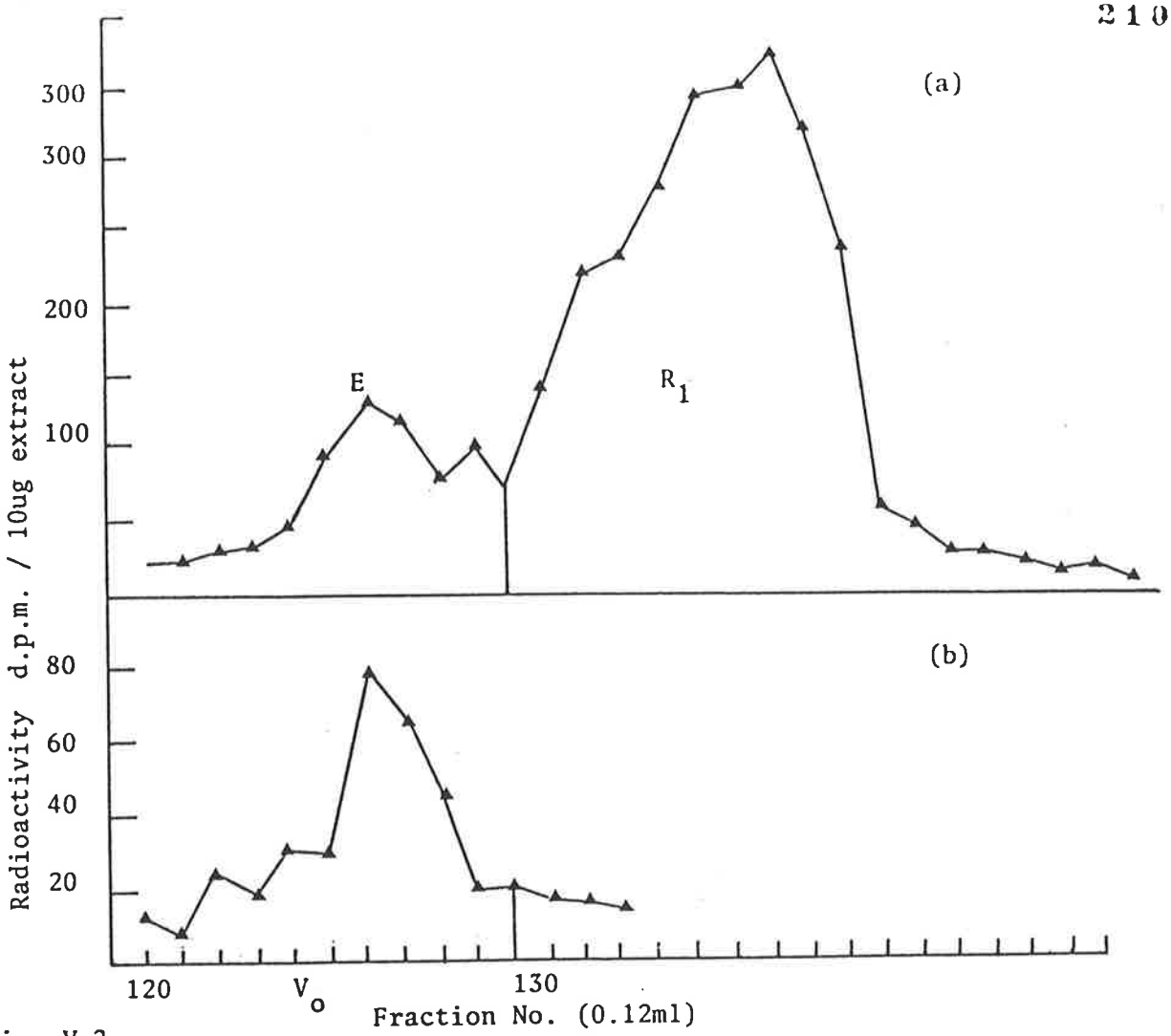


Fig. V-2

Gel chromatography of macromolecular material extracted from gingival connective tissue. Gingival connective tissue was incubated for 24h in nutrient medium containing (^{3}H) -acetate and extracted with 4M-guanidinium chloride This material was applied to a column of Sepharose 2B-CL and eluted with 0.5M sodium acetate (a). Material eluted at the void volume (E) was further separated in 4M-guanidinium chloride (b).

(^{3}H) - acetate ◄——►

Column size (30cm x 0.9cm); fraction size 0.12ml.

of residual hyaluronic acid, or the repeated concentration of the R_{1D} fractions did not result in further self aggregation of that fraction. Nevertheless, the return of those proteoglycans which do not self aggregate to the void volume of a Sepharose 2B-CL separation in the presence of HA under associative conditions indicated that these molecules had the capacity to aggregate with HA.

Further, radioautographic studies by WIEBKIN & THONARD (1983) have shown inhibition of both the synthesis and the localization of sulphated PGs by gingival epithelial slices incubated in the presence of HA. THONARD & SCHERP (1962) were unable to remove all intercellular PG from fixed histological sections of gingival tissue by testicular hyaluronidase. The dramatic loss of tissue integrity when hyaluronidase was added to gingival epithelial organ cultures has been described (Chapter IV). In contrast to the effects observed on fixed tissue matrix by particular enzymes, the turnover of intercellular material by metabolizing tissue would provide for cryptic enzyme cleavage points to be intermittently exposed. Evidence that gingival epithelial PG are capable of forming both intercellular aggregates with HA and self aggregated material (Fig. V-1), accords with the view that, due to the stocheiometry of these aggregates, enzyme cleavage points remain cryptic in epithelium in the early stages of periodontal disease. Such PG aggregates have not been identified in the underlying connective tissue which is more susceptible to loss of tissue integrity in disease (Fig. V-2). Elucidation of the fundamental differences between the aggregatability of various intercellular PG may also lead to a better understanding of the interaction of epithelium and connective tissue under both normal and pathological (e.g.) neoplastic conditions.

CHAPTER VI

FINAL DISCUSSION AND PROGNOSTICATIONS.

Conceptual Retrospection.

In the absence of our current knowledge on the chemistry of extracellular matrix components which has been accumulating over the past 20 years, GROBSTEIN in 1954 described the matrix as a"labile intercellular continuum, locally alterable in penetrability....endowed with a high degree of specificity ....providing (complex bio-physicochemical) boundaries and interfaces...".
In spite of this assertion, the simplistic view that the intercellular substances of the epithelium, particularly of the gingivae, merely functioned as a cement, was and still is to be found in the literature. Indeed since that time little more than GAG analysis of the gingivae as a whole has been investigated in this regard.

Several classes and species of macromolecule can be selectively extracted or digested from epithelium, some of which have been described in the previous Chapters. Such heterogenicity of molecular structure allows for a wide range of supra-molecular patterns with the potential for high chemical specificity and hence would be coded for a large amount of information content (GROBSTEIN 1974). Thus, GROBSTEIN emphasized that there are two general kinds of intercellular matrix effects: one being physical in emphasis, the matrix being both a substrate and a biologically defined factor in cell cohesiveness; while the other is biochemical in emphasis. This would affect cell metabolism and tissue maintenance.

Variability in Supramolecular patterns: the significance to gingival epithelium and to periodontal disease.

Examples of supramolecular conformations of gingival PG have been investigated. In a recent study the relative extractability in increasing ionic concentrations of PG labelled with (^{35}S)-sulphate from cultures of gingival tissue revealed that several PG pools could be characterized by their susceptibility to dissolution in increasing salt concentrations (WIEBKIN, BARTOLD,YELLAND & THONARD, 1980). Moreover, these differences did not bear direct relationship between molecular weight (gel exclusion) and ionic strength of the aqueous extractant. (BARTOLD, WIEBKIN & THONARD,1982).

Proportionately, greater amounts of newly synthesized (^{35}S)-material requiring 4M guanidinium chloride could be extracted from the epithelium than could be extracted from connnective tissue under similar conditions. Furthermore, a greater percentage of the intercellular component could be extracted under associative conditions by $CaCl_2$ from connective tissue than from the epithelium in respect of the total extractable labelled material. The amount of larger molecular weight macromolecules was greater in the epithelium than in the connective tissue - with the connective tissue having more of the smaller molecular weight, sulphated component.

The differences between the molecular classes based on mol.Wt.(gel chromatography) and conformational nature (relative extractability) of macromolecules in epithelium as opposed to those in the underlying connective tissue, together with their relative proportions, further highlight the importance of the intercellular matrix in chemically effecting the biosynthetic regulation of constituent cells.

The significance of the results becomes relevant when considering the pathogenesis of human disease. Histological observations on periodontally diseased tissue indicate a breakdown of connective tissue and changes in the integrity of the epithelium (STAHL,SANDLER & SUBEN, 1958; QUINTARELLI,1960; MELCHER, 1967). Early workers in this area such as SCHULTZ-HAUDT & LUNDQVIST, 1962 , believed that the progression of periodontal disease was via the sulcular epithelium (Fig.VI-1) to the underlying connective tissue because "the sulcular epithelium is thin and not keratinized. It forms, therefore, per se, no formidable barrier against penetration by bacterial products or invasion of bacteria". However, this has never been conclusively shown to be the case. A current consensus of opinion (e.g. McDOUGALL, 1971,1972) maintains that the initiating stimulus for periodontal disease arises in the sulcus and permeates to the connective tissue via the junctional epithelium - this implies that the sulcular epithelium remains relatively intact during the early stages of periodontitis. Hence the nature of the epithelial extracellular matrix and its susceptibility to degradation could be important in determining the early stages of periodontal disease ,(Fig.VI-1).

Data obtained in the course of this work indicate that biochemical differences between the intercellular matrices of gingival epithelium and connective tissue, especially the molecular size of the constituent PG, may be significant in maintaining tissue integrity. Since the extracellular non-fibrous macromolecules of the connective tissue are smaller than molecules of a similar molecular species in the epithelium, their stoicheiometry could be less complex. Hence, the connective-tissue matrix would be more susceptible to bacterial and endogenous enzyme degradation and to inflammatory cell

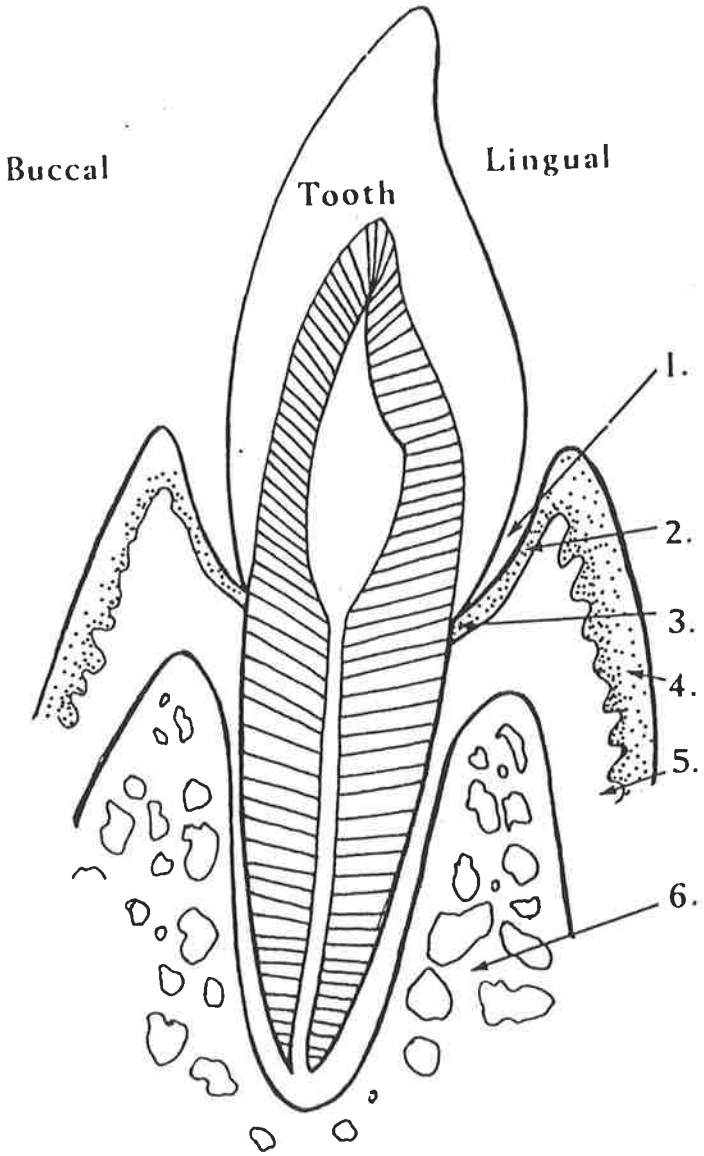


Fig. VI-1.

Anatomy of the periodontium. A vertical section through a tooth and its supporting structures.

1. Gingival Sulcus
2. Sulcular Epithelium
3. Junctional Epithelium
4. Oral Epithelium
5. Connective Tissue
6. Alveolar Bone

invasion than the more concentrated and complex network of the epithelial extracellular matrix.

Previously, experiments which have been described earlier in this thesis and elsewhere (WIEBKIN & THONARD 1977; WIEBKIN, BARTOLD & THONARD 1979), had foreshadowed the emphasis on the conformational and interactive nature of gingival PG. At least two PG species extracted from gingival epithelium under dissociative conditions can form massive molecular aggregates. One preparation required HA for aggregation, while the other fraction readily dissociated in the absence of HA ("self" aggregates).

The Fine Chemistry of PG Structure.

It is disappointing to note that, despite the exciting advances made in the structural studies of intercellular matrix components within the last decade, the state of knowledge about gingival and periodontal tissue PG is still lacking. BUTLER (1975), LIMBACK, SODEK & BRUNETTE (1978) and others have continued to investigate the collagenous components of the periodontium while DZIEWIATKOWSKI, LAVALLEY & LAVALLEY (1977) have emphasized both the functional and structural importance of PGs, as opposed to the GAGs, by describing the respective molecular sizes estimated on the ultracentrifuge and on gel chromatography. Although, more recently, EMBERY, OLIVER & STANBURY, (1979), have compared PGs and GAGs in inflamed and non inflamed gingivae the research emphases have nonetheless generally concentrated on the isolation of GAGs from whole gingival tissue (eg. CIANCO & MATHER, 1971; HIRAMATSU, ABE & MINAMI, 1978 and SAKAMOTO, OKAMOTO & OKUDA, 1978).

Following on from the earlier work described in this thesis, collectively we have carefully characterized the proportions of specific GAGs in epithelium and connective tissues separately (BARTOLD, WIEBKIN & THONARD,1981). The molecular weights of the sulphated GAGs are similar for the two gingival tissue types (BARTOLD, WIEBKIN & THONARD, 1982), whilst the HA is twice as large in the epithelium as in the connective tissue. Extensive PG composition studies have also been achieved by us (BARTOLD, WIEBKIN & THONARD,1983) and for periodontal ligament by GIBSON & PEARSON, (1982). Nevertheless we know very little about the variations in the intra-molecular structure with respect to the degree of microheterogeneity of the saccharide sequences within the carbohydrate chains, (cf. hen's egg glycoprotein- NEUBERGER, 1968; CUNNINGHAM, CLOUSE & FORD, 1963), and our understanding of the integral structural role of the oligosaccharides (cf. AXELSSEN & HEINEGÅRD,1979; GREGORY,1981), is unexplored in gingival PGs. To date alternatives in the conformation of the pyranose rings of the GAGs have not been established, eg. the C_1 chair of iduronic acid is not necessarily universal in tissue specific DS chains. Iduronidase preparations appear to recognise conformational differences by preferentially selecting one electrophoretically distinct DS band from another. The selective reactivity of the enzyme and substrate, leaving some of the substrate undegraded, may be interpreted as conformational limiting, (HOPWOOD, 1979). Such stoicheiometric alternatives may have a direct relationship to the formation of co-polymers of PG containing DS or (and) ChS, by providing optimum interactive molecular structures and may be integral in elucidating a "self" aggregating DS-PG. Despite these shortcomings in our knowledge about the bulk of gingival intracellular PG, the structural role of HA in the intercellular matrix and the specific biosynthetic roles have been addressed.

High Chemical Specificity of HA in Epithelial PG Interaction: its parallels in other biological systems.

The apparently specific interaction of HA with the protein cores of some PG has been well established (eg HASCALL & SAJDERA, 1970). Furthermore, only cells of a few tissues have been shown to synthesize such PGs i.e. Chondrocytes, epithelial cells and aortic endothelial cells, (eg, WIEBKIN & MUIR; 1977; WIEBKIN BARTOLD & THONARD, 1979; WIEBKIN & THONARD, 1981).

Biologically, the interaction of small amounts of HA with the surfaces of at least some of these cell types, eg, chondrocytes, results in regulation of the secretion and subsequent synthesis of PG,(WIEBKIN & MUIR, 1973, and TOOLE, 1973). De novo cartilage nodules, derived from isolated adult chondrocytes responded poorly to macromolecular HA; the culture required pretreatment with EDTA(WIEBKIN & MUIR, 1977b). On the other hand HANDLEY & LOWTHER,(1976), have demonstrated that PG synthesis by dense multilayers of chick embryonic chondrocytes were indeed affected by HA.

The autoradiographic demonstration that HA prevented secretion of (^{35}S)-sulphate-PG by gingival epithelial cell, as well as observations that other secretory or synthetic processes could be affected by short term incubations of gingival slices with material resembling those of the extracellular matrix, were early indications that extracellular matrix components played a regulator role on the secretion and synthesis of other matrix material by cells.

In fact the responses appear to be highly specific for each of the particular extracellular materials. Indeed there is specific binding of fluorescein labelled HA* to approximately 3% of isolated gingival epithelial cells which had been cultured for at least 4 days, (Fig.VI-2). Much of this binding is transient and is blocked by preincubation in non-fluorescent HA. Where HA was observed on the cells, surface mobility was not observed. In previous studies on cell surface binding of lectins, (WIEBKIN & FAULK 1979) and fluorescent HA, (WIEBKIN, 1981) by isolated chondrocytes in suspension culture, receptors appeared to be highly specific. In respect of fluorescent HA, quantitative studies revealed that there were two HA binding sites; one was a trypsin insensitive, avid affinity receptor while the other showed only transient binding and was sensitive to mild trypsin treatment.* Binding to only the latter affected PG secretion and synthesis. These findings were in accord with earlier studies with (^{14}C)-HA (WIEBKIN & MUIR, 1975). Similar quantitation is being performed with fluorescein labelled HA and epithelial cells.

Mild trypsinization can remove uronic acid containing material from cells, (both N-sulphated; KRAMER & SMITH, 1974; and Ch-S; CARLSTEDT, CÖSTER, MALMSTRÖM & FRANSSON, 1979;) as did Cathepsin D from cultured adult chondrocytes, (WIEBKIN & MUIR, 1975). In the latter <u>in vitro</u> experiments both trypsinization and pretreatment with Cathepsin D abolished the inhibitory effect of extraneous HA on subsequent PG synthesis by chondrocytes. Thus the binding of HA to the cell surface protein cores of specifically interactive PG by those cells which synthesize that specific PG, has been suggested.

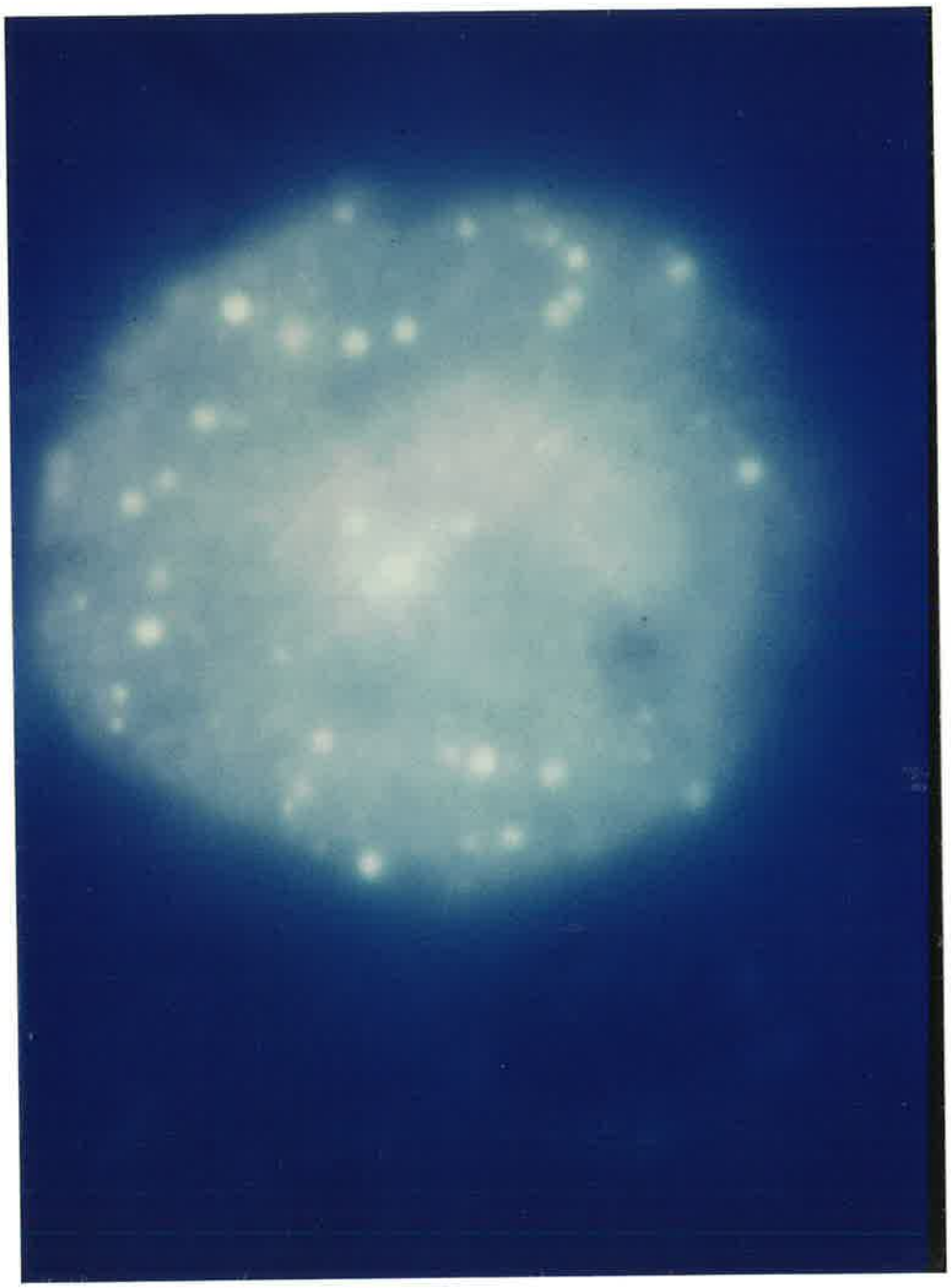


Fig. V I-2

The binding of fluorescein labelled HA* to epithelial cells isolated from human gingivae. Cells were cultured as suspensions for 4 days. No capping was observed over 12 h but some label was lost to the medium. (HA: Mol Wt = 1 x 10^6; degree of substitution of fluorescein = 1.25 disacch.)

With respect to the studies on amnion cells described in this thesis, chemical analysis showed some associated protein in the 0.5M NaCl eluants of CPC precipitated macromolecular uronate. The supposition to be further investigated is that there is an interactive role of that protein as a "link" fraction, analogous to the PG-protein cores of cartilage macromolecular PG:HA aggregates, or to the "glycoprotein links" described by GREGORY,(1973). Another possibility to explain the existence of protein in the NaCl fraction is a PG binding protein similar to that recently described by PAULSSEN & HEINEGÅRD,(1979) at the cell surface, or an acute phase reactant,(eg. haptoglobin) (SMITH,1975).

The Regulation of the Biosynthesis and Secretion of Proteoglycan by Cells in Culture.

Epithelial cells were capable of synthesizing different pools of PG which appeared to contain different species of GAG chains which themselves were sulphated to varying degrees. Such differences were related to the source from which the culture was derived and the conditions under which they were incubated; for example amnion cells, gingival cells, degree of confluence serum content of media etc.

Not only did the amount of ChS-rich intercellular material appear to be important in regulating PG synthesis, but HA levels varied as epithelial cells became associated one with another, either as monolayers (++++ rating) or as gingival epithelial cell clumps (Category III) as discussed in Chapter II.

Analogies with cell systems from other tissues, and the cell responses to extracellular influences may be relevant. For example, HANDLEY & LOWTHER (1979) have recently shown that chondrocytes were sensitive to extracellular PG sub-units. The synthesizing enzymes involved with elongation of the GAG chains and those associated with endoplasmic reticulum were reduced. Furthermore, both proteoglycanases from polymorphonuclear leucocytes,(LOWTHER,SANDY & BROWN,1979) and HA oligosaccharides, (WIEBKIN & MUIR, 1977b) were potentially capable of inhibiting PG synthesis by chondrocytes in rabbit articular cartilage or embedded within the extracellular matrix at the de novo "cartilage nodules" respectively.

More important perhaps is the fact that cartilage slices, freshly isolated from an animal and incubated in nutrient medium, initially secreted large amounts of PG with different physical properties from that which was subsequently synthesized in vitro; (after 4 days) these cells were described as activated cells,(SANDY, BROWN & LOWTHER, 1979).

The previous studies on gingival epithelial cells described in Chapter II are consistent with the concept that there are cell/tissue specific responses to microenvironmental changes. In contrast to the response by cartilage slices, the total GAGs associated with cells isolated from human gingival epithelium decreased more than 10 fold during the first 4 days of culture (Table II-6), despite some total dry weight increase of cell mass, before being replaced with newly synthesized GAGs during the ensuing 4 days. This indicated that gingival cells, subjected to culturing, were also responding to their new extracellular environment by modifying their PG synthetic processes.

SANDY et al (1979) deduced from their system, where there was up to a 5 fold increase in GAG synthesis by cartilage slices after 5 days of in vitro incubation, that the cells were responding to an initial loss of PG into the medium. The chondrocyte response was characterized by synthesis of a GAG with a two fold increase in size and an increase in the ChS/KS ratio. The quality of the GAGs which specified the gingival epithelial cell response related to (a) time in culture, (b) cell/cell contact and (c) culture condition, was observed to vary both in the levels of sulphation and in the critical electrolyte concentration required for elution for CPC precipitates. Apart from the caution needed to compare data from various experimental regimes implied by the use of these parameters, it is important to note an intrinsic spectrum of responses which cells can exhibit to their microenvironment.

The Relation Between Intercellular Material and Periodontal Disease.

The importance of establishing the PG sizes in freshly excised gingival epithelium separately from those extracted from tissue sustained in prolonged culture is thus obvious. This data, together with knowledge of the different rates of synthesis and turnover of epithelial PGs from cultured tissue excised either from healthy patients or from those with periodontal disease, it may be possible to understand the importance of molecular size, and stoicheiometric conformation in the maintenance of gingival tissue integrity. Ultrastructural studies of the gingivae as a whole have revealed that, with

respect to the extracellular matrix, the PGs of the epithelial intercellular spaces are different from those associated with the fibrous proteins of the connective tissue (Fig.VI-3). These differences underlie the different roles of the two tissues. While epithelial PGs provide an appropriate medium for the transport of proteins, nutrients, etc., between cells, in vitro studies showed that the epithelial PG of the gingivae were less susceptible to enzyme degradation than those of the underlying connective tissue matrix,(cf. Fig. IV-4 a,b& c : and Fig.IV-7).

Resistance to degradation of healthy tissue may be due to stoicheiometry of cryptic cleavage sites on those molecules which have aggregated into a protective configuration. Such an allosteric mechanism would be an advantage in the maintenance of tissue integrity in epithelium which is, of course, subjected to both bacterial and mechanical abuse. Conversely, those PG associated with the fibrous proteins of connective tissue are part of a mechanically stable structure.

Indeed such hypotheses are circumstantially supported by the various observations of incomplete substrate elimination by specific enzymes on fixed and unfixed histological sections of gingival tissue,(THONARD & SCHERP, 1962). Conversely the observations (Fig. IV-4) that viable tissue incubated in the presence of hyaluronidase, was grossly disrupted would indicate that the state of flux and fluidity of intercellular matrices provide increased opportunity for exposure and interaction of enzyme active sites with substrate cleavage sites(Chapter IV)

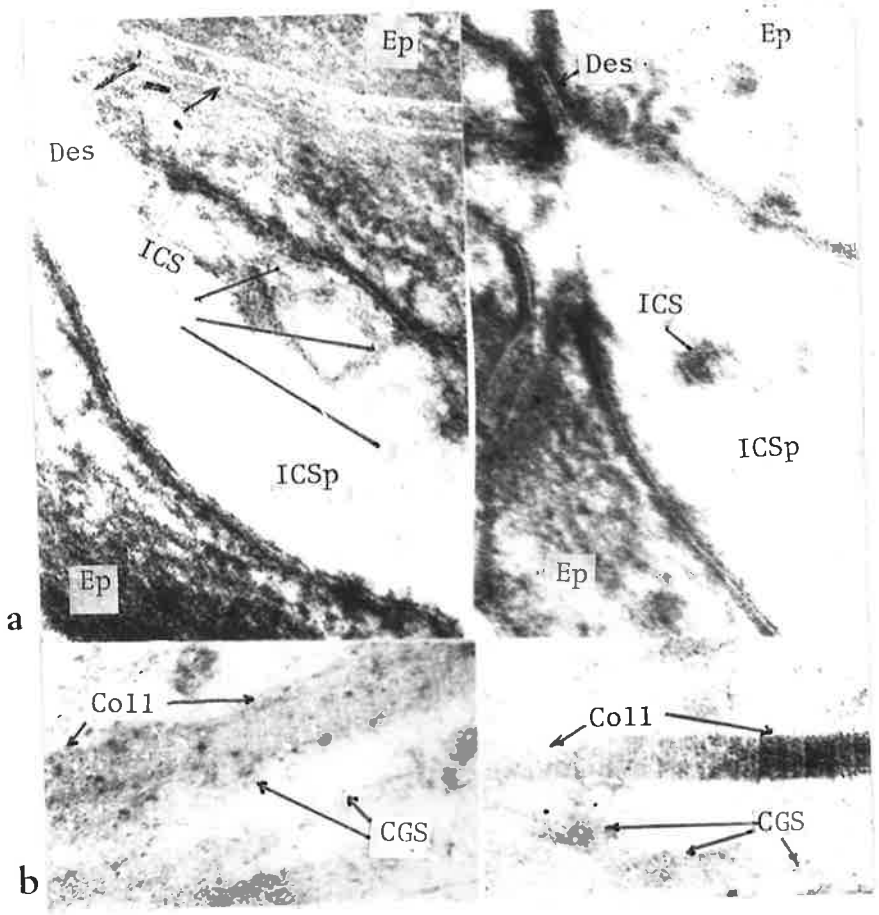


Fig. VI-3

Electron Micrograph of (a) gingival epithelial intercellular spaces and (b) gingival connective tissue matrix (Mag. x 40K).

1. Fixed: Glutaraldehyde.
2. Fixed: Glutaraldehyde/Formaldehyde.

Note: There is less loss of intercellular material from gingival epithelium in 1a. than 2a.

Ep, epithelium; ICS, intercellular substance: ICSp, intercellular space; Des, desmosome; Coll, Collagen fibre; CGS, connective tissue ground substance, (WIEBKIN, BARTOLD, YELLAND & THONARD, 1980).

Inevitably the effect of trypsin on the protein cores, of PG will be limited by the external carbohydrate character of subunit PG attached to them. Indeed some of the neighbouring lysine or arginine cleavage sites may be further protected by the conformations of the linkage region of the GAG.

In vitro degradation of some PGs with trypsin into octets of GAGs and subsequent digestion with chymotrypsin (cleavage sites at aromatic amino acids phenylalanine, tyrosine and tryptophan if any) yields doublets of GAGs. Thus the cleavage sites for trypsin between the linkage regions of GAG chains do not appear to be abundant. As shown by the protein core analysis of BARTOLD, WIEBKIN & THONARD, (1983).

Intercellular PG which will interact with HA or "self aggregate" through iduronic acid periods of DS, or bind with other components such as collagen or a matrix binding protein, (PAULSSON & HEINEGÅRD, 1979) may, under normal healthy conditions, protect the tissue as a whole against loss of integrity in the presence of degradative enzymes.

In contrast to the hypothesis that aggregated molecules of the healthy intercellular matrix are not conformationally susceptible to enzymatic degradation, it is interesting to speculate that the intramolecular components of the massive PG aggregates, such as the glycoprotein links, or the matrix binding protein of cartilage, may contain proteoglycanase activity. Specific activation of such

enzymes, or the existence of enzyme inhibitors may be diagnostic of healthy tissue. Indeed, discounting free radical depolymerization, LEVY, NEVO & FEINSTEIN (1981) have discovered what they believe to be enzyme activity embodied in the purified PG molecule itself! It is clear however, that if trypsin or some other extracellular degradative enzyme, for example cathepsin or neutral protease were allowed access to the cell surfaces, the glycocalyx would be solubilised. Cells respond to localized denuding of glycocalyx by a rapid resynthesis of uronic acid containing macromolecular material.* (WIEBKIN & MUIR, 1975).

Such intercellular matrix components synthesized under aberrant stimulation (activation), may vary in quality similarly to those synthesized under the various conditions described in this thesis or as elegantly demonstrated for activated chondrocytes in cartilage slices by SANDY et al.(1979). We may then argue that apart from the specific activation of degradative enzymes, or the existence of enzyme inhibitors, the quality of the intercellular PG network itself may be diagnostic of healthy tissue.

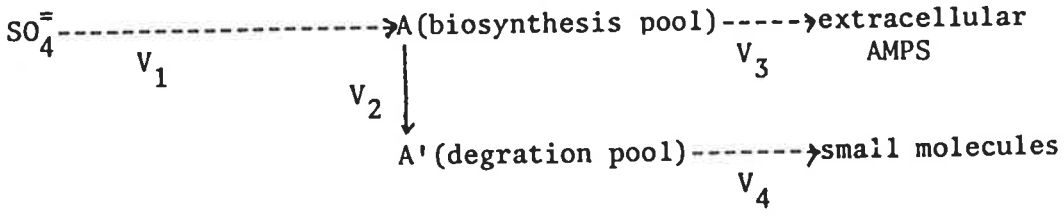
Biosynthetic Implications for Future Study.

The important implications of the in vitro GAG turnover in fibroblasts to the overall control of extracellular matrix synthesis were postulated earlier by FRATANTONI, HALL & NEUFELD, (1968). They commented that the kinetics of secretion of AMPS revealed that the bulk of the intercellular AMPS was not a precursor of secreted material. Cells continued to accumulate radio-active labelled precursors long after the rate of secretion of macromolecules had become linear, (at which time the

secretory pool would have reached constant specific activity).

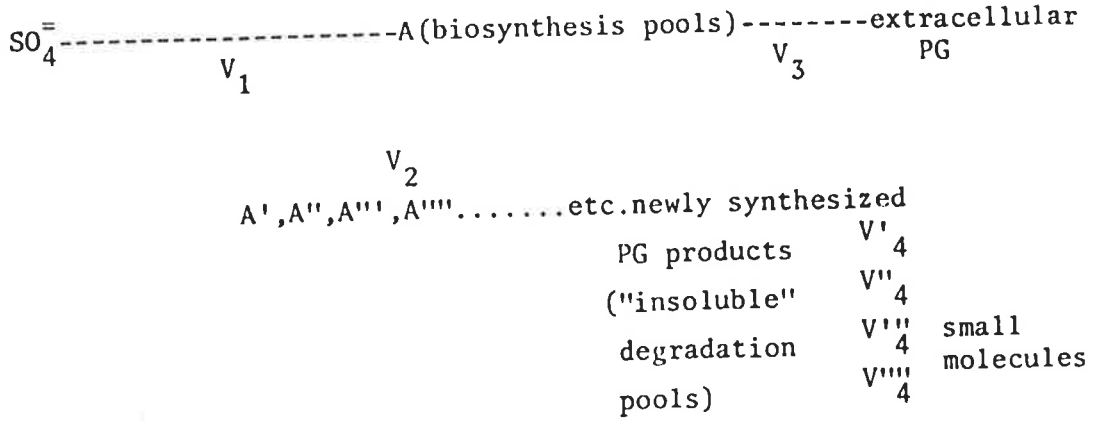
The incorporation of (^{35}S)-sulphate into materials not immediately destined for PG synthesis described in Chapter III (UDP-hexN (^{35}S), oligosacch-(^{35}S) & nucleotide(^{35}S)), may contribute in part to such incorporation into an intracellular pool. By analogy, these intracellular products are possible effectors of feedback inhibition. KORNFIELD, KORNFIELD & O'BRIEN (1964) and KORNFIELD (1967) have described feedback inhibition of N-acetyl glucosamine-6-phosphate from fructose-6-phosphate and glutamine by UDP N-acetyl glucosamine. Indeed although glutamine is an essential amido-group donor in the synthesis of GAG,(STEIGHT, HANDLEY & LOWTHER, 1978), its use may be inhibited by a number of feedback mechanisms. Moreover, STOOLMILLER & DORFMAN,(1969) have reported that UDP-N-acetyl glucosamine is interconvertible with UDP-N-acetyl galactosamine, by an equilibrium reaction, thus highlighting further the important feedback implications.

The effects of degradation products, and the intracellular regulatory mechanisms on the rates of reactions are important if we are to understand the biochemical dynamics of the intercellular matrix. FRATANTONI et al.(1968) postulated an AMPS biosynthetic scheme for fibroblasts as shown:



where V_1-V_4 represents the different rates of reaction. The A' pool may of course not be a "breakdown dump" but may contain PG moieties, their nucleotides, etc. which are never destined to be secreted as

extracellular structural matrix PGs. Indeed a scheme which includes additional biosynthetic pools, A', A'', A''', A'''',.....etc., may prove to be more meaningful in establishing the role of matrix components in controlling PG synthesis.



FRATANTONI et al.(1968) commented that the "insoluble" cell fraction of GAG may contain material destined for intracellular degradation. KRESSE,TEKOLF,von FIGURA & BUDDECKE (1975) reported that about 40% of the total pericellular GAG synthesized by bovine artery cells was pinocytosed and degraded intracellularly. It is difficult to visualize how such large hydrated molecules as PG or HA can be internalized by cells but the GAG chains following protease activity may, on association with other molecules, be pinocytosed. We have recently dissociated a protein-rich fraction from a uronic acid-rich fraction of a "self" associating pig gingival DS-PG. In the absence of the protein rich fraction and under isotonic conditions, about 80% of the DS-PG formed a highly insoluble precipitate. This material is thought be represented by the highly sulphated GAGs described in this thesis. The effect of this material on cell metabolism is to

be investigated, but does raise the question, how metabolically significant is the intercellular matrix of the gingival tissue compartments which is often quoted as an "inert, amorphous intercellular cementing substance"?

Other PG interactions may be related to tissue structure, (co-polymerization and collagen binding), to affinity binding for other metabolic activities, (cf. Hep and blood clotting) and to biosynthetic control similar to the interactive formation of HA:PG aggregates at the cell surfaces.

Circumstantially the surface interactive HA receptor sites described earlier, appeared to be represented by trypsin or cathepsin susceptible PG moieties, which are fundamental to biosynthetic control.

However, the problem of chemically separating these macromolecular pools from each other in order to understand their inter-relationships in biosynthetic control is proving to be difficult, particularly in tissues such as the gingivae which is small and anatomically multistructural.

Any criteria for future investigations of gingival PGs and their role in tissue integrity must recognise the importance of the PG as a basic structure. They must take account of the following:

1. Extracellular PGs are derived from more than one intracellular biosynthetic pool.
2. There appears to be no precursor-product relationship between soluble PG of different size.
3. Within the insoluble (limited extractablity) metabolically

idle pools, (A',A",A'",A""......), there is an intracellular PG fraction which has a rapid turnover and which is the precursor of extracellular pools.

4. There is a positive correlation between PG solubility (or extractability) and metabolic turnover; the milder the conditions required for extraction, the more rapid the turnover.

5. The longer the GAG chains (eg. ChS) are synthesized more rapidly than shorter chains, (GALLAGHER, 1977).

Indeed the implications from fundamental research, broadly based on some of these concepts will hopefully provide more defined understanding in the whole gamut of cell/matrix interactive influences.

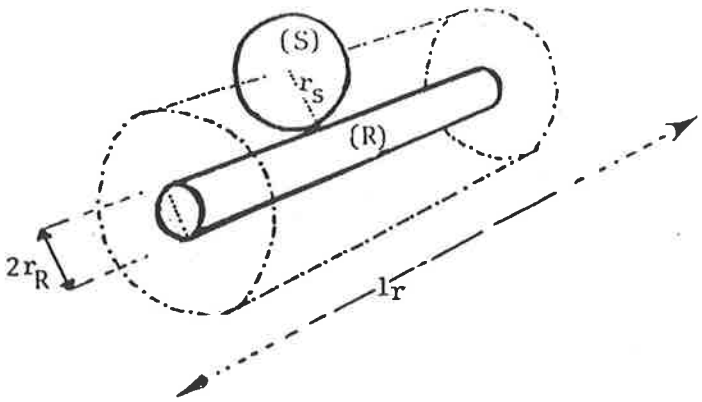
Indeed, if doing nothing else, this study has indicated that the term which is commonly used in standard texts to describe the inter-cellular matrix as "inert, amorphous cementing substance" is inaccurate and misleading. The intercellular matrix is specific and highly structured, these structures being responsible for particular dynamic responses by the cells embedded in it. In consequence of the initiating work done between 1967 and 1970 and described in this thesis, the data accumulated by our group on gingival epithelium and connective tissue intercellular matrix materials separately, endorses the hypothesis that intercellular PGs of the gingival epithelium play a fundamental role in maintaining tissue integrity or in the susceptibility of the periodontium to disease*.

APPENDIX. I.

Parenthetic notes to all items, phrases or sentences designated * are listed below according to pagination.

Page

21. Chondrocytes were isolated from adult pig larynges and cultured in suspension or as monolayers for upto 21 days (WIEBKIN & MUIR, 1977). The cells and media were recovered. Hyaluronate was solubilized from CPC precipitates of the total extracted GAG in 0.5M NaCl, assayed and expressed as a percentage of total CPC precipitable uronic acid. The lipid was extracted from the cells by suspending them (or the media) in $CHCl_3$:MeOH:H_2O, (1:2:0.8) overnight and separating an enriched $CHCl_3$ "lower" fraction by adding an additional 1vol of $CHCl_3$ and 1vol. of H_2O (this was then washed in $CHCl_3$:MeOH:H_2O (2:2:1.8) and using the "upper" layer). The lipid extracts of cells and media were spotted (in ether) onto silica thin layer plates (Keiselgel H.F. Nach Stahl Typ 60 Merck) and chromatographed in developing solvent, Hexane:Ether:Acetic acid (65:35:1). Cells from cultures which assayed between 8-10% 0.5M NaCl soluble uronic acid (HA) yielded cholesterol esters, triglycerides and free fatty acids. Cells which assayed 7% or less of 'HA' contained no free fatty acids and cells with uronic acid content representing HA of less than 1% contained no triglyceride. Some free fatty acids and triglyceride were found in all media but only trace amounts of cholesterol ester was detected in cultures of low HA values (1% in the cells). Dr M. Gurr who advised us in this work assumed that all were phospholipids.



34 The steric exclusion of a sphere by a rod

Rod-shaped particle (R) excludes a sphere (S), the largest exclusion effect (calculated as volume excluded for S per mass unit of (R) is obtained when R is very long and thin.

The centre of S cannot come closer to R than r_s i.e. its own radius. The volume excluded for the sphere S is equal to the cylinder marked with the dotted line. The cylinder has radius $r_s + r_R$ where r_R is the radius of the rod. Disregarding end effects of a rod length l_r the volume excluded for the sphere by the rod is equal to $\pi . l_r (r_s + r_R)^2$

If the density of the rod is d then its mass will be $\pi . l_r . r_R^2 d$ and the excluded volume V_{excl} per unit length of R is expressed as:

$$V_{excl.} = \frac{\pi . l_r (r_s + r_R)^2}{\pi . l_r . r_R^2 . d} = \frac{1}{d}\left(\frac{r_s}{r_R} + 1\right)^2$$

The exclusion increases with increasing radius of S and decreasing radius or increasing asymmetry of R. If a number of rods are distributed randomly throughout a space, their total exclusion of a spherical particle is also influenced by the overlapping of the exclusion regions of the individual rods. Ogston (1958) has calculated the fraction Kav, of the total volume available to a sphere of radius r_s in a space containing randomly distributed long rods of radius r_R as,

$$K_{ave} = \exp\left/ -\pi L (r_s + r_R)^2 \right/$$

where L is sum of the lengths of all rods in a unit volume. The concentration of the rods (L) and the square of the radius of sphere (r^2) are the only parameters which determine the available volume when

$$r_R \ll r_S$$

Kav, the volume fraction of a system available to a substance, has turned out to be a useful parameter for defining exclusion properties of a system. It is related to the exclusion volume by the relationship

$$V_{excl.} = \frac{1 - Kav}{c_R}$$

c_R represents the concentration of R expressed as weight per volume.

36. In discussion following a lecture by Dr. John Blackwell, Polysaccharide-polypeptide systems as models for heparin interactions. Fed. Proc. (1977) 36.101.

44. Medium 199. Supplied by the Commonwealth Serum Laboratories, Melbourne, Australia. The concentration of all components except antibiotics was as recommended by SALK, YOUNGER and WARD (1954), Amer. J. Hyg. 60:214. Anti-biotics used were normally streptomycin 100 ug/ml., penicillin 200 units/ml. Polymyxin and Neomysin B Sulphate were already included in the preparation at concentrations of 2 and 1 unit/ml. respectively.

47. Constituents of Hank's Balanced Salt Solution

	mg/litre
$CaCl_2$	140
$MgSO_4.7H_2O$	200
KCl	400
KH_2PO_4	60
$NaHCO_3$	350
NaCl	8000

47. Hank's Balanced Salt Solution (Hank's B.S.S.) Supplied by Commonwealth Serum Laboratories. Final salt concentration as recommended by HANK and WALLACE (1949). Proc.Soc. exp. Biol. 71:196.

47. Filter through Gelman Membranes which contain small amounts of detergent caused alkalinity; therefore, pH control was achieved after sterilisation.

54. Alcian Blue. Supplied by Gurr.

54. Xam. Supplied by Gurr. Polystyrene mountant.

55. Sudan Black. Supplied by Gurr.

55 Titration of potassium dichromate. Method adopted by WILLARD and FURMAN, Elementary Quantitative Analysis, pp. 246-248 , Van Nostrand Co. Inc., 1947, with diphenylamine sulphonate as indicator.

56. Hyaluronidase E.C.4.2.99.1. Testicular hyaluronidase from Sigma Chemical Corporation, Turbidity Reducing Unit estimations were carried out using bovine albumin (Fraction V) DORFMAN, A. Methods in Enzymology 1.166 (1955) and measured specific activity .01mg= 1TRU in the sample used.

56. RNAase. EC.2.7.7.16. Supplied by Sigma Chemical Corporation.

59. Nuclear Fast Red and Light Green counter staining Kernechrot 0.1 gm/ 100 mls. of 5% aqueous aluminium sulphate-stain for 45 minutes (1 crystol thymol); Light Green 0.0004 gm./100 mls - stain for 30 seconds, dehydrate, etc. and mount.

65. Keisleguhr. Supplied by Drug Houses of Australia, Anax Division. This diatomaceous earth did not bind mucopolysaccharides, but assisted the sedimentation of MPS/CPC complexes.

65. ANTONOPOLOUS et al. (1964), Biochim. Biophys. Acta. 83:1-19. The column that these authors used was attached to a manifold as demonstrated in Figure A.1 and was found to be effective for larger quantities of tissue extract separations.

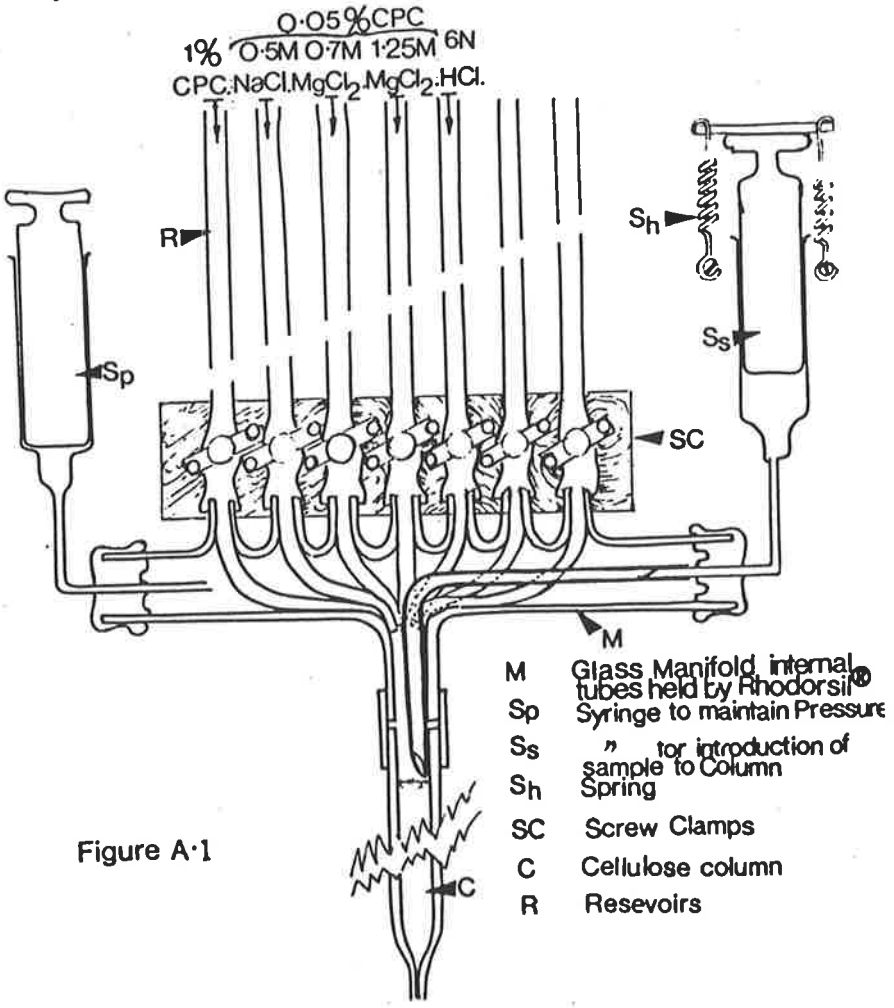
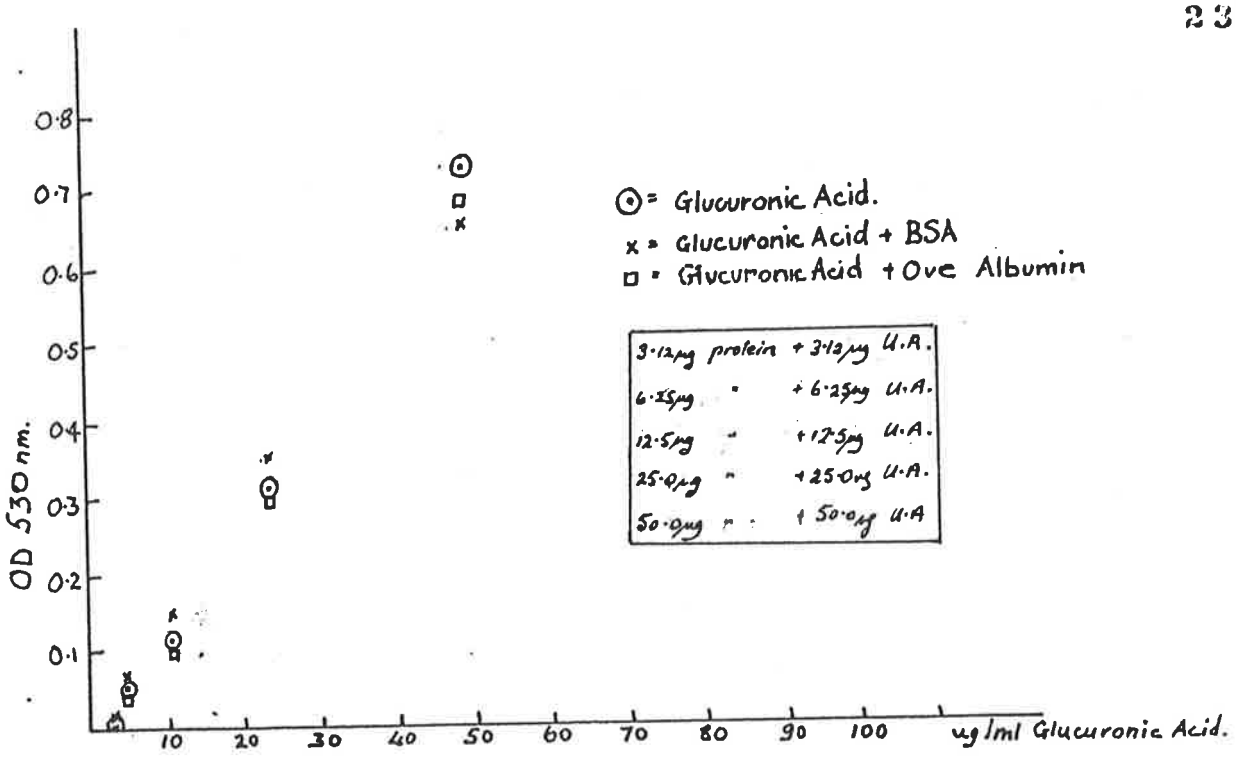
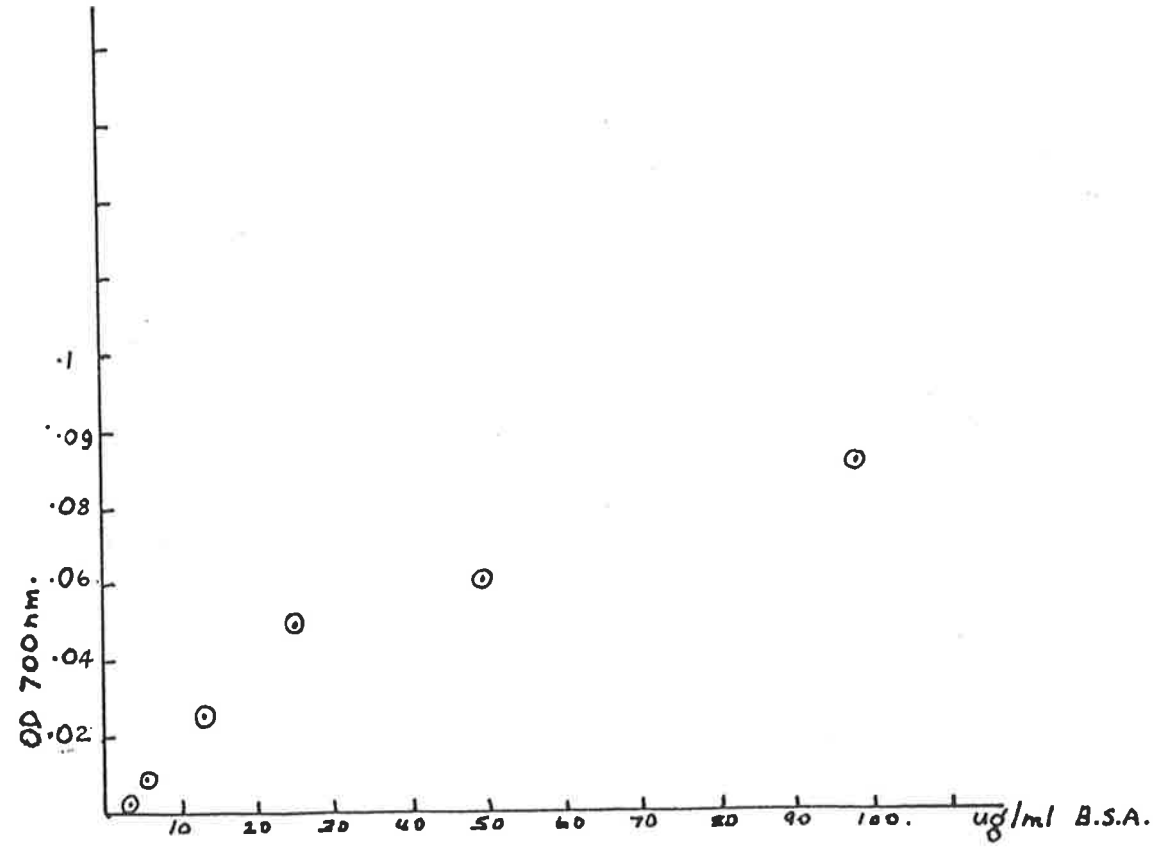


Figure A·1

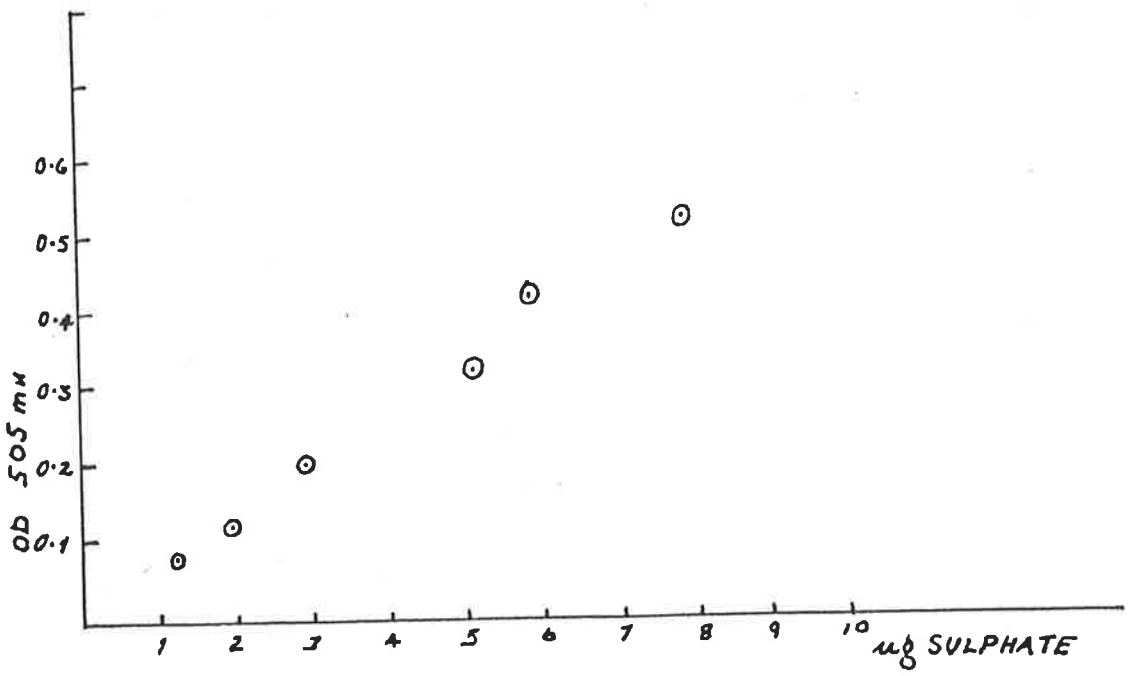
Comparison of elution from CPC/AMPS complexes by neural $MgCl_2$ ~~reveals~~ and acid $MgCl_2$ reveals that the criteria for neutral salt elution is not mol. wt., but relies on the degree of sulphation, and proportion of L-iduronic acid or D-glucuronic acid in the macromolecule (FRANSSON et al Carbohydrate Res., (1970) 15;73.).



66. Uronic acid standard curve. Figure A.2.



67. Protein standard curve. Figure A.3.



69. Sulphate standard curve. Figure A.4.

70. Sialic Acid Standard curve. See Figure A.5.

Method as follows: (a) Periodate: 25mM-per-iodic acid in 0.125N H_2SO_4 (pH 1.2); (b) Sodium arsenite: 2% solution of sodium arsenite: in 0.5N HCl; (c) Thiobarbituric acid 0.1M solution of 2-thiobarbituric acid in H_2O, adjusted to pH 9.0 with NaOH. It keeps well for about a month in a dark bottle at 4^oC; (d) Acid butanol: butan-1-01 containing 5% (v/v) of 12N HCl. A solution of the sample blank or standard (containing 5-40ug of N-acetyl neuraminic acid) in 0.5 ml H_2O is treated with 0.25 ml. of the periodate reagent for 30 minutes in a water bath at 37^o. The excess of periodate is then reduced with 0.2mls. of sodium arsenite. As soon as the yellow colour of the liberated iodine has disappeared (1-2 minutes), 2 mls, of thiobarbituric acid reagent is added and is then heated for 7.5 minutes. The coloured solutions are then cooled in ice water, and shaken with 5 mls. of acid butanol. The separation of the two phases is facilitated by the short centrifugation. Intensities of the colour in the butanol

layer are read at 549 nm, Despite the apparent discolouring on standing, the extinctions are constant for 2 hours, but they decline appreciably in 20 hours.

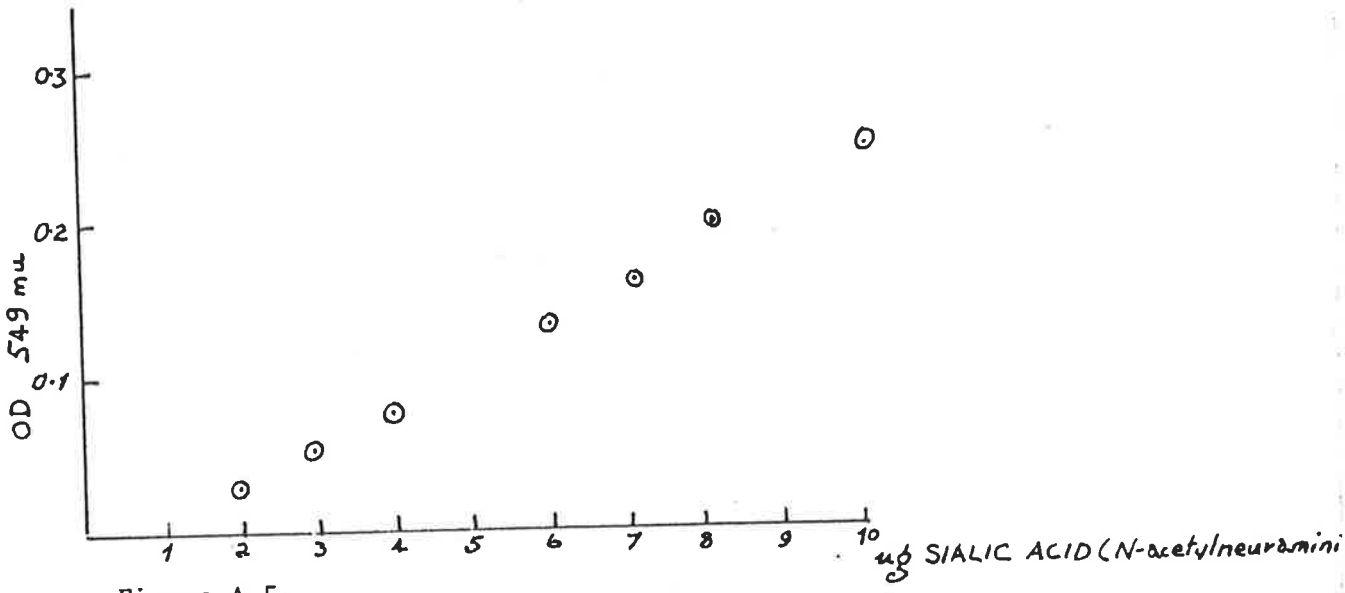


Figure A.5.

70. Electrophoresis on starch gel or cellulose acetate with Buffer - 0.01M Citric acid, adjusted with saturated NaOH to pH 3.5, cf. Brookhart, J.H., J. Chromatogr. (1965) 20:191-193.

Sepraphore III. Supplied by Gelman.

Size 30 cm. x 2.5 cm. Application was achieved with a Gelman applicator delivering 1.5ul.

108. Sialic acid in biosynthesis and transport (WAGNER and CYNKIN, (J. Biol.Chem. (1971) 264:143-151) have made the following comments.

The results of several investigations suggest that the carbohydrate units of the glycoprotein, which the non-detergent precipitable bound sialic acid may be, are assembled by the step wise transfer of monosaccharides to the completed polypeptide. Evidence indicates that the first sugar attached may be transferred directly to the ribosome bound polypeptide (MOLNAR, ROBINSON and WINZIER (1965), J.

Biol.Chem. 240:1882; LAWFORD and SCHACTER (1966), J. Biol.Chem. 241:548; HALLINAN, MURTY and GRANT (1968) Arch. Biochem.Biophys. 125:715), while additional sugar residues are being transferred, subsequent to the ribosomal stage of protein synthesis, as nascent glycoprotein travels through the channels of the rough and smooth endoplasmic reticulum, en route to excretion from the cells; perhaps via the Golgi apparatus. Thus, it is possible that the N-acetyl glucosamine, galactose and N-acetylneuraminic acid residues of the terminal trisaccharides, for example, are transferred to the glycoprotein within the golgi apparatus after the core proteins have been inserted at other sites within the E.R.

125. Radio isotope source. Supplied by the Radiochemical Centre, Amersham, Buckinghamshire, England. Sulphur-35 at pH 6-8. Tritiated acetic acid, sodium salt, specific activity 500mCi/mM purified by decarboxylation of malonic acid-T, steam distilled and filtrated with NaOH; M.W. = 82. Uridine-5-diphosphoglucose is prepared biosynthetically from glucose-^{14}C (U) and purified by ion exchange and paper chromatography; specific activity: 204mc/mH., pH 7.

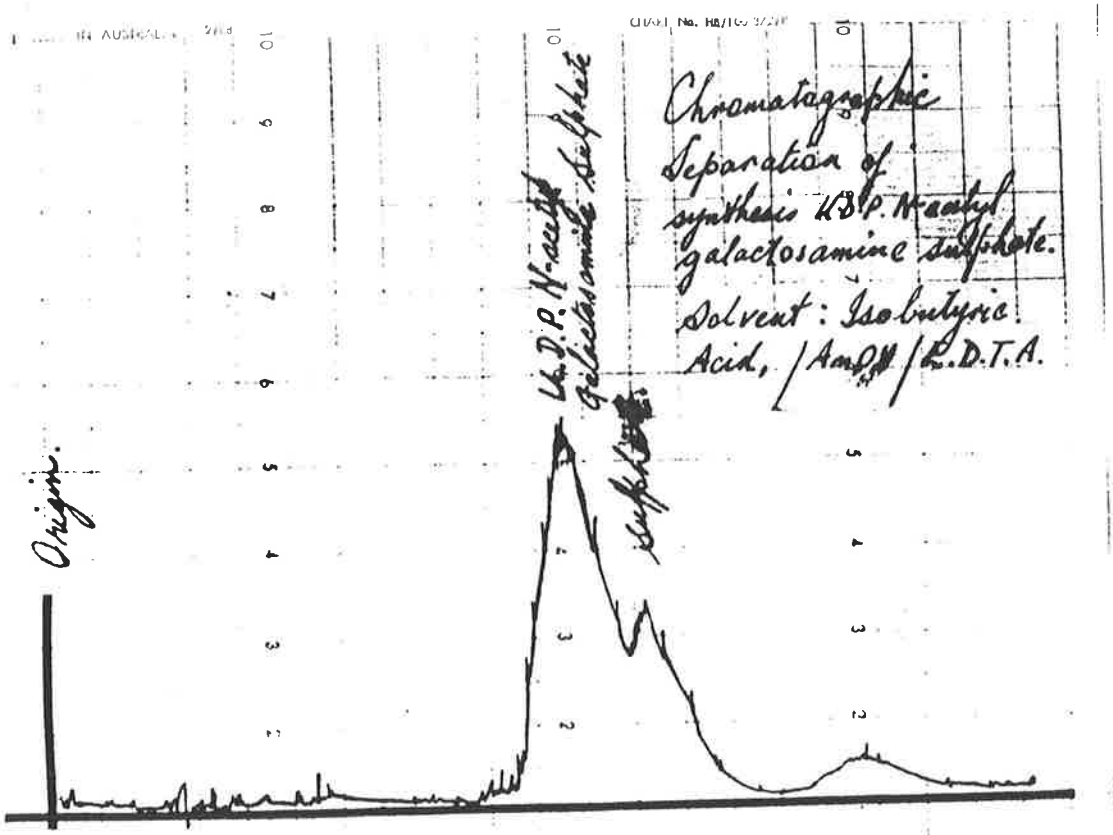


Figure A.6. Synthetically prepared Controls

128. Uridine diphospho-N-acetyl galactosamine (^{35}S) - sulphate

Synthetically prepared controls were applied to paper and chromatographed individually or in a mixture with other biosynthetically labelled material Figures A.6.& A.7.

128. 3'-phosphoadenosine 5' phosphosulphate (PAPS). ^{35}S - sulphate was prepared by the method of ROBBINS in Methods in Enzymology, ed. Colowick and Kaplan, Volume V.pp966-968, using bakers yeast, and purification was verified on high voltage electrophoresis. Fig.III-2. and Figure A.7.

128. Cellogel. Supplied by Chemetron, Milan, Italy. Size 14 cm x 2.5 cm.

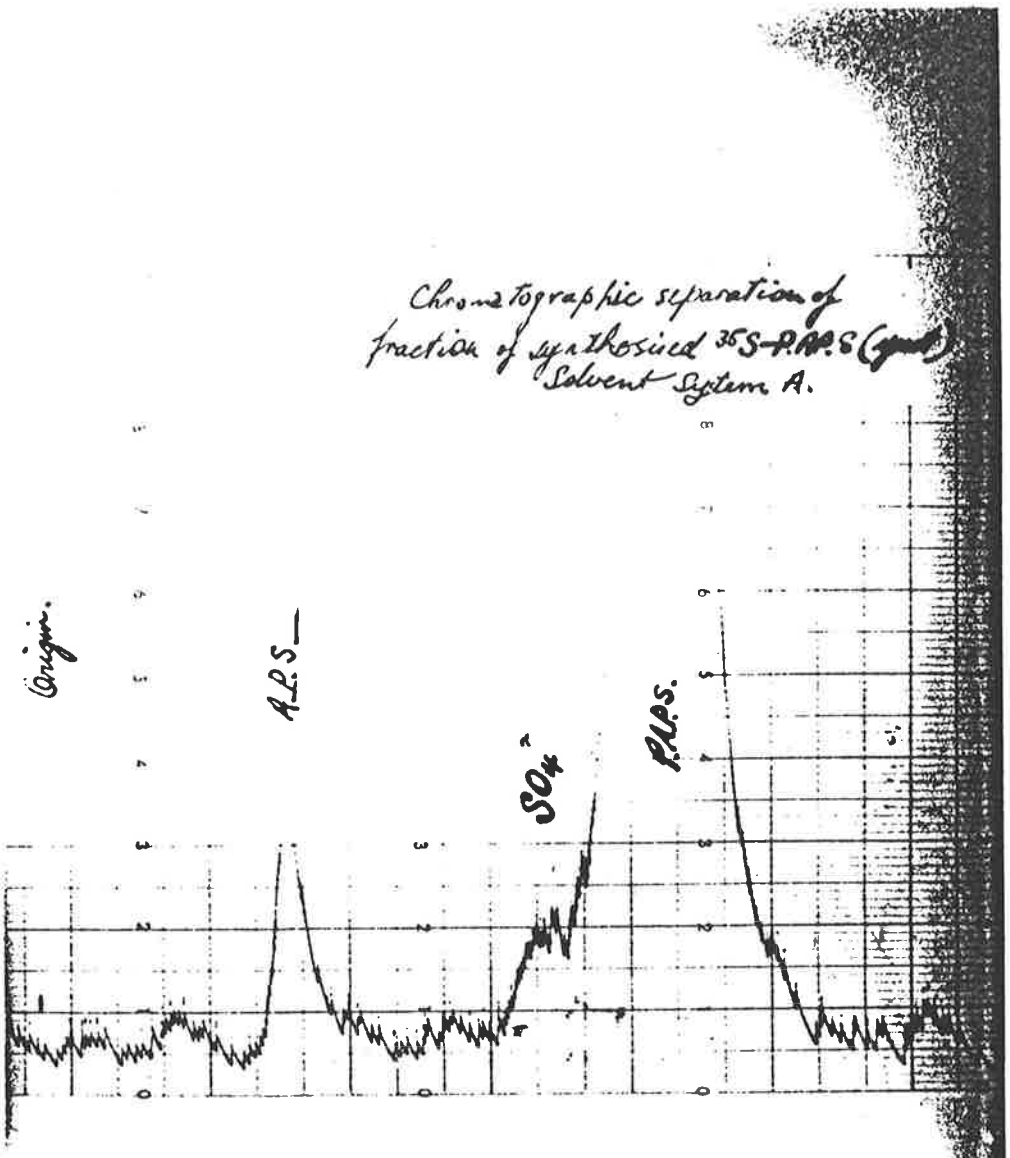


Figure A.7. Yeast synthesised Sulphated Nucleotides

Methods of High Voltage Electrophoresis:

9. Buffer, 0.1M Na Citrate at pH 5.2 was diluted to final concentration of 0.05M. Carbontetrachloride was used as a coolant. A 5 x 57 cm strip of 3MM Whatman filter paper was cut as shown to ensure good separation, and accurate matching with authentic controls. The paper was wetted with buffer and lightly blotted to remove surface moisture. The supernatant fractions (0.02 mls.) were applied slowly with an autopipette as a band leaving 1 cm. clear at the edge. The paper was committed to the ceramic tank and a current of 1,500 volts. 230ma applied for 60 minutes.

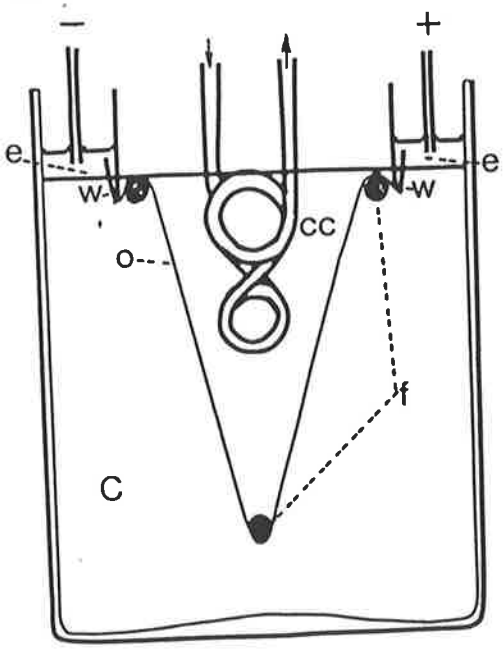
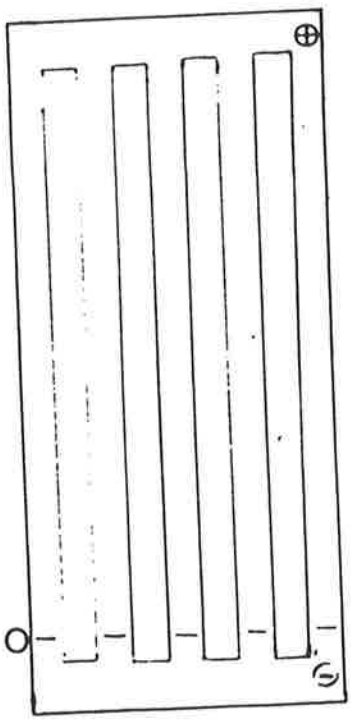


Figure A.8. High voltage electrophoresis
e. electrolyte chambers; w. wicks; f. glass and polyethylene support frame; o. origin on electrophoresis paper; C. carbontetrachloride coolant; cc. cooling coil.



Method of cutting electrophoresis paper

129.

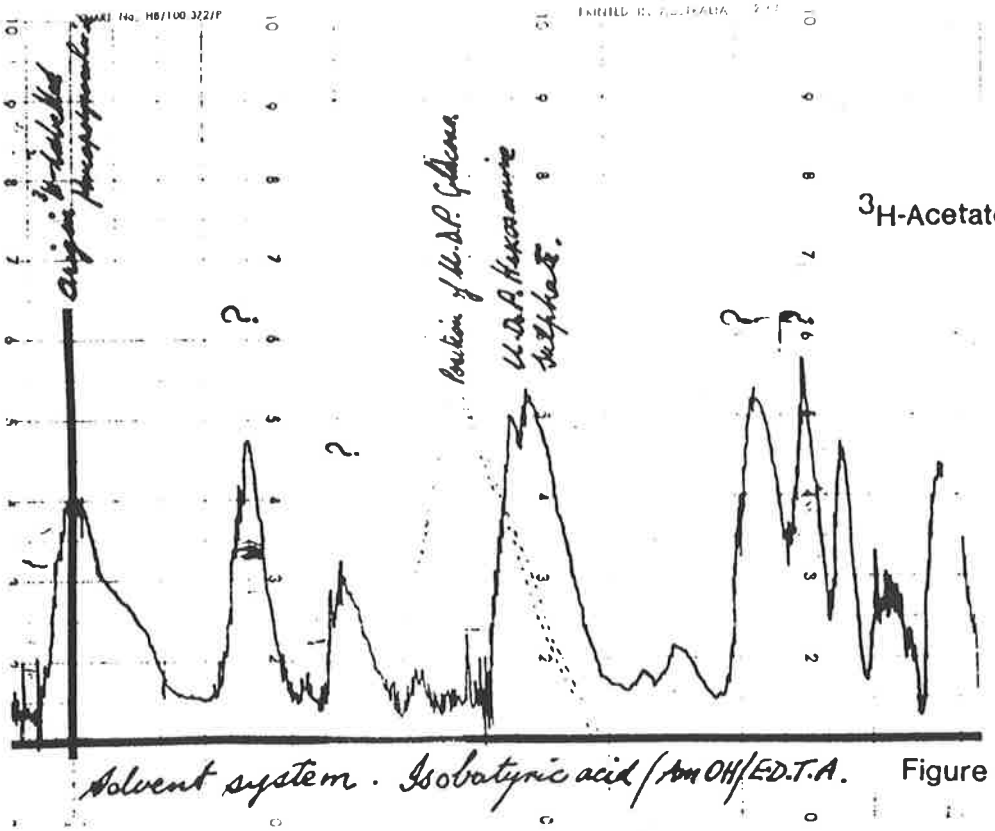


Figure A.9

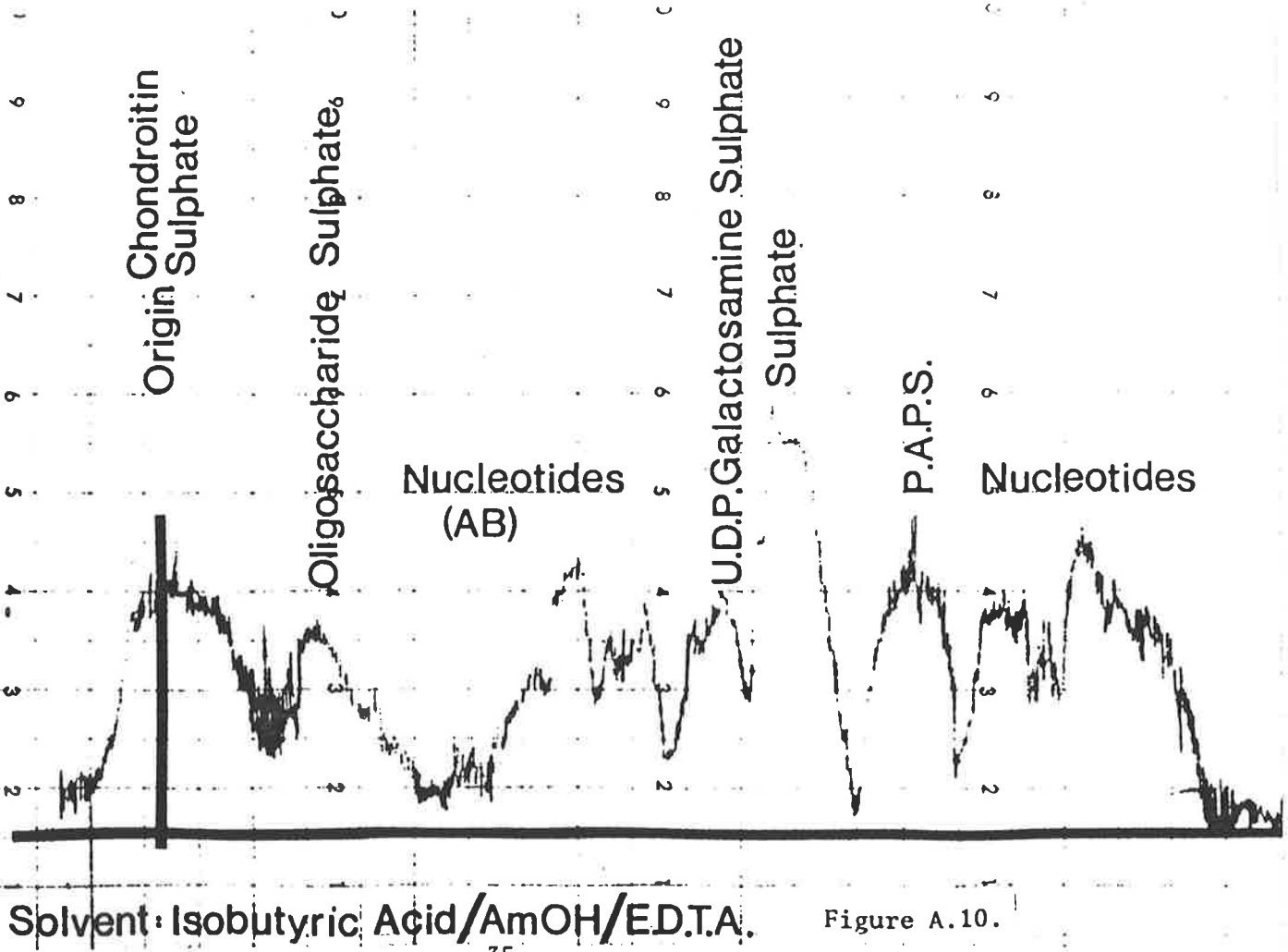


Figure A.10.

Typical separation of (^{35}S)-sulphate labelled extract from amnion cells (++++ rating) after 25 min 'radio isotope free chase' on paper chromatography using solvent system A and scanned with Actiscan III, Nuclear Chicago.

133. Liquid scintillation counting. Samples from the electrophoresis separation were cut out and placed in Packard vials. Scintillation fluid (0.5 ml.) consisting of PPO (0.3% w/v) which were then placed into the Packard Tri-Carb liquid scintillation spectrophotometer (Model 13375). The red channel was adjusted to count ^{35}S with high efficiency above a low background. Thus, the gain was set at 12% and the two discriminators at 40 and 1000 respectively. The gain of the blue channel was the same but the two discriminators were set at 40 and 150 resp. Samples were counted for 5 to 10 minutes, depending on their activity. Under these conditions, when the Packard series of ^{14}C quenched standards were counted, the ratio of net counts per minute (above background) in the red and blue channels increased linearly as the efficiency of counting decreased. Since ^{14}C- emissions are of almost the same energy as ^{35}S, in most cases about 86% of the disintregation emitted by ^{35}S on paper was detected.

161. Hyaluronidase E.C.4.2.99.1. Testicular hyaluronidase from Sigma Chemical Corporation, Turbidity Reducing Unit estimations were carried out using bovine albumin (Fraction V) DORFMAN, A. Methods in Enzymology 1.166 (1955) and measured specific activity .01mg = 1TRU in the sample used.

164. It is realised that tissue culture, Medium 199, with added foetal calf serum is not carrier free, with respect to sulphate nor free of other "indigenous" added "test" materials.

182. Assume the cross-section of cultured cells of a confluent monolayer adhering to a conventional surface is a hexagon, this will provide a "good" approximation to epithelial morphology. The dimensions are shown Figure A.11a.
In general, if the volume of a columnar cell with a hexagonal base remains the same, and its height increases by ℓ , B would be increased by $\frac{1}{\sqrt{\ell}}$ and the length of the hexagonal side x would equal $\sqrt{\left(\frac{B}{3\sqrt{\ell}}\right)^2 + \left(\frac{B}{2\sqrt{\ell}}\right)^2}$

$$= \frac{\sqrt{4B^2\ell + 9B^2\ell}}{36\ell^2}$$

$$= \frac{\sqrt{13\ell}}{6\ell} B$$

Total area of six sides of a column L high

$$= \frac{6\sqrt{13\ell}\,BL}{6}$$

$$= \frac{\sqrt{13\ell}}{\ell} B$$

Surface area ratio change of column increased in height by

$$= \frac{\sqrt{13\ell}\,BL}{\ell\ 13\ BL}$$

$$= \frac{\ell}{\ell}$$

Since we can fit 'ℓ' times more cells, overall intercellular surface contact $= \ell/\sqrt{\ell}$

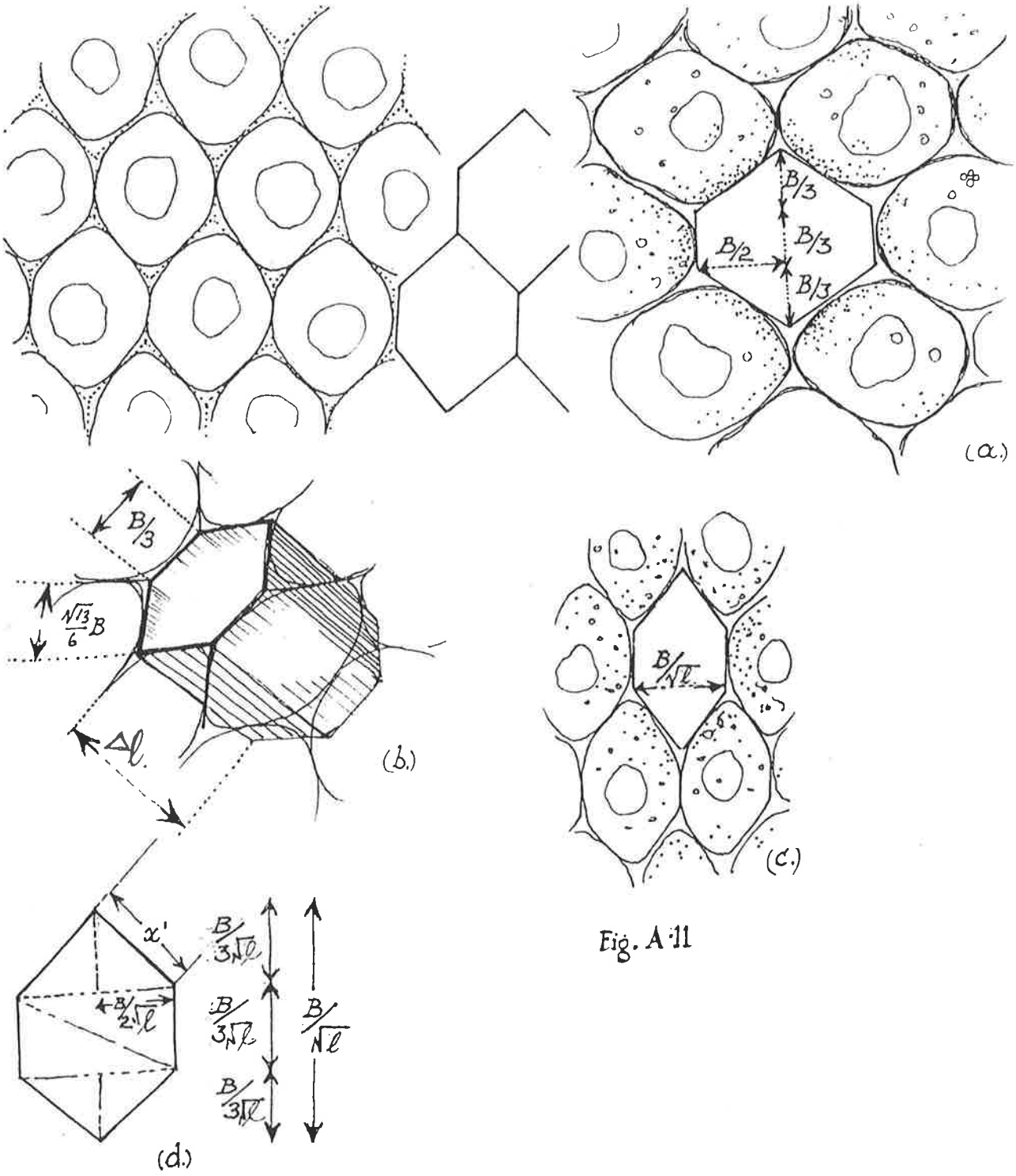


Fig. A·11

195. SAITO & UZMAN,(1970) have confirmed that AMPS are reingested whole by cells and recent data from NEUFELD (personal communication) indicate that PG are reutilized in preference to GAGs. If increased molecular weight (or hydrodynamic size) is a criteria for pinocytosis, and the molecular weight of rat skin heparin is 1.1×10^6, one qualification at least, is fulfilled for the pinocytosis of Hep.

219 HA prepared and generously supplied by Ove Wik (Pharmacia) Fluoresceinamine labelled with a degree of substitution (d.s.) of 0.002. On receipt of the material, it was further purified on Sepharose 2B-CL and then asample was tested for distribution of label by incompletely digesting it with hyaluronidase and the oligosaccharides were separated on G50 Sephadex. The constancy of Fln:UA ratios in all fractions indicated even distribution of the label along the polysaccharide. Moreover the material was tested for the ability to inhibit PG synthesis by chondrocytes.

202 Constituents of media L-15

	mg/litre
L-alanine	225.0
L-arginine	500.0
L-asparagine	250.0
L-cysteine	120.0
L-glutamine	300.0
Glycine	200.0
L-histidine	250.0
L-isoleucine	125.0
L-leucine	125.0
L-lysine	75.0
L-methionine	75.0
L-phenylalanine	125.0
L-serine	200.0
L-threonine	300.0
L-tryptophan	20.0
L-valine	300.0
D-Ca pantothenate	100.0
Choline Chloride	1.0
Folic acid	1.0
i-inosotol	1.0
Nicotinamide	2.0
Pyrodoxine. HCL	1.0
Riboflavin-5-Phosphate Na	0.1
Thiamine monophosphate	140.0
$CaCl_2$	900.0
D (+) Galactose	196.0
$MgSO_4$	196.0
KCl	400.0
KH_2PO_4	60.0
NaCl	8000.0
Na_2HPO_4	190.0
Na Pyruvate	550.0
Phenol red Na	10.0

227. The Incorporation of (^{35}S)-sulphate by adult chondrocytes is affected by denuding the cells of their glycocalyx with trypsin. Metabolic recovery takes at least 18 h, the criteria being the ability to respond to extraneous HA in the culture medium of suspension cultures. (Figure A.12)

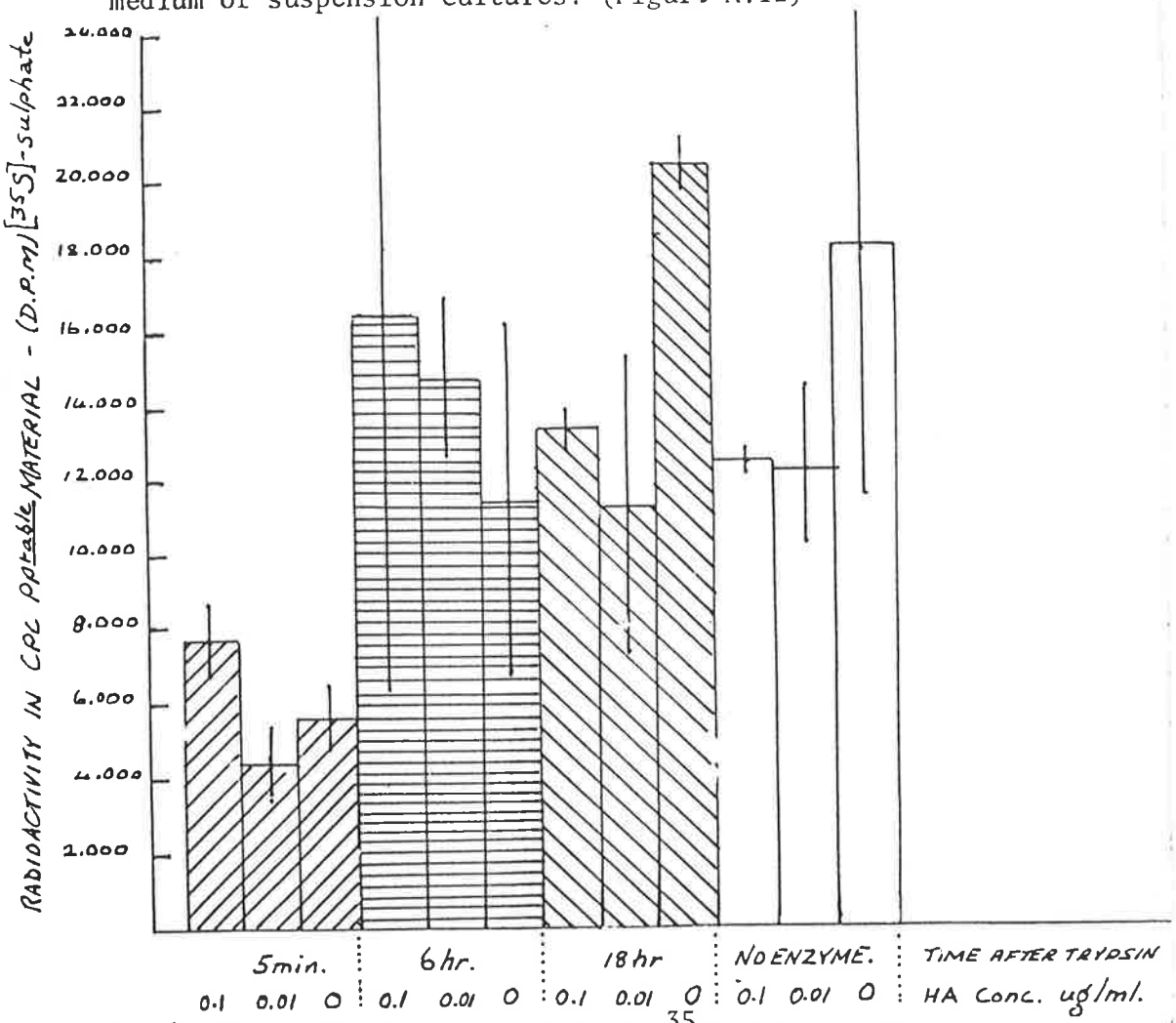


Fig. A.12. The incorporation of (^{35}S)-sulphate into a suspension culture of isolated adult chondrocytes in the presence of various amounts of HA, and following pre treatment with mild trypsinization of their cell surfaces.

231. Critical reviews have been submitted to Lab. Invest. and to Medical Hypotheses. In summary, they cite evidence which indicates that the intercellular matrix of the gingivae is highly ordered, even following inflammatory invasion. And it is this order which regulates diffusion, molecular reactivity (rates of reactions of enzyme degradation) and cellular responses.

REFERENCES

ABBOTT, J. and HOLTZER, H. (1966) J.Cell Biol., 28;473.

ADAMS, J.B. (1960) Biochem. J. 76; 520.

ALDRICH, B.I. (1958), Biochem.J., 70;236.

AMINOFF, D.(1961). Biochem.J., 81, 384.

ANDERSON, J.C., MATSUHASHI, M., HASKIN, M and STROMINGER, J.L.(1965) Proc . Natl. Acad. Sci. U.S. 53;881.

ANTONOPOULOS, C.A. (1962). Acta Chem.scand., 16;1521.

ANTONOPOULOS, C.A., GARDELL, S., SZIRMAI, J.A. and VAN BOUEN-DeTYSSONSK, E. (1964) Biochim. Biophys. Acta, 83;1.

ARMSTRONG, P.B. and JONES, D.P., (1968). J.exp. Zool., 167; 275.

ARNOTT, S., GUSS, J.M., HUKINS, D.W.L., DEA, I.C.M. and REES, D.A. (1974). J.Mol.Biol.88;175.

ARNOTT, S., GUSS, J.M. and WINTER, W.J. (1975). In Extracellular Matrix Influence on Gene Expression, Ed. Slavkin, H. and Greulich, R.C. Pub. Academic Press. N.Y. p.209.

ATKINS, E.D.T. and SHEEHAN, J.K. (1973). Science. 179;562.

ATKINSON, A., GATENBY, A.D. and LOWE, A.G. (1971) Nature, New Biol. 233;145.

ATTRAMADAL, A. (1964). D. Norske Tannl-f-Tid., 74;11.

AUSTIN, J., ARMSTRONG, D., STUMPF, D., LUTTENEGGER, T. and DRAGOO, M. (1969) Biochim. Biophys. Acta, 192;29.

AXELSSON, I. and HEINEGARD, D. (1975) Biochem. J. 145;491.

AXELSSON, I. and HEINEGARD, D. (1978). Biochem.J., 169;517.

BAKER, J.R., RODEN, L. and STOOLMILLER, A.C. (1972). J.Biol.Chem., 247;3838.

BAKER, J.R., RODEN, L. and YAMAGATA, S. (1971). Biochem. J. 125;93P.

BALAZS, E.A. (1974) In Disorders of the Knee. Ed. Helfet, A. Pub. Lippincott, Phil.

BALAZS, E.A. and DARZYNKIEWICZ, Z. (1973). In: Biology of Fibroblast. Ed.: Kulonen, E. & Pikkarainen, J.Acad. Press, 237.

BARRETT, A.J. (1975). In: Dynamics of Connective Tissue Macromolecules. Ed.: Burleigh, P.M.C. & Poole A.R. North Holland Press, Amsterdam, 219.

BARTOLD, P.M., WIEBKIN, O.W. and THONARD, J.C. (1982). Arch.oral Biol., 27;1.

BARTOLD, P.M., WIEBKIN, O.W. and THONARD, J.C. (1981). Connect. Tiss. Res. 9;99.

BARTOLD, P.M., WIEBKIN, O.W. and THONARD, J.C. (1982) Connect. Tiss. Res. 9;165.

BARTOLD, P.M., WIEBKIN,O.W. and THONARD, J.C. (1983). Biochem J. 211; (in press).

BAXTER, E. AND FRASER, J.R.E. (1980). Connect.Tiss.Soc.Aust. & N.Z. Prog.Abst. Papers; 7.

BAXTER, E., FRASER, J.R.E. and HOLMS, M. (1978). Connect.Tiss.Soc.Aust. & N.Z. Prog.Abst. Papers; 20.

BAXTER, E. and MUIR, H. (1975) Biochem. J. 149;657.

BAZIN, S. and DELAUNAY, A. (1968). Ann. Inst.Pasteur, 115;899.

BELANGER, L.F. (1954). Canad. J. Biochem.Physiol., 32;161.

BETTLEHEIM, F.A. and PLESSY, B. (1975). Biochim. Biophys. Acta. 381;203.

BHAVANANDAN, V.P. and DAVIDSON, E.A. (1975). Proc. Natl. Acad. U.S. 72;2032.

BHAVANANDAN, V.P. and MEYER, K. (1968). J. Biol. Chem. 243;1052.

BLACK, L. and BERENBAUM, M.C. (1964). Exp.Cell Res., 35;9.

BLACKWELL, J., SCHODT, K.P. and GELMAN, R.A. (1977). Fed.Proc., 36;98.

BOSMANN, H.B. (1969). Proc.Roy.Soc. (Lond.Ser.1B) 169;399.

BÖSTRÖM, H., RODÉN, L. (1966) in The Amino Sugars, Vol. IIB. ed. Jeanloz and Balazs, Academic Press, N.Y.

BRACHET, J. (1940). Compt.Rend.Soc.Biol. 133;88.

BRACHET, J. (1953) Quart.J.Micr.Sci., 94;1.

BRANDT, K.D., TSIGANOS, C.P. and MUIR. H. (1973). Biochim. Biophys. Acta. 320;453.

BRAY, B.A., LIEBERMANM R. and MEYER, K. (1967). J. Biol. Chem. 242;3373.

BROOKHART, J.H. (1965). J.Chromatogr., 20;191.

BRIMACOMBE, J.S. and WEBBER, (1964). The Mucopolysaccharides. Elsevier Pub., Amsterdam.

BRUNGENBURG, DE Jong, H.G. (1949). Colloidal Science, Vol. 2, Kruyt, ed. Elsevier.

BUTLER, W.T., BIRKEDAL-HANSEN, H. and TAYLOR, R.E. (1975). In: Extracellular Matrix Influences on Gene Expression. Ed: Slavkin, H.C., Greulich, R.C. Acad. Press. NY., 371.

CABIB, S., LELOIR, L.F. and CARDINI, C.E. (1953). J.Biol.Chem., 203; 1055.

CARLSTEDT, I., CÖSTËR, L., MALMSTRÖM, A. and FRANSSON, L-Å. (1979). Biochem. J. 178;257

CASTOR, C.W. (1959). Arthritis Rheum., 2;259.

CASTOR, C.W. and FRIES, F.F. (1961). J.lab. Clin.Med., 57;393.

CASTOR, L.N. (1968) J.Cell Physiol. 72;161.

CHEN, L.B., GUDOR, R.C., SUN, T.T., CHEN, A. and MOSESSON, M.W. (1977) Science, 197; 776.

CHOI, H.U. and MEYER, K. (1975). Biochem.J., 151;543.

CHOTINER, G., SMITH, J.G. and DAVIDSON, E.A. (1968). Anal.J.Biochem., 26;146.

CHRISTNER, J.E., BROWN, M.L. and DZIEWIATKOWSKI, D.D. (1977). Biochem. J. 167;711.

CHRISTNER, J.E., BROWN, M.L. and DZIEWIATKOWSKI, D.D. (1979). J.Biol.Chem., 254;12303.

CIANCIO, S.G. and MATHER, M.L. (1971) J.Periodont.Res., 6;188.

CIFONELLI, J.A. and DORFMAN, A. (1960). J.Biol.Chem. 235;3283.

CIFONELLI, J.A. and MATHEWS, M.B. (1972). Connect.Tiss.Res., 1;121.

CIMASONI, G. and HELD, A.J. (1963). Helv. odont. Acta, 7;50.

COHEN, L. (1968). Arch.Oral Biol.. 13;163.

CÖSTER, L. and FRANSSON, L-Å. (1977). Uppsala J.Med.Sci., 82;126.

CUNNINGHAM, L.M., CLOUSE, R.W. and FORD, J.D. (1963). Biochim. Biophys. Acta, 78;379.

CURRAN, R.C., CLARK. E.A. and LOVELL, D. (1965). J.Anat. 99;427.

D'AMBAMO, F. and LIPMANN, F. (1957). Biochim. Biophys.Acta, 25;211.

DEA, I.C.M., MOORHOUSE, R., REES, D.A., ARNOTT, S., GUSS, J.M. and BALAZS, E.A. (1973). Science. 179;560

DeLUCA., S., LOHMANDER, L.S., NILSSON, B., HASCALL, V.C. and CAPLAN, A.E. (1980). J.Biol.Chem., 254;1614.

DeLUCA, S. and SILBERT, J.E. (1968). J. Biol. Chem. 243;2725.

DERGÉ, J.G. and DAVIDSON, E.A. (1972). Biochem.J., 126;217.

DISCHE, Z. (1965) in the Amino Sugars, Vol. IIA. ed. Jeanloz and Balazs, Academic Press, N.Y. Chapter 22, p.115.

DORFMAN, A. (1958). Pediatrics, 22;576.

DOUGHTERTY, T. F. and DOLOWITZ, D.A. (1964).Amer.J. Cardiol. 14;18.

DUNSTONE, J.R. (1962). Biochem.J., 85;336.

DUTTON, G.J. and STOREY, I.D.E. (1953). Biochem.J., 53;xxxvii.

DZIEWIATKOWSKI, D.D. (1951). J. Biol.Chem., 189;187.

DZIEWIATKOWSKI, D.D. (1961). Mineral Metabol., Vol.2B.eds.Comar, C.L. and Bonner, F., Academic Press, N.Y.

DZIEWIATKOWSKI, D.D. (1962). J.Cell Biol., 13;359.

DZIEWIATKOWSKI, D.D. (1961), with CAMPO, R.D. J.Biophys. Biochem. Cytol.9;401

DZIEWIATKOWSKI, D.D. (1964). Clin.Orthop. Rel.Res., 35;189.

DZIEWIATKOWSKI, D.D. (1966). with HIRSCHMAN,A. Science, 154;393.

DZIEWAITKOWSKI, D.D., LAVALLEY, S.J. and LAVALLEY, J.A. (1977). J.Dent.Res., 56; Sp.Iss. A.327 (Abs.).

EICHWALD, E. (1965). Ann.Chem.Pharm., 134;177.

EMBERY,G., OLIVER, W.M. and STANBURY, J.B. (1979). J. Periodont. Res. 14;512

FITTON-JACKSON, S. (1967). Biochem.J., 104;13P.

FITTON-JACKSON, S. (1970). Proc. Roy. Soc. Lond. B. 175;405

FLORKIN, M. and STOTZ, E.H. (1969). Comprehensive Biochemistry Vol.17 Carbohydrate Metabolism. Elsevier Publishing Co., Amsterdam.

FRANSSON, L-Å. (1973) In: Biology of Fibroblast. Ed: Kulonen, E. & Pikkarainen, J.Acad.Press;

FRANSSON, L.-Å. and CÖSTER, L. (1979). Biochim. Biophys. Acta 582;132.

FROME, H.G. BUDDECKE, E., von FIGURA, K. and KRESSE, H. (1976) Exp. Cell Res. 102; 445.

FRANSSON, L-Å. and CÖSTER, L. (1979).

FRANSSON, L.-Å. and RODÉN, L. (1967). J.Biol.Chem., 242;4170.

FRATANTONI, J.C., HALL, C.W. and NEUFIELD, E.F. (1968) Proc.Natl. Acad.Sci. (U.S.), 60;699.

FRATANTONI, J.C., HALL, C.W. and NEUFIELD, E.F. (1968). Science, 162;570.

GALLAGHER, J. (1977). Biochem. Soc. Trans. 5;402.

GASIC, G.J. and BERWICK, L. (1963). J.Cell Ciol., 19;223.

GASIC, G.J., BERWICK, L. and SORENTINO, M. (1968). Lab.Invest. 18;63.

GELMAN, R.A. and BLACKWELL, J. (1973). Connect.Tiss.Res., 2;31.

GELMAN, R.A. and BLACKWELL, J. (1974). Biochim.Biophys.Acta., 342;254.

GHALAMBOR, M.A. and JEANLOZ, R.W. (1974) Fed. Proc. 33;1368.

GIBSON ,G. and PEARSON, C.H. Connect. Tiss. Res. 10;161.

GILLARD, G.C., BIRNBAUM, P., REILLY, H.C., MERRILEES, M.J. and FLINT, M.H. (1979). Biochim.Biophys.Acta., 584;520.

GILLARD, G.C., MERRILEES, M.J., BELL-BOOTH, P.G., REILLY, H.C. and FLINT, M.G. (1977). Biochem.J., 163;145.

GODMAN, G.C. and LANE, N. (1964). J.Cell.Biol. 21;353.

GOLDHARBER, P. (1965). Science, 147;407.

GREBNER, E.E., HALL, C.W. and NEUFELD, E.F. (1966). Arch. Biochem.Biophys., 116;391.

GREBNER, E.E, HALL, C.W. and NEUFELD, E.F. (1966). Biochem.Biophys.Res.Commun., 22;672.

GREGORY, J.D. (1973). Biochem J. 133; 383.

GREGORY, J.D. (1981). In Glycoconjugates. Proc. 6th. Int. Symp. on Glycoconjugates, Tokyo, Japan, Ed. Yamakalva T., Osawa,T. & Handa, S.

GREGORY, J.D. and ROBBINS, P.W. (1960). Ann.Rev.Biochem., 29;347.

GREGORY, J.D. and RODEN, L. (1961). Biochem. Biophys. Res. Commun. 5;430.

GREILING, H. and STUHLSATZ, H.W. (1966) Hoppe-Seyler's Z. Physiol. Chem. 345;236.

GREULICH, R.C. (1962). Internl.Assoc.Dent.Res. 40th Ann.Gen. Meet., St.Louis, Miss., Abs.304.

GROBSTEIN, C. (1954). In Aspects of Synthesis and Order in Growth ed. Rudnick, D., Princetown Uni. Press, p.233.

GROBSTEIN, C. (1975). In Extracellular Matrix Influences on Gene Expression. Ed. Slavkin,H. and Greulich, R.C. Pub. Academic Press. N.Y. p. 809.

GUSS, J.M., HUKINS, D.W., SMITH, P.J.C., WINTER, W.T., ARNOTT, S., MOORHOUSE, R. and REES, D.A. (1975). J. Mol. Biol. 955;359.

HANDLEY, C.J. and LOWTHER, D.A. (1976) Biochim.Biophys. Acta, 444, 69-74.

HANDLEY, C.J. and LOWTHER, D.A. (1977). Biochim.Biophys.Acta., 500;132.

HARDINGHAM, T.E., FITTON-JACKSON, S. and MUIR. H. (1972). Biochem.J., 129;101.

HARDINGHAM, T.E. and MUIR, H. (1972). Biochim.Biophys.Acta., 279;401.

HARDINGHAM, T.E. and MUIR, H. (1973). Biochem.J. 135, 905-908.

HARDINGHAM, T.E. and MUIR, H. (1973a). Biochem.Soc.Trans., 1;282.

HARDINGHAM, T.E. and MUIR, H. (1974). Biochem. J. 139, 565

HARRIETT, R.M. (1961). Quoted in Experimental Immunochemistry. eds.Kabat, E.A. and Mayer,M.M. (Charles C. Thomas, Publisher, Springfield, Ill.) 2nd Edn,Chap.26.

HASCALL, V.C. and RIOLO, R.L. (1972). J. Biol. Chem. 247;4529-

HASCALL, V.C. and SAJDERA, S.W. (1970). J.Biol/Chem., 245;4920.

HAUG, A. and LARSEN, B. (1971) Carbohyd. Res., 17; 297.

HEINEGÅRD, D. (1972). Biochim.Biophys.Acta., 285;185.

HEINEGÅRD, D. (1973) Chim. Scripta, 4, 199-201.

HEINEGÅRD, D. and GARDELL, S. (1967) Biochim. Biophys. Acta. 148;164.

HELTIN, T. (1971). J.Biol.Chem., 246;815.

HELTING, T. and RODÉN, L. (1968). Biochim.Biophys.Acta, 170;301.

HELTING, T. and RODÉN, L. (1968). Biochim.Biophys.Res.Commun., 31;786.

HENNING, F.R., WIEBKIN, O.W. and THONARD, J.C. (1970). J. Dent. Res. 49;658. (Abst.).

HIRAMATSU, M., ABE, I. and MINAMI, N. (1978). J.Periodont.Res., 13;224.

HIRSCHMAN, A. and DZWIEWIATKOWSKI, D.D. (1964). Anat. Rec. 148;291.

HOLLEY, R.W. and KIERNAN, J.A. (1968). ProcNatl.Acad.Sci(U.S.), 60;300.

HÖÖK, M., LINDAHL, U. and IVERIUS, P.-H. (1974). Biochem.J., 137;33.

HOPWOOD, J. (1979). Personal Communication.

HOPWOOD, J.J. and DORFMAN, A. (1977). Biochem. Biophys. Res. Commun. 75;472.

HOPWOOD, J.J. and ROBINSON, H.C. (1974a). Biochem. J. 141; 57.

HOPWOOD, J.J. and ROBINSON, H.C. (1974b). Biochem. J. 141; 517.

HOPWOOD, J.J. and ROBINSON, H.C. (1975). Biochem. J. 151;581.

HORNER, A.A. (1971). J.Biol.Chem. 246;231.

HORNER, A.A. (1977). Fed. Proc. 36;35.

HORWITZ, A.L. (1972). PhD Thesis, University of Chicago.

HORWITZ, A.L. and DORFMAN, A. (1968). J.Cell.Biol., 38;358.

HORWITZ, A. and DORFMAN, A. (1977) unpublished, cited by Hopwood & Dorfman, (1977).

HOSKINS, J.M., MAYNELL, G.G. and SAUNDERS, F.K. (1956). Exp.Cell Res., 11;297.

HUTTERER, F. (1966). Biochem. Biophys.Acta, 115:312.

JACOBSON, B. and DAVIDSON, E.A. (1962). J.Biol.Chem., 237;638.

JOHNSON, A.H. and BAKER, J.R. (1973). Biochim. Biophys. Acta. 320;341.

JORPES, J.E. and GARDELL, S. (1948). J.Biol. Chem. 176;267.

KANNO, Y. and MATSUI, Y. (1968). Nature, 218;775.

KATSURA, N. and DAVIDSON, E.A. (1966). Biochim. Biophys. Acta. 121;120.

KEMPSON, G.E., MUIR,H., SWANSON, S.A.V. and FREEMAN, M.A.R. (1970) Biochim. Biophys. Acta. 215;70.

KERBY, G.P. and EADIE, G.S. (1953). Proc. Soc. Exp. Biol. Med., 83;111.

KNUTSON, C.A. and JEANES, A. (1968). Anal.Biochem., 24;470 and 490.

KORN, E.D. (1959). J.Biol.Chem., 234;1325.

KORN, E.D. (1959). J.Biol.Chem., 234;1647.

KORNFIELD, R. (1967). J. Biol.Chem., 242;3135.

KORNFIELD, S., KORNFIELD, R. NEUFELD, E.F. and O'BRIEN, P.J. (1964) Proc.Natl.Acad.Sci. (U.S.), 52;371.

KOSHER, R.A., LASH, J.W. and MINOR, R. (1973). Develop. Biol. 35;210.

KRAMER, P.M. (1971a). Biochemistry, 10;1437.

KRAMER, P.M. (1971b). Biochemistry, 10;1445.

KRAMER, P.M. and SMITH, D.A. (1974). Biochem.Biophys.Res.Commun., 56;423.

KRESSE, H., TEKOLF, W., Von FIGURA, K. and BUDDECKE, E., (1975). Z.Physiol.Chem., 356;929.

KRUNKENBURG, C.F.W. (1884). Z.Biol., 20;307.

KUETTNER, K.E. (1977) Arthritis and Rheum. 20;5124.

KUETTNER, K.E., RAY, R.D., CROXEN, R.L. and EISENSTEIN, R. (1967) J. Cell Biol. 44;329.

LAMPEN, J.O. (1966). 16th Symp.Soc.Gen.Micro.(Cambridge University Press) Interference by Polyenic Antifungal Antibiotics.

LANGKAMP, H.H. PLATT, D. and THONARD, J.C. (1968). Internl. Assoc. Dent. Res. 46th Ann.Gen.Meet., San Francisco, Abst.416.

LAURENT, T.C. (1964). Nature, 202;1334.

LAURENT, T.C. (1968). The Chemical Physiol. of Mucopolysaccharides. ed. Quintarelli, p.153.

LAURENT, T.C. (1977). Fed. Proc. 36;24.

LAURENT, T.C. and ANSETH, A. (1961) Exp. Eye Res. 1;99.

LELOIR, L.F. (1951) Arch. Biochem., 33;186.

LELOIR, L.F., STANELONI, R.J., CARMINATTI, H. and BEHRENS,N.H. (1973). Biochem. Biophys.Res. Commun. 52;1285.

LEVITT, D. and DORFMAN, A. (1973). Proc. Natl. Acad. Sci. U.S.70;2201.

LEVY, ., NEVO, Z. AND FEINSTEIN, . (1981). Proc. Symp. Connect. Tiss. Macromol. Jerusalem.

LIMEBACK, H.F., SODEK, J. and BRUNETTE, D.M. (1978). Biochem.J., 170;63.

LINDAHL,U., BÄCKSTRÖM, G. MALMSTRÖM, A and FRANSSON, L.-Å. (1972). Biochem. Biophys. Res. Commun.,46;985.

LINDAHL, U., HÖÖK, M., BÄCKSTRÖM, G., JACOBSSON, I., REISENFELD, J., MALMSTRÖM, A., RODÉN, L. and FEINGOLD, D.S. (1977). Fed.Proc., 36;19.

LINDAHL, U. and RODEN, L. (1972). In: Glycoprotein Ed: Gottschalk, Elsevier Amsterdam;491.

LIPPMANN, M.M. and MATHEWS, M.B. (1977). Fed.Proc., 36;55.

LOHMANDER, L.S., DeLUCA, S., NILLSON, B., HASCALL, V.C., CAPUTO, C.B., KIMURA, H. and HEINEGÅRD, D. (1980). J.Biol.Chem., 255;6084.

LOWTHER, D.A. (1978). Lecture to Joint Meeting of Aust. Orthopaedic Soc. & Connect. Tissue Soc. of Aust. & N.Z. Control of synthesis and degradation of cartilage matrix.

LOWTHER D.A., SANDY, J.D. and BROWN, H.L.G. (1979). Proc. of Connect. Tiss. Soc. of Aust. & N.Z. (abst.)

LUBIN, M. (1967). Nature, 213;451.

LUBIN, M. and ENNIS, H.L. (1964). Biochim.Biophys.Acta, 80;614.

LUSCOMBE, M. and PHELPS, C.F. (1967). Biochem. J. 102;110.

MACKENZIE, I.C., DABELSTEEN, E. and ROED-PETERSEN, B. (1979). Scand.J.Dent.Res., 87;234.

McCORD, J.M. (1974). Science, 185;529.

McDOUGALL, W.A. (1971). J. Periodont. Res. 6;89.

McDOUGALL, W.A. (1972). J. Periodont. Res. 7;304.

McNEIL, J., WIEBKIN, O.W., BETTS, H. and CLELAND, L. (1982) Proc.Symp.Connect.Tiss. in Health & Dis., Melbourne, Aust.

MACIEIRA-COELHO, A. (1967). Exptl. Cell Res., 47;193.

MAGNUSSEN, B. (1968). Trans.Roy.Sc.Dent., Stockholm and Umea. Vol.14.

MAROUDAS, A. (1974). Articular Cartilage. Ed. Freeman, M.A.R., Pitman, Lond.

MARSH, J.M., MAINI, R.N., WIEBKIN, O.W. and DUMOND, D.C. (1978). J. Rheum. 5;148

MARSH, J.M., WIEBKIN, O.W., GALE, S. MUIR, H. and MAINI, R.N. (1979). Annals Rheum. Dis. 38;166.

MATHEWS, M.B. (1971). Biochem. J. 125;37.

MATHEWS, B.M. (1975). Molecular Biology and Biophysics, Vol. 19.

MATHEWS, M.B. and CIFONELLI, J.A. (1965). J.Biol.Chem. 240;4140.

MEEZAN, E. and DAVIDSON, E.A. (1967). J. Biol.Chem. 242;4956.

MELCHER, A.H. (1967). J. Periodont. Res. 2;127.

MERRILEES, M.J. and SCOTT, L. (1980). Develop. Biol. 76;396.

MEYER, K. (1938). Cold Spring Harbor Symp. Quant. Biol., 6;91.

MOLNAR, J. CHAO, H., IKEHARE, Y. (1971). Biochim. Biophys. Acta 239;401.

MONIS, B. and ZAMBRANO, D. (1966). Proc. 3rd Internl. Meet. Nephrology, Washington, D.C., ABS. II, 245.

MONIS, B. and ZAMBRANO, D. (1968). Zeits.Zellfors., 85;165.

MORNER, R.C.T. (1889). Skand.Arch.Physiol., 1;210.

MOWRY, R.W. (1978). Stain Technol. 53;141.

MILLER, and GOLDER, (1950). Arch. Biochem. 29;420.

MULDER, G. 1838. Bull Sci. Phys. Neerlande, 172.

NEUBERGER, A. (1968). Personal communication. Also in The Glycoproteins. ed. Gottschalk (1966). Pub. Elsevier. Chapter 7 to 11, Pages 158-314.

NEUBERGER, A. WILSON, B.M. (1967). Nature, 251;524.

NEUFIELD, E.F. and HALL, C.W. (1965). Biochem.Biophys.Res. Commun., 19;456.

NEVO, Z. and DORFMAN, A. (1972). Proc.Nat.Acad. Sci. USA, 69, 2069-2072.

NEVO, Z. HOROWITZ, A.L. and DORFMAN, A. (1972). Develop. Biol. 28, 219-228.

NICHOLAS, D.J.D. (1970). Personal Communication.

OGSTON, A.G. and PHELPS, C.F. (1961). Biochem. J.78;827.

OKAYAMA, M., KIMURA, K. and SUZUKI, S. (1973). J.Biochem(Tokyo).74;1069.

OKAYAMA, M., TAKAGAKI, KAJI, TOOLE, B.P. (1977).

PAGE, R.C. and SCHROEDER, H.E. (1976). Lab Invest. 33;235.

PAULSSON, M. and HEINEGÅRD, D. (1979). Biochem. J. 183;539.

PEDLER, J. (1979). Arch.oral Biol., 24;777.

PERLIN, A.S. (1977). Fed.Proc., 36;106.

PERLMAN, R.L., TELSER, A. and DORFMAN, A. (1964) Chem., 239;3623.

PICARD, J. (1962). J.Chromat., 7;223.

PICARD, J. and GARDAIS, A. (1964). In Connective and Skeletal Tissue, eds. Fitton Jackson et al p. 338.

PITA, J.C., MULLER, F. and HOWELL, D.S. (1975) In Dynamics of Connective Tissue Macromolecules. Ed. Poole, A.R. & Burleigh, P.M.C. Pub North Holland Press.

PRESTON B. N. (1977). Sympos. on Biol. Connect. Tiss. Uppsala,92.

PRESTON, B.N., LAURENT, T.C., COMPER, W.D. and CHECKLEY (1980) Nature,Lond., 287;499.

PRESTON, B.N. and SNOWDEN, McK. (1973). In: Biology of Fibroblast. Ed: Kulonen, E. & Pikkarainen, J. Acad.Press;

PRINZ,R., SCHWERMANN., BUDDECKE, E. and von FIGURA, K. (1978) Biochem. J. 176;671.

QUINTARELLI, G. (1960). Arch. oral Biol. 2;271 & 277.

QUINTARELLI, G. (1968). In The Chemical Physiology of Mucopolysaccharides. ed. Quintarelli, p. 199. pub. Little, Brown and Company.

RICHMOND, M.E., DeLUCA, S. and SILBERT, J.E. (1973). Biochemistry, 12; 3898 & 3904.

RILEY, J.F. (1959). The Mast Cell. pub.Livingstone.

RILEY, J.F. (1962). Lancet, 7245;40.

RINGERTZ, N.R. and REICHARD, P. (1960). Acta Chem. Scand., 14;303.

ROBBINS, P.W. (1962). Methods in Enzymology V eds. Colowick, S.P. and Kaplan, N., Academic Press, N.Y., P. 964.

ROBINSON, H.C. (1965). Biochem.J., 94;687.

ROBINSON, H.C. (1969). Conference on Calcium and Calcified Tissue Metabolism. Royal Dent. Hosp., Melbourne. Abs.4

ROBINSON, H.C. and DORFMAN, A. (1969). J. Biol.Chem., 244;348.

ROBINSON, H.C., HORNER, A.A., HOOK, M., OGREN,S. and LINDAHL,U. (1978) J.Biol. Chem. 253;6687.

ROBINSON, H.C., TELSER, A. and DORFMAN, A. (1966). Proc. Natl. Acad.Sci. (U.S.)., 56;1859.

RODÉN, L. (1956). Arkiv.Kemi., 10;333.

RODÉN, L. (1965). In:Structure and Function pf Connective and Skeletal Tissues. Ed: Fitton-Jackson, S., Harkness, R.D. Partridge, S.M., & Tristram, G.R. Butterworths, London;141.

RODÉN, L. (1970). In Metabolic Conjugation and Metabolic Hydrolysis. Ed. Fishman, W.D. Pub. Academic Press N.Y. 2;345.

RODÉN, L. (1970). In:The Chemistry and Molecular Biology of the Intercellular Matrix. Ed: Balazs, E.A. Acad. Press Vol. 2.

RODÉN, L. and DORFMAN, A. (1958). J.Biochem., 233;1030.

RODÉN, L. and SMITH, R. (1966). J.Biol.Chem., 241;5949.

ROLLET, A. (1858). Cited by Van Lier, 1909.

ROPES, M.W. BENNETT, G.A. and BAUR, W. (1939). J.clin. Invest., 18;351.

ROSENBERG, L., PAL, S. and BEALE, R.J., J.Biol.Chem. 248;3681.

SAITO, H. and UTZMAN, B.G., (1970). Exp.Cell Res., 60;301.

SAITO, H., YAMAGATA, T. and SUZUKI, S. (1968). J.Biol.Chem., 243;1536.

SAKAMOTO, N., OKAMOTO, H. and OKUDA, K. (1978). Arch.oral Biol., 23;983.

SANDY,J.D., BROWN, H.L.G. and LOWTHER, D.A. (1979). Proc. of Connect. Tiss. Soc. of Aust. & N.Z. (abst.)

SAVAGE, J. and BERNARD, G.W. (1969). Internl. Assoc. Dent. Res., 47th Ann.Gen.Meet., Houston, Tex., Abs. 654.

SCHERER, J.J. (1846). Ann.Chem.Pharm., 57;196.

SCHILLER, S., MATHEWS, M.B., CIFONELLI, J.A. and DORFMAN, A. (1956). J.Biol.Chem., 218;139.

SCHILLER, S., SLOVER, G.A. and DORFMAN, A. (1964). Biochem.Biophys. Res.Comm., 15;250.

SCHNEIDERBERG, O. (1891) Arch.Exptl.Path.Pharmakol., 28;355.

SCHOENBERG, M.D. and MOORE, R.D. (1958). Arch.Path., 65;115.

SCHULTZ-HAUDT, S.D. (1958). Odont.Tids., 66;No.1.

SCHULTZ-HAUDT, S.D. and LUNDQUIST, C. (1962). Int. Dent. J. 12;180.

SCHWARTZ, N.B., DORFMAN, A. and RODEN, L. (1975) In Extracellular Matrix Influences on Gene Expression. Ed, Slavkin H.C. and Greulich, R.C. Pub. Acad. Press. N.Y. p. 197.

STALLARD, R.E., DIAB, M.A. and ZANDER, H.A. (1965). J.Periodont., 36;130.

STARK, S.M. (1950). Amal.Chem., 22;1158.

STOCKWELL, R.A. and SCOTT, J.E. (1967). Nature, 215;1376.

STOKER, M. and RUBIN, A. (1967). Nature 215;171.

STOOLMILLER, A.C. and DORFMAN, A. (1967). Fed.Proc., 26;346.

STOOLMILLER, A.C. and DORFMAN, A. (1969). J.Biol.Chem., 244;236.

STOOLMILLER, A.C. and DORFMAN, A. (1969). In Comprehensive Biochemistry. eds. Florkin, M. and Stotz, E.H., Carbohydrate Metabolism, Vol. 17.

STOOLMILER, A.C., HORWITZ, A.L. and DORFMAN, A. (1972) J. Biol. Chem. 247; 3525.

STOREY, I.D.E., DUTTON, G.J. (1955). Biochem.J. 59;279.

SUGAHARA, K., SCHWARTZ, N.B. and DORFMAN, A. (1977) unpublished, cited by Hopwood & Dorfman, (1977)

SUNBLAD, L. and BALAZS, E.A. (1966). In Amino Sugars. In Amino Sugars, Ed. Jeanloz, R.W. and Balazs, E.A., Pub. Acad. Press Vol IIB;229

SUZUKI, S., SAITO, H., YAMAGATA, T., ANNO, K., SENO, M., KAWAI, Y. and FURUHASHI, T. (1968). J.Biol.Chem., 243;1543.

SUZUKI, S. and STROMINGER, J.L. (1967). J.Biol.Chem., 242;2288.

SUZUKI, S., TRENN, R.H. and STROMINGER, J.L. (1961). Biochim.Biophys. Acta, 50;169.

TELSER, A., ROBINSON, H.C. and DORFMAN, A. (1966) Arch. Biochem. Biophys. 116;458.

THEODORE De SAUSSURE, N. (1835). Historical.

THONAR, E.J.-M.A. and SWEET, M.B.E. (1979). Biochim.Biophys.Acta., 584;353.

THONARD, J.C. HEFFLIN, C.M. and STEINBERG, A.I. (1965). J.Bact., 89;924.

THONARD, J.C. and SCHERP, H.W. (1962). Arch.Oral Biol., 7;125.

THONARD, J.C. and WIEBKIN, O.W.(1973). J.Perio. Res. 8;101.

TODO, N. and SENO, N. (1970). Biochim.Biophys.Acta, 208;227.

SCOTT, J.E. (1960). Methods Biochem.Anal., 8;145.

SCOTT, J.E. (1968). In: The Chemical Physiology of the Mucopolysaccharides. Ed: Quintarelli, G. Little, Brown & Co., Boston;216.

SCOTT, J.E. (1975). Phil. Trans. Roy. Soc. Lond. B. 271;235.

SCOTT, J.E. and DORLING, J. (1973) In: Biology of Fibroblast. Ed: Kulonen, E. & Pikkarainen, J., Acad. Press Lond., 421.

SCOTT, J.E. and DORLING, J. (1965). Histochemie., 5:215.

SCOTT, J.E. and STOCKWELL, R.A. (1967). J.Histochem. Cytochem., 15;111.

SCOTT, J.E. and TIGWELL, M. (1978). Biochem. J. 173;103.

SCOTT, J.E., TIGWELL, M.R. and SAJDERA, S.W. (1972). Histochem.J., 4;155.

SENO, N., MEYER, K., ANDERSON, B. and HOFFMAN, P. (1965). J. Biol. Chem. 240;1005.

SILBERT, J.E. (1962). Biochem.Biophys.Acta, 9;266.

SILBERT, J.E. (1963). J. Biol.Chem., 238;3542.

SILBERT, J.E. (1964). J.Biol. Chem.239;1310

SILBERT, J.E. (1967). J.Biol.Chem., 242;5146.

SIMON, E.R. (1977). Fed.Proc., 36;9.

SISCA, R.F., LANGKAMP, H.H. and THONARD, J.C. (1971). Histochemie. 27;173.

SLAVKIN, H. and GREULICH, R.C. (1975) Extracellular Matrix Influences on Gene Expression. Pub. Academic Press N.Y.

SMITH, J.V. (1975). PhD Thesis, Sydney New South Wales, Aust.

SMITH, E.E.B. and MILLS, G.T. (1954). Biochim.Biophys. Acta, 13;386.

SMULOW, J.B. and GLICKMAN, I. (1966). Arch.Oral Biol., 11;1143.

SOLURSH, M. VAEREWYCK, S.A. and REITER, R.S. (1974). Develop.Biol. 41, 233-244.

SONNENSCHEIM, C. and SMULOW, J.B. (1966). Arch. Oral Biol., 43;633.

SPEIGHT, G., HANDLEY,C.J. and LOWTHER, D.A. (1978). Biochim. Biophys. Acta 540;238.

STAHL, S.S., SANDLER, H.C. and SUBEN, E. (1958). J. Periodontol. 29;183.

TKACZ, J.S. and LAMPEN, J.O. (1975). Biochem. Biophys. Res. Commun. 65;248.

TOOLE, B.P. (1973). Amer. Zool., 13;1061.

TOOLE, B.P. (1973b). Science, 180;302.

TOOLE, B.P. (1972). Develop. Biol. 29; 321.

TOOLE, B.P. (1980). Personal communication at Intern. Symp. on Connect. Tiss. Jerusalem.

TOOLE, B.P., JACKSON, G. and GROSS, J. (1972) Proc. Natl. Acad. Sci. USA 69;1384.

TSIGANOS, C.P. and MUIR, H. (1967). Biochem. J. 104;26c.

TUDBALL, N.N., ARONSON, J. and DAVIDSON, E.A. (1967). Fed. Proc. 26;856.

TURCO, S.J. and HEATH, E.C. (1976). Fed. Proc. 35;Abst. 104.

VAN LIER. (1909). Historical.

WAETCHER, C.J., LENNARTZ, W.J. (1976). Ann.Rev.Biochem. 45;95.

WARREN,L. (1960). Nature, 186;237.

WASTESON, A. and LINDAHL, U. (1971). Biochem. J. 125;903.

WEIDMAN, S.M. (1963). Intern. Rev. Connect. Tiss. Res. Vol.I, Ed. Hall, D. Pub. Academic Press. N.Y. p.33.

WIEBKIN, O.W. (1969). J.Dent. Res. 49;657.

WIEBKIN, O.W. (1981). Aust. N.Z. J. Med., 11;715.

WIEBKIN, O.W., BARTOLD, P.M. and THONARD, J.C. (1979). Biochem J., 183;467.

WIEBKIN, O. W. BARTOLD, P.M. HATAKEYAMA, K. and THONARD, J.C. (1983). Lab. Invest. (submitted).

WIEBKIN, O.W., BARTOLD, P.M., YELLAND, E. and THONARD, J.C. (1980). In Fibrous Proteins, Scientific, Industrial and Medical Aspects. Eds. Parry, DAD, & Creamer K. Pub. Academic Press, N.Y. p.121.

WIEBKIN, O.W. and FAULK, P.W. (1979). J.Dent. Res. 58;2234.

WIEBKIN, O.W., HARDINGHAM, T.E. and MUIR, H. (1975a). In Dynamics of Connect. Tiss. Macromols. Eds. Burleigh, P.M.C. & POOLE, A.R. Pub. North-Holland Pub. Co. p.81.

WIEBKIN, O.W., HARDINGHAM, T.E. and MUIR, H. (1975b). In Extracellular Matrix Influences on Gene Expression Ed. Slavkin, H. & Greulich, R.C. Pub. Academic Press. N.Y. p.209.

WIEBKIN, O.W. and MUIR, H. (1973a) FEBS Letts. 37, 42-46.

WIEBKIN, O.W. and MUIR, H. (1973b) in Biology of Fibroblasts (Kulonen, E. and Pikkarainen, J., eds.) pp 231-236, Academic Press, New York.

WIEBKIN, O.W. and MUIR, H. (1975a) Phil. Trans. R. Soc. Lond. B. 271, 283-291.

WIEBKIN, O.W. and MUIR, H. (1977a). Biochem. J. 174;269.

WIEBKIN, O.W. and MUIR, H. (1977b). J.Cell Sci., 27;199.

WIEBKIN, O.W. and THONARD, J.C. (1968). Proc. Aust. Soc. Med. Res. 2;190.

WIEBKIN, O.W. and THONARD, J.C. (1969). J.Dent.Res., 48;1084 Abst.

WIEBKIN, O.W. and THONARD, J.C. (1978) Uppsala. J. Med. Sco. 82;161.

WIEBKIN O.W. and THONARD, J.C. (1980) Proc. of Connect. Tissue Soc. of Aust. & N.Z. (abst.)

WIEBKIN, O.W. and THONARD, J.C. (1981). J. Periodont. Res. 16;600.

WIEBKIN, O.W. and THONARD, J.C. (1982). J.Periodont.Res., 17;626.

WILT, J.C., MILLER, D. and RUITER, J. (1964). Can. J. Micro., 10;170.

WINTER, W.T., SMITH, P.S.C. and ARNOTT, S. (1975). J.Mol.Biol., 99;219.

WOOD, G.C. (1960). Biochem. J., 75;605.

WOODARD, C. and DAVIDSON, E.A. (1968). Proc.Natl. Acad.Sci. (U.S.), 60;201.

WUSTEMAN, F.S. and DAVIDSON, E.A. (1975). Connect. Tiss. Res. 3;123.

APPENDIX II.

PUBLICATIONS

1. Wiebkin, O.W. & Thonard, J.C. Mucopolysaccharide elaboration in primary cultures of gingival epithelium and an established amnion cell line. Proc. Aust. Soc. Med. Res. 2:190, 1968.

2. Wiebkin, O.W. & Thonard, J.C. A preliminary comparison of mucopolysaccharide elaboration of a human gingival epithelial and an amnion cell line. J. Dent. Res. 48:1084, 1969.

3. Wiebkin, O.W. Gingival epithelial intercellular polysaccharides; Functional and Structural Significance. J. Dent. Res. 49:756, 1970.

4. Henning, F.R., Wiebkin, O.W. & Thonard, J.C. Radioautographic demonstration of gingival mucopolysaccharides. J. Dent. Res. 49:657, 1970.

5. Thonard, J.C. & Wiebkin, O.W. Localisation of mucopolysaccharides in epithelial-like cells cultured in vitro. J. Perio. Res. 8:101, 1973.

6. Wiebkin, O.W. & Muir, H. Factors affecting the biosynthesis of matrix constituents by chondrocytes in short term maintenance culture isolated from adult tissue. In The Biology of Fibroblast. Academic Press. ed. E. Kulonen, J. Pikkarainen 1973 pp. 231-236.

7. Wiebkin, O.W. & Muir, H. The inhibition of hyaluronic acid of sulphate incorporation in isolated adult chondrocytes. FEBS Lett 37:41, 1973.

8. Wiebkin, O.W., Hardingham, T.E. & Muir, H. The interaction of proteoglycans and hyaluronic acid and the effect of hyaluronic acid on proteoglycan synthesis by chondrocytes of adult cartilage. In Dynamics of Connective Tissue Macromolecules. North Holland Press, ed. Burleigh, P., Mary, C. & Poole, A.R. 1975, Ch. 4.

9. Wiebkin, O.W., Hardingham, T.E. & Muir, H. Hyaluronic acid-proteoglycan interaction and the influence of hyaluronic acid on proteoglycan synthesis by chondrocytes from adult cartilage. In Extracellular Matrix Influences on Gene Expression. ed. Slavkin, H. & Greullch, R. Academic Press 1975, pp. 209-223.

10. Wiebkin, O.W. & Muir, H. The effect of hyaluronic acid on proteoglycan synthesis and secretion by chondrocytes of adult cartilage. Phil. Trans. R. Soc. Lond. B. 271:283, 1975.

11. Marsh, J., Spencer, S., Wiebkin, O.W., Maini, R.N., Dumonde, D.C. & Muir, H. Heterogenicity of human synovial cell cultures. Annals Rheum. Dis. 33:578, 1974.

12. Wiebkin, O.W. & Muir, H. Proteoglycan synthesis by cultures of isolated chondrocytes and the effect of hyaluronic acid in the medium. Biochem. J., 164:269, 1976.

13. Marsh, J., Wiebkin, O.W., Gale, S., Muir, H. & Maini, R.N. Synthesis of sulphated proteoglycans by rheumatoid and normal synovial tissue in long term culture. Annals Rheum. Dis., 38:166, 1979.

14. Wiebkin, O.W. & Faulk, W.P. Lectin and hyaluronic acid receptor sites on chondrocytes and fibroblasts. J. Exp. Med. (in preparation).

15. Wiebkin, O.W. & Muir, H. Synthesis of proteoglycans by suspension and monolayer cultures of adult chondrocytes and de novo cartilage nodules - The effect of hyaluronic acid. J. Cell Sc., 27:199, 1977.

16. Marsh, J.M., Maini, R.N., Wiebkin, O.W., Dumonde, D.C. Rheumatoid and normal synovial tissue in long-term culture: Evidence of cell heterogeneity and an approach to quantitation of populations and the effects of passage. J. Rheum., 5:148, 1978.

17. Wiebkin, O.W., Thonard, J.C. The effect of hyaluronic acid and chondroitin sulphate on the localisation of proteoglycan in gingival organ cultures. Uppsala J. Med. Res. 82:161, 1978.

18. Marsh, J.M., Wiebkin, O.W., Gale, S., Muir, H. & Maini, R.N. Synthesis of sulphated Proteoglycans by Rheumatoid & Normal Synovial Tissue in Culture. Annals Rheu. Dis. 38:166-170, 1979.

19. Wiebkin, O.W., Bartold, P.M. & Thonard, J.C. Proteoglycans from human gingivae. Biochem. J. 183:467-470, 1979.

20. Wiebkin, O.W., Bartold, P.M., Yelland, E. & Thonard, J.C. Functional and Structural Differences in the Proteoglycans isolated from Gingival Epithelium and from Underlying Connective Tissue. In Fibrous Proteins: Scientific, Industrial & Medical Aspects. Ed. D.A.D. Parry & K. Creamer, Academic Press 121-132.

21. Wiebkin, O.W. & Thonard, J.C. (1981) Mucopolysaccharide localisation in gingival epithelium. I. Autoradiographic demonstration. J. Periodontal Res. 16:600-610.

22. Bartold, P.M., Wiebkin, O.W. & Thonard, J.C. (1982) Proteoglycans in Human Gingivae. I. Molecular size distribution in epithelium and connective tissue. Arch. Oral Biology 27:1.

23. Bartold, P.M., Wiebkin, O.W. & Thonard, J.C. (1981) Glycosaminoglycans of Human Gingival Epithelium and Connective Tissue. Connective Tiss. Res. 9:99-106.

24. Bartold, P.M., Wiebkin, O.W. & Thonard, J.C. (1982) Molecular weight determination of sulphated glycosaminoglycans from gingival epithelium and connective tissue. Connective Tiss. Res. 9: 165-172.

25. Wiebkin, O.W. & Thonard, J.C. (1982) Mucopolysaccharide Localisation in Gingival Epithelium. II. Factors Affecting Biosynthesis of Sulphated Proteoglycans in Organ Cultures of Gingival Epithelium. J. Perio. Res. 17: 626-639.

26. Hirsch, R.S., Wiebkin, O.W. & Thonard, J.C. (1983) Proteoglycan and Collagen in Porcine Gingivae: The limitation of tissue sample size in obtaining quantitatively significant data. J. Periodont. Res. 18; 92-100

27. Bartold P.M., Wiebkin,O.W. &Thonard, J.C. (1983) Proteoglycans in human gingival epithelium and connective tissue, Biochem, J. 211:

28. Wiebkin,O.W., Bartold, P.M., Hatakeyama,K. & Thonard, J.C. (1983) The nature of proteoglycans of pig gingivae- its role in periodontal disease. . Lab. Invest.

APPENDIX III.

Some publications which have direct relevance to the work described in this Thesis, and some publications which have borne out the hypotheses expressed in this work. Each publication represents a significant contribution by this Author.

Wiebkin, O.W., Bartold, P.M., Thonard, J.C., (1979) Proteoglycans from adult human gingival epithelium.
Biochemical Journal, v. 183 (2), pp. 467-470.

Wiebkin, O.W., Bartold, P.M., Yelland, E., and Thonard, J.C., (1980) Functional and structural differences in the proteoglycans isolated from gingival epithelium and from underlying connective tissue.
In Parry, D.A.D., and Creamer, L.K., *Fibrous Proteins: Scientific, Industrial, and Medical Aspects,* Academic Press, N.Y., pp. 121-132.

Wiebkin, O.W., Muir, H., Leaback, D.H., and Stockwell, R.A., (1975) The effect of hyaluronic acid on proteoglycan synthesis and secretion by chondrocytes of adult cartilage.
Philosophical Transactions B: Biological Sciences, v. 271 (912), pp. 283-291.

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SYNTHESIS OF PROTEOGLYCANS BY SUSPENSION AND MONOLAYER CULTURES OF ADULT CHONDROCYTES AND *DE NOVO* CARTILAGE NODULES – THE EFFECT OF HYALURONIC ACID

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SUMMARY

Chondrocytes were isolated from adult laryngeal cartilage by an enzymic procedure that included 6 h digestion with collagenase. The level of $^{35}SO_4^{2-}$ incorporation into cetylpyridinium chloride-precipitable material by these cells depended upon the subsequent culturing conditions. Suspension cultures incorporated more $^{35}SO_4^{2-}$/cell than monolayer cultures. Hyaluronic acid in the medium inhibited $^{35}SO_4^{2-}$ incorporation only when the cells were in primary suspension cultures. It had no effect on monolayer cultures, or monolayers organized into nodules, or suspension cultures derived from monolayers. Mild pretreatment with EDTA, however, rendered these susceptible to hyaluronic acid inhibition. In contrast EDTA abolished the inhibitory effect of hyaluronic acid on primary suspension cultures. Oligosaccharides, derived from hyaluronidase digestion of hyaluronic acid that were larger than decasaccharide, had some inhibitory effect on $^{35}SO_4^{2-}$ incorporation by monolayer cultures.

The total $^{35}SO_4^{2-}$ incorporation was less in primary suspension cultures of chondrocytes isolated after 12 h than after 6 h digestion of cartilage and the inhibition by hyaluronic acid was also less. These differences persisted during 12 days of culture.

It is suggested that the method of isolating chondrocytes and subsequent culture conditions may modify the cell surface and mask or abolish specific binding sites for hyaluronic acid.

INTRODUCTION

Low concentrations of hyaluronic acid (HA) added to the media, specifically inhibited the incorporation of $^{35}SO_4^{2-}$ and [^{3}H]glucosamine into proteoglycan (PG) by cultures of chondrocytes isolated from various cartilage sites and species (Wiebkin & Muir, 1973*a*, 1975*a*; Solursh, Vaerwyck & Reiter, 1974). Toole, Jackson & Gross (1972) observed that cellular aggregation of cultured embryonic chondrocytes was inhibited in the presence of HA and suggested that by interacting with the cell surface, HA may regulate cell surface activity. Subsequently Wiebkin & Muir (1975*b*) showed that HA binds to cell surfaces of chondrocytes, and could be removed with mild trypsin treatment. While bound to the cell surface, HA inhibited PG synthesis, but this effect was lost over 30 min when the cells that had been pre-incubated with HA, were transferred to media free of HA. These experiments were performed on suspension cultures of chondrocytes.

However, HA is a quantitatively minor component of cartilage most of which is present not as free HA but bound to PG in aggregates (Hardingham & Muir, 1973). Wiebkin & Muir (1975*b*) showed that PG–HA complexes do not inhibit PG synthesis.

Whether chondrocytes in monolayer cultures or those organized into cartilage nodules could be affected by the addition of HA to the culture media was examined here and also whether the capacity to synthesize $^{35}SO_4^{2-}$-labelled macromolecular material by primary cultures was influenced by different culture conditions. Suspension cultures derived from monolayer cultures were more active in synthesis than the parent equivalent monolayer cultures. Earlier, Srivastava, Malemud & Sokoloff (1974) showed that spinner cultures of rabbit articular chondrocytes, derived from monolayers synthesized 10 times more PG than did the parallel monolayer culture. Differences in synthetic rates may be a function both of the intimate micro-environment at the 'fuzzy coat' cell surface and of the accessibility of HA to the appropriate binding site. Understanding of how HA influences proteoglycan synthesis by chondrocytes might throw light on control mechanisms operating in cartilage *in vivo*. In adult cartilage the cells, which are not in direct contact with the circulation, are sparsely distributed in a firm matrix in which the diffusion of all but small solutes is very limited.

MATERIALS AND METHODS

The cells

Chondrocytes were isolated from laryngeal cartilage by enzymic digestion of the matrix and cultured as described later. Cartilages of the thyroid plate of bacon pigs were obtained fresh from the slaughterhouse, scraped free of connective tissue and of perichondrium and cut into small pieces (1 mm × 1 mm). The cartilage from each animal was treated separately. The cells were isolated by a modification of the method of Green (1971) in which each lot, about 5–7 g was suspended with stirring in a sterile double chamber (100 ml), the inner being fitted with a nylon mesh base through which cells that were freed from the digested matrix could pass. The cartilage samples were first digested at 37 °C for 10 min with 5–7 ml of a solution containing 500 TRU/ml of testicular hyaluronidase (EC. 3.2.1.35) in Tyrode's solution. After washing, the samples were incubated at 37 °C for 30 min with 5–7 ml of a solution of trypsin (EC. 3.4.21.4) in Tyrode's solution, containing 640 u/ml.

Finally, after washing, the cartilage was incubated for 6 h with 15–20 ml of a solution containing 125–200 u/ml of bacterial collagenase (EC. 3.4.4.24.3) in Leibovitz L-15 medium enriched with 8% foetal calf serum. Initial washings required the cells to be centrifuged at about 1500 *g*; subsequently when the medium contained no digested matrix, the cells were centrifuged at 900 *g*. Since the tissue was not obtained under sterile conditions, Amphotericin (2·5 μg/ml), Kanamycin (100 μg/ml) and Gentamycin (200 μg/ml) were added to the collagenase solution. Cells released from the cartilage were removed with a pipette, washed and suspended in 10 ml Leibovitz L-15 medium (8% FCS). Such cells are referred to as 6-h isolates (Fig. 1, *a–d*).

In some isolation preparations a considerable amount of undigested cartilage remained after 6 h. In such cases digestions were allowed to continue for a total of 12 h at 37 °C which gave an improved yield of cells. The cells thus released are referred to as 12-h isolates (Fig. 1, *e*).

Cells from representative isolations were initially cultured as suspensions or as monolayers and cell viability was assessed by the exclusion of trypan blue.

Suspensions. Cells intended as suspension cultures were washed twice in Tyrode's solution (10–15 ml) and resuspended in 10 ml of Leibovitz L-15 medium (8% FCS) in hydrophobic polystyrene 'Universals' (25-ml) at a concentration of 2–5 × 10^6 cells/10 ml. The cells were maintained in culture for at least 4 days before use (Fig. 1, *a*).

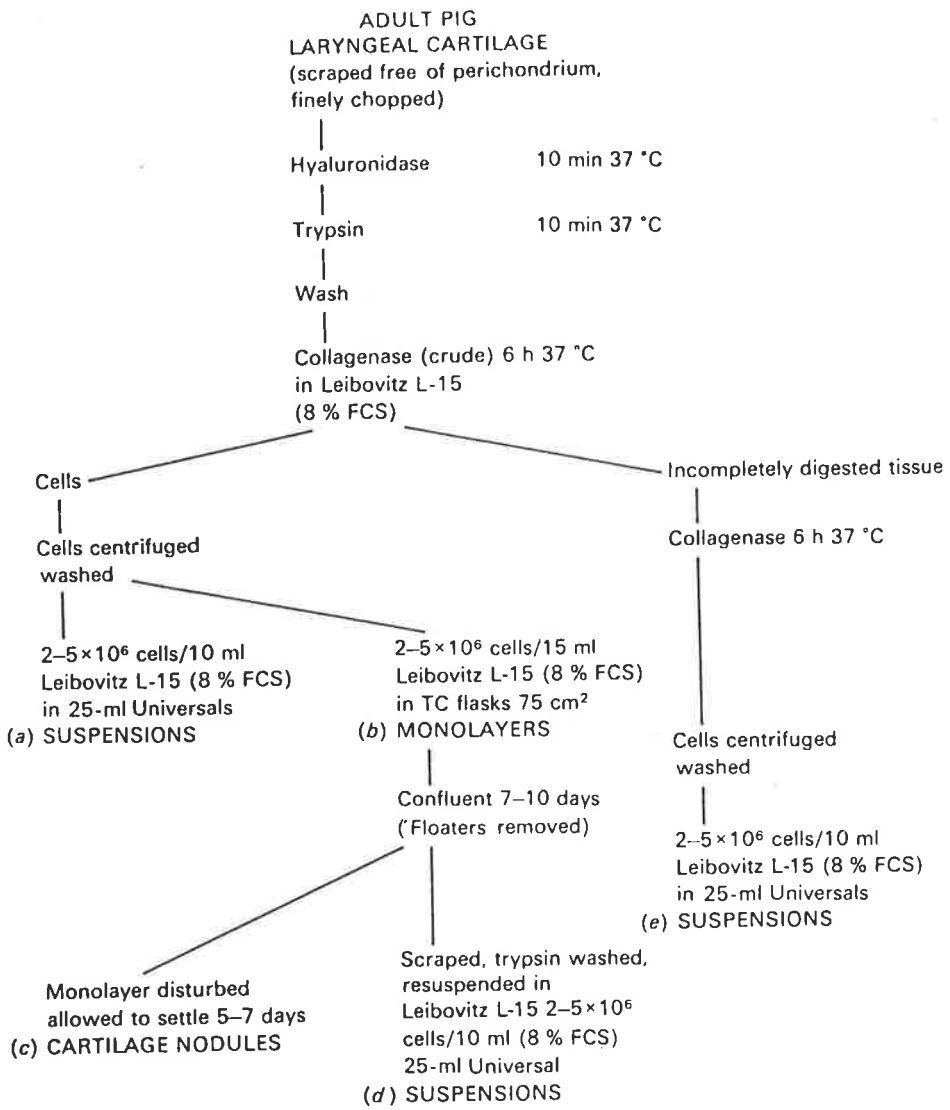

Fig. 1. Derivation of chondrocyte cultures.

Monolayers. Cells ($2–5 \times 10^6$ cells) from 6-h isolates to be cultured as monolayers were washed twice in Tyrode's solution (10–15 ml) suspended in 15 ml of Leibovitz L-15 medium (8 % FCS) and transferred into hydrophilic coffin-shaped tissue culture flasks (75-cm²). The medium was changed 3 times a week. The monolayers became confluent in 7–10 days (Fig. 1, *b*).

Cartilage nodules (de novo). Confluent sheets of cells in the above medium were disturbed by sharp shaking or folding with a sterile glass rod. These cultures were then left for several days without further disturbance. Where the cell sheets had aggregated together, a nidus for the formation of cartilage nodules developed (Fig. 1, *c*).

Suspensions derived from monolayers. Other monolayers were dispersed by incubation for 2–3 min with 0·25 % trypsin solution, washed in Leibovitz L-15 medium (10 % FCS) and resuspended in Universals at a density of 2×10^6 cell/10 ml in Leibovitz L-15 medium (8 % FCS). Since it required at least 18 h to replace cell surface components removed by mild

trypsin treatment from chondrocytes (Wiebkin & Muir, 1975*a*) the suspension cultures were allowed to recover for at least 24 h before use (Fig. 1, *d*).

'*Floaters.*' When the media of the monolayer cultures were changed, the cells in the spent media were spun down. These are referred to as 'floaters'.

The incorporation of $^{35}SO_4^{2-}$ by chondrocytes and the effect of hyaluronic acid

A standardized test system for measuring $^{35}SO_4^{2-}$ incorporation was used (Wiebkin & Muir, 1973*a*, *b*). In all experiments where the incorporation of $^{35}SO_4^{2-}$ was measured, test and control groups of cells in pairs were compared using cells from the same animal or culture to take account of the variations between animals or culture conditions.

Cell suspension. Cells were transferred to Tyrode's solution in which $MgCl_2$ replaced $MgSO_4$ but which contained no additive other than 5 μCi/ml of carrier free $^{35}SO_4^{2-}$; where the inhibitory effects of HA or of oligosaccharides derived from HA were to be tested these were included in the Tyrode's solution. The final concentration of HA was 0·1 μg/ml and of oligosaccharide 1 μg/ml of uronic acid respectively. Cells were incubated for 2 h and were then washed gently twice in Tyrode's solution containing no radioisotope at 4 °C. The supernatants from incubations and washings were pooled. In experiments where $^{35}SO_4^{2-}$-labelled material on the cell surface was to be determined, washed cells were incubated in a mixture of trypsin (258 u/ml) and DNase (EC. 3.1.4.5 18 u/ml) at 37 °C for exactly 30 min (Kraemer, 1971). This procedure provides a useful operational definition of cell surface material, although there is some unavoidable contamination by material from within the cell.

Monolayers and cartilage nodules. These cultures were rinsed with Tyrode's solution which was then replaced with 10 ml of Tyrode's solution containing radioactive isotope (with or without HA or HA oligosaccharides). After 2 h, the monolayer was either carefully scraped off and resuspended for haemocytometer counting with a fine-nozzled Pasteur pipette or trypsinized (Hayflick & Moorhead, 1961) retaining the trypsin and washings for determination of the total radioactivity incorporated.

Incorporation of $^{35}SO_4^{2-}$ into cetylpyridinium chloride-precipitable material

Cells and their culture media (and where applicable, cell surface material) were treated with 2% cetylpyridinium chloride (CPC) to a final concentration of 1% in Tyrode's solution containing 0·1 M Na_2SO_4 (the final volume of the cell pellet was 2 ml). A small amount of Keiselguhr was added to each tube to aid precipitation and the samples stirred vigorously with a glass rod, particularly the cell samples, to ensure that the cells were entirely disrupted (verified by microscopic inspection). The samples were then left to precipitate for at least 2 h at 37 °C shaken and centrifuged at 3650 g. The pellets were washed exhaustively in 0·5% CPC containing 0·1 M Na_2SO_4 and after draining were transferred quantitatively with 2 ml of 70% propanol into vials containing 10 ml of scintillation fluid.

The scintillation fluid contained per litre of toluene:2 methoxyethanol (3:2 v/v), 80 g of naphthalene and 4 g of 2,5-bis-(5-*t*-butylbenzo oxozol-2-yl) thiophen. The Keiselguhr was left to settle for at least 15 min and the temperature allowed to equilibrate with that of the counter. Radioactivity was determined using a Packard Tricarb liquid scintillation spectrophotometer. Counting rates were corrected for quenching using a channels ratio method with an external standard (Wiebkin & Muir, 1973*a*, 1975*a*). In most experiments the radioactivity was directly related to cell numbers counted in an haemocytometer, in others DNA was occasionally measured by the method of Hill & Whateley (1975) using the binding capacity of Mithracin to double-stranded DNA.

Cell division. The mitotic indices were determined by the standardized technique of [^{3}H]thymidine-pulsed incorporation (Simnett, 1975).

The pre-treatment of cultures with EDTA. Suspensions, monolayer cultures and cartilage nodules, were washed with Ca^{2+}- and Mg^{2+}-free Hanks' solution. These cultures were then incubated in 10 mM EDTA in Ca^{2+}- and Mg^{2+}-free Hanks' solution for 5 min at 37°C. This regime was selected to release equivalent amounts of uronic acid-containing material as after the mild trypsin treatment of Kraemer described above. Cells treated with EDTA were placed

in Leibovitz L-15 medium (8% FCS) for 5 min and then tested for their ability to incorporate $^{35}SO_4^{2-}$ into CPC-precipitable material and their susceptibility to inhibition by HA.

Preparation of oligosaccharides derived from hyaluronic acid. Fifty milligrammes of the purified HA were dissolved together with 700 μg of hyaluronidase (250 TRU) in 5 ml of 0·1 M sodium acetate, pH 5·5 and incubated at 37 °C for 16 h. The entire digestion was applied to a column (120 cm × 1·2 cm) of Sephadex G-50 equilibrated and eluted with acetate/pyridine buffer (1·4% glacial acetic acid in water adjusted with pyridine to pH 6·0). Fractions of 2 ml were collected and analysed for uronic acid by an automated procedure (Heinegård, 1973). Fractions were pooled to represent 8 samples of oligosaccharide. Each sample was rechromatographed separately on the same Sephadex G-50 column under the same conditions and representative fractions pooled, dialysed and evaporated to dryness. Oligosaccharides with K_d* less than 0·2 were pooled as were those of K_d greater than 0·2; thus approximately separating the oligosaccharides into those lesser than from those greater than decasaccharides.

Aliquots of stock solutions were diluted in Tyrode's solution to 100 μg/ml.

Materials

All enzymes, including hyaluronidase, trypsin (twice crystallized bovine pancreas Type 1) and collagenase (crude), for isolation procedures were supplied by Sigma Chemical Co. Ltd., Surrey, U.K. Tissue culture media, foetal calf serum and antibiotics were purchased from Flow Laboratories, Ayreshire, U.K. and Tyrode's solution from Difco Laboratories, Surrey, U.K. Tissue culture vessels were from Corning, Scientific Supplies Ltd., London EC1, U.K. Cetylpyridinium chloride from Fisons Ltd., Loughborough, Leicestershire, U.K., [^{3}H]thymidine (sp. act. 18·4 Ci/mol) and $^{35}SO_4^{2-}$ were supplied by the Radiochemical Centre, Amersham, Bucks, U.K. Sepharose 2B and Sephadex G-50 were purchased from Pharmacia (GB), London W5, U.K. and Mithracin from Pfizer Ltd., Sandwich, Kent, U.K.

RESULTS

Suspension cultures (Fig. 1, *a*)

Cells isolated from laryngeal cartilage over a period of 6 h or 12 h of digestion with collagenase and maintained as a suspension culture for 4 days showed no marked differences in ultrastructure (Fig. 2A, B). Nevertheless, 12-h isolates had only a third of the capacity to incorporate $^{35}SO_4^{2-}$ into macromolecular material as had 6-h isolates (Table 1). Moreover, this difference in the synthetic rate did not change over the following 12 days. The proportion of total radioactivity which was incorporated as cell bound material in 6-h isolates was about half that retained by the 12-h isolates and they exported five times more material into the medium (Table 2). The inhibitory effect of HA on the incorporation of $^{35}SO_4^{2-}$ into macromolecular material by these cultures was also different. In the presence of HA the incorporation by 12-h isolates was reduced by 50% of controls whereas with the 6-h isolates the incorporation was reduced by as much as 86% (Table 1). This inhibitory effect of HA was abolished by pretreatment with EDTA. It is noticeable however, that pretreatment of both 6-h and 12-h isolates with EDTA reduced the total incorporation and caused more label to be retained by the cells (Table 3), this effect being more marked with 6-h isolates.

Monolayer cultures and suspensions derived from monolayer cultures (Fig. 1, *b*, *c*)

Confluent monolayer cultures were prepared only from 6-h isolates; 12-h isolates seldom adhered to hydrophilic surfaces. These monolayer cultures incorporated

* K_d = coefficient of distribution of a molecule in gel chromatography which is a function of molecular size (Wheaton & Baumann, 1953).

slightly less $^{35}SO_4^{2-}$ than did 6-h isolates in primary suspension culture and the proportion of the label associated with the cells in monolayer was 4% of the total which was similar to the suspension cultures (Table 4). Suspensions were derived from these monolayers (Fig. 1*c*) by mild trypsin dispersal, or by scraping and further

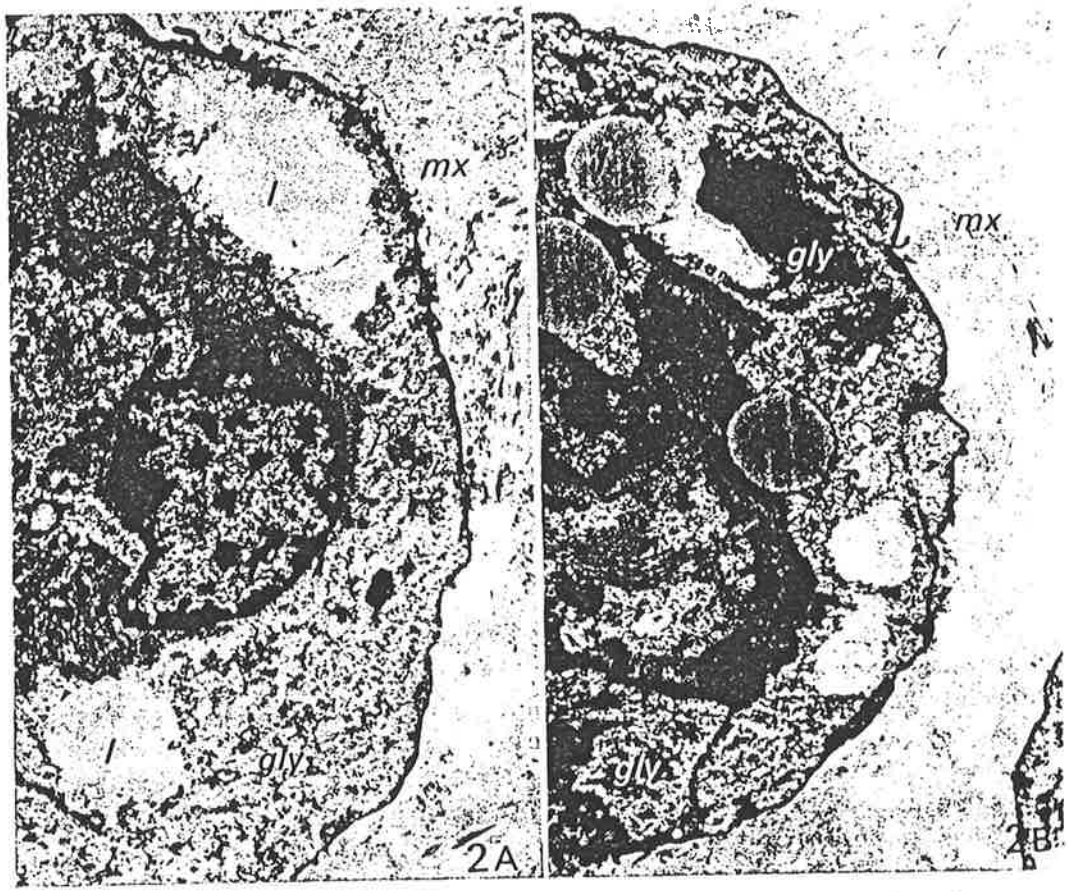

Fig. 2. Ultrastructure of chondrocytes isolated from adult pig larynx and cultured for 4 days in Leibovitz L-15 (8% FCS). × 10000. Fixed with glutaraldehyde followed by osmium tetroxide; stained with uranyl acetate and lead citrate. A, chondrocyte isolated by an enzymic digestion procedure including 6 h in collagenase (Fig. 1, *a*). B, chondrocyte isolated as in procedure (*a*) above but digested for 12 h instead of 6 h in collagenase (Fig. 1, *e*). Cells from both isolation procedures secreted some matrix material (*mx*), developed lipid vacuoles (*l*) and retained glycogen-like material (*gly*).

separation by expulsion through a fine nozzle of a pipette. They were incubated overnight under the same conditions as for the primary suspensions. The incorporation of $^{35}SO_4^{2-}$ per cell was twice that of the monolayer from which they had been derived. The cells retained as much as 18% of the total label, of which most (83%) was associated with cell surface since it was solubilized by trypsin. There was no inhibitory effect of HA on $^{35}SO_4^{2-}$ incorporation by monolayers or cell suspensions

Table 1. *The effect of hyaluronic acid upon the 2-h incorporation of* $^{35}SO_4^{2-}$ *into macromolecular material by suspensions of adult chondrocytes* ($\pm$ = *range*)

Cell isolation	Concentration of HA, μg/ml	Radioactivity, dpm/10^6 cells	% of total radioactivity retained by cells	% of control radioactivity	No. of experiments
6 h	0	5907 ± 109	4·2	100	15
	0·1	827 ± 18	29	14	15
12 h	0	1721 ± 70	32·8	100	15
	0·1	842 ± 15	50	49	15

Table 2. *The incorporation during 2 h of* $^{35}SO_4^{2-}$ *into macromolecular material by primary suspensions of adult chondrocytes*

Cell isolation	Days in culture	Cell body dpm	Cell* surface dpm	Medium dpm	Total dpm	% of total radioactivity retained by cells	No. of experiments
6 h	5	153	79	5892	6124	3·8	10
	12	177	71	5680	5907	4·2	10
12 h	5	420	98	1273	1791	28·9	10
	12	450	108	1160	1721	32·4	10

* Cell surface is defined as radioactive material solubilized by trypsin (Kraemer, 1971) and then precipitated by cetylpyridinium chloride.

Table 3. *The effect of EDTA upon the 2-h incorporation of* $^{35}SO_4^{2-}$ *into macromolecular material by primary suspensions of adult chondrocytes* ($\pm$ = *range*)

Cell isolation	Concentration of EDTA, mM	Radioactivity, dpm/10^6 cells	% of total radioactivity retained by cells	% of control without EDTA	No. of experiments
6 h	0	5907 ± 109	4·2	100	6
	10	1045 ± 53	22·5	17·8	6
	0 + 0·1 μg HA	1203 ± 32	23·0	20·4	6
12 h	0	1700 ± 69	32·8	100	6
	10	1600 ± 70	40·0	94·5	6

derived from them, although, after pretreatment with EDTA, HA had some inhibitory effect on $^{35}SO_4^{2-}$ incorporation (Table 5*A*, *B*). Cells brought into suspension from monolayers with only a 2-h period of recovery, when tested for $^{35}SO_4^{2-}$ incorporation had a limited capacity for synthesis and retained less $^{35}SO_4^{2-}$ than when allowed to recover for 16 h (Table 6). Mild treatment of monolayers with EDTA produced an immediate increase in incorporation of $^{35}SO_4^{2-}$ (Table 6). There was no significant

difference however, in the distribution of the radioactivity between cells and medium of control and EDTA-treated monolayers.

Cartilage nodules

Cartilage nodules incorporated 50% less $^{35}SO_4^{2-}$ per cell than did suspension cultures derived from them and only between 1–3% of the labelled material was

Table 4. *The incorporation of $^{35}SO_4^{2-}$ into macromolecular material during 2 h by primary suspension culture and by monolayer culture* (± = *range*)

Cell culture	Radioactivity, dpm/10^6 cells	% of total radioactivity retained by cells and matrix
6-h isolate, primary suspension	5907 ± 109	4·2
6-h isolate, confluent monolayer	4800 ± 92	4·0

Table 5. *Effects of EDTA pretreatment on $^{35}SO_4^{2-}$ incorporation*

(*A*). Mild EDTA pretreatment for 30 min on incorporation during 2 h of $^{35}SO_4^{2-}$ by monolayer cultures of adult chondrocytes

EDTA concentration, mM	Radioactivity, dpm/10^6 cells	Radioactivity, % of untreated controls
0	4800	100
10	8400	175

(*B*). Mild EDTA pretreatment for 30 min on incorporation of $^{35}SO_4^{2-}$ by monolayer cultures in presence of hyaluronic acid

EDTA concentration, mM	HA concentration, μg/ml	Radioactivity, dpm/10^6 cells	Radioactivity, % of untreated controls
0	0	4800	100
	0·1	6000	125
10	0	8400	175
	0·1	6062	126

associated with the cells and matrix the remainder (97–99%) was in the medium (Table 7). In contrast the distribution of total uronic acid between cells and medium of overnight cultures was 1:2, i.e. only 66% in the medium. The inhibitory effect of HA on $^{35}SO_4^{2-}$ incorporation by nodules was insignificant but after pretreatment with EDTA some inhibition was noted (Table 7).

Oligosaccharides added to the media of monolayer cultures

The larger oligosaccharides derived from HA reduced the incorporation of $^{35}SO_4^{2-}$ by 50–80% of 6-h isolates but only by 10–15% in 5 out of 6 monolayer cultures (Table 8). Larger oligosaccharides were those fractions with a K_d of between 0·12 and 0·2 when eluted from Sephadex G-50.

Table 6. *Comparison of 2-h incorporation of $^{35}SO_4^{2-}$ into macromolecular material by monolayer cultures and by suspensions derived from them* (± = *range*)

Cell culture	Radioactivity, dpm/10^6 cells	% of total radioactivity retained by cells and matrix
Monolayer	4800 ± 92	4
Suspension derived from monolayer allowed to recover for 2 h	2964 ± 83	10
Suspension derived from monolayer allowed to recover for 16 h	9209 ± 102	18

Table 7. *Comparison of the incorporation of $^{35}SO_4^{2-}$ during 2 h in the presence and absence of hyaluronic acid by (a) primary suspensions of 6 h isolates, and (b) 'cartilage nodules', and the effect of pretreatment with EDTA*

Cell culture	EDTA, mM	HA concentration, μg/ml	Radioactivity, dpm/10^6 cells	% of control incorporation
(*a*) primary suspension	0	0	5907	100
	10	0	1045	18
	0	0·1	827	14
	10	0·1	1203	204
(*b*) 'cartilage nodules'	0	0	2691	100
	10	0	3001	112
	0	0·1	2731	98
	10	0·1	2074	77

Distribution of CPC-precipitable material, as % of total uronic acid, from 16-h incubations of 'cartilage nodules' in complete Leibovitz L-15 medium: cells/matrix, 23·2; medium, 66·7.

DISCUSSION

The isolation of chondrocytes by Smith (1965) and by Manning & Bonner (1967) initiated many *in vitro* studies on the biosynthesis of cartilage matrix components by chondrocytes. Subsequently various expressions of phenotype were considered, notably by Coon (1966), Ham & Sattler (1968) and in a series of publications by

Holzer's group exemplified by Abbott & Holzer (1966), as well as by others. More recently, the effects on chondrocyte biosynthesis, of material extracted from, or resembling those found in cartilage matrix, have been reported (Nevo & Dorfman, 1972; Nevo, Horowitz & Dorfman, 1972; Wiebkin & Muir, 1973*a*, *b*; Handley & Lowther, 1976). However, most of these studies rely on arbitrary test systems using particular culture procedures.

Table 8. *The effect of oligosaccharides derived from HA on the incorporation of* $^{35}SO_4^{2-}$ *during 2 h into macromolecular material by cultured chondrocytes (4 experiments were performed with oligosaccharide)*

Culture	Additive HA oligosaccharides	Uronic acid concentration, μg/ml	Radioactivity, dpm/10^6 cells	% of control radioactivity
6-h isolate (suspensions)	—	0	5310	100
	K_d 0·42	1·0	5291	99·6 ± 0·6
	K_d 0·2–0·12	1·0	2502	47·1 ± 1·2
	HA	0·1	1082	20·4 ± 2·2
Monolayers	—	0	4273	100
	K_d 0·42	1·0	4011	94·9 ± 4·4
	K_d 0·2–0·12	1·0	3341	78·1 ± 3·3
	HA	0·1	3996	93·1 ± 4·4

Sokoloff (1976) has reported that species differences are important for the successful culture of articular chondrocytes and Hough & Sokoloff (1975) have surveyed some of the sources of the cartilage material currently in use. That report provided some information on the likelihood of contamination by other cell types that may invalidate the final evaluation of the results attributed to chondrocyte biosynthesis *per se*. Indeed Bryan (1968) demonstrated the effect of contamination of non-cartilage cells in cultures of chondrocytes.

In the present study, chondrocytes were derived from a source that consisted entirely of cartilage, as verified by Hough & Sokoloff (1975). They were cultured under a variety of conditions, a primary suspension, primary monolayer and as a suspension derived from a monolayer (Fig. 1). Other studies have shown that chondrocytes incorporate $^{35}SO_4^{2-}$ into cetylpyridinium chloride (CPC)-precipitable material which on Sepharose 2B gel chromatography and equilibrium density gradient centrifugation in CsCl, behaved like proteoglycan (Wiebkin & Muir, 1977). Hyaluronic acid has a specific inhibitory effect on the synthesis of proteoglycan by chondrocyte cultures derived from immature cartilage (Toole, 1973*a*, *b*; Solursh *et al.* 1974; Handley & Lowther, 1976) and from mature cartilage as primary suspensions (Wiebkin & Muir, 1973*a*, *b*). The hyaluronic acid appears to bind to cell surfaces (Wiebkin & Muir, 1975*b*). It should be emphasized that the inhibitory effect of hyaluronate on chondrocytes derived from mature cartilage has so far only been observed with primary suspension cultures. These cultures showed no cell

division and the matrix material which they secreted passed into the medium and was not laid down as a dense matrix limiting diffusion.

In contrast, monolayer cultures not only divide but also secrete a dense metachromatic material that, itself, limits the access of macromolecules from the medium. However, there are reports that the matrix produced by monolayer cultures differs in glycosaminoglycan composition from that of the original cartilage (Srivastava *et al.* 1974; Green & Ferguson, 1975). The data described here suggest that chondrocytes in monolayers or organized into cartilage nodules are surrounded by a matrix barrier that may be removed or reduced by EDTA. HA cannot penetrate this matrix but oligosaccharides of HA may be small enough to do so. Such oligosaccharides have been found to inhibit $^{35}SO_4^{2-}$ incorporation by chondrocyte suspensions (Wiebkin, & Muir, 1975*b*; Wiebkin, Haringham & Muir, 1975).

The incorporation of $^{35}SO_4^{2-}$ and its inhibition by hyaluronic acid by cells in primary suspension culture differ according to the duration of the isolation procedure. Moreover, these differences persisted for 12 days. This suggests that the longer exposure to collagenase during the 12-h isolation procedure permanently damaged the cells so that their capacity to synthesize proteoglycans was reduced and they no longer divided.

Suspension cultures differed in their synthetic capacity depending on whether they were derived from monolayer cultures or from the original cartilage. They also differed in their response to hyaluronic acid and to EDTA. Hyaluronic acid inhibited protoeoglycan synthesis by primary cultures, an effect that was abolished by EDTA, whereas, suspensions derived from monolayers were unaffected by hyaluronic acid unless pretreated with EDTA. Thus culture conditions appear to have modified the mechanisms that control proteoglycan synthesis. Furthermore, Srivastava *et al.* (1974) using rabbit articular chondrocytes have shown that the relative synthesis of keratan sulphate, chondroitin sulphate etc. varied with the culture conditions.

Since there is little or no cell division in adult cartilage, it is assumed that cells derived from adult cartilage, when they start to divide and form monolayers, have changed their behaviour in this respect. Most studies reporting the inhibitory effect of hyaluronic acid on proteoglycan synthesis used monolayer cultures of chondrocytes isolated from embryonic cartilage. In contrast chondrocytes in monolayer derived from adult cartilage were not affected by hyaluronic acid unless pretreated with EDTA.

The inhibitory effect of hyaluronate on proteoglycan synthesis results from its interaction with the cell surface (Wiebkin & Muir, 1975*b*) by a mechanism that has many similarities with the specific interaction of hyaluronate with proteoglycans involved in proteoglycans aggregation (Hardingham & Muir, 1974). The loss of response to hyaluronate by cells derived from monolayer cultures of cells isolated from adult cartilage may be due to loss or blocking of binding sites for hyaluronic acid or in the case of monolayers themselves to the lack of penetration by hyaluronic acid.

In conclusion, the data from this study suggest that chondrocyte characteristics may be lost or modified during prolonged isolation procedures and subsequent

culture condition. Nevertheless, many chondrocyte characteristics may be retained to a greater or lesser extent. By choosing appropriate conditions the mechanisms that control proteoglycan synthesis may be better understood.

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REFERENCES

Abbott, J. & Holtzer, H. (1966). The loss of phenotypic traits by differentiated cells. 3: The reversible behaviour of chondrocytes in primary cultures. *J. Cell Biol.* **28**, 473–487.

Bryan, J. (1968). Studies on clonal cartilage strains. I. Effect of contaminant non-cartilage cells. *Expl Cell Res.* **52**, 319–326.

Coon, H. G. (1966). Clonal stability and phenotypic expression of chick cartilage cells *in vitro*. *Proc. natn. Acad. Sci. U.S.A.* **55**, 66–73.

Green, W. T. (1971). Behaviour of articular chondrocytes in cell culture. *Clin. Orthopaed.* **75**, 248–260.

Green, W. T. & Ferguson, R. J. (1975). Histochemical and electron microscopic comparison of tissue produced by rabbit articular chondrocytes *in vivo* and *in vitro*. *Arthritis Rheum.* **18**, 273–280.

Ham, R. G. & Sattler, G. L. (1968). Clonal growth of differentiated rabbit cartilage cells. *J. cell. Physiol.* **72**, 109–114.

Handley, C. J. & Lowther, D. A. (1976). Inhibition of proteoglycan biosynthesis by hyaluronic acid in chondrocytes in cell culture. *Biochim. biophys. Acta* **444**, 69–74.

Hardingham, T. E. & Muir, H. (1973). Binding of oligosaccharides of hyaluronic acid to proteoglycans. *Biochem. J.* **135**, 905–908.

Hardingham, T. E. & Muir, H. (1974). Hyaluronic acid in cartilage and proteoglycan aggregation. *Biochem. J.* **139**, 565–581.

Hayflick, L. & Moorhead, P. G. (1961). The limited *in vitro* lifetime of human diploid cell strains. *Expl Cell Res.* **37**, 614–636.

Heinegård, D. (1973). Automated procedures for the determination of protein, hexose and uronic acid in column effluents. *Chim. Scripta* **4**, 199–201.

Hill, B. T. & Whateley, S. (1975). A simple, rapid microassay for DNA. *FEBS Letters, Amsterdam* **56**, 20–23.

Hough, A. J. & Sokoloff, L. (1975). Tissue sampling as a potential source of error in experimental studies of cartilage. *Conn. Tiss. Res.* **3**, 27–31.

Kraemer, P. M. (1971). Heparan sulphates of cultured cells. I. Membrane associated and cell-sap species in Chinese hamster cells. *Biochemistry, N.Y.* **10**, 1437–1451.

Manning, W. K. & Bonner, W. H. (1967). Isolation and culture of chondrocytes from human adult articular cartilage. *Arthritis Rheum.* **10**, 235–239.

Nevo, Z. & Dorfman, A. (1972). Stimulation of chondromucoprotein synthesis in chondrocytes by extracellular chondromucoprotein. *Proc. natn. Acad. Sci. U.S.A.* **69**, 2069–2072.

Nevo, Z., Horowitz, A. L. & Dorfman, A. (1972). Synthesis of chondromucoprotein by chondrocytes in suspension culture. *Devl Biol.* **28**, 219–228.

Simnett, J. D. (1975). The correlation between DNA synthesis and tritiated thymidine uptake in organ culture. In *Laboratory Manual of Cell Biology* (ed. D. Hall & S. Hawkins), pp. 29–32. London: The English Universities Press.

Smith, A. U. (1965). Survival of frozen chondrocytes isolated from cartilage of adult mammals. *Nature, Lond.* **205**, 782–786.

Sokoloff, L. (1976). Articular chondrocytes in culture: matrix production and hormonal effects. *Arthritis Rheum.* **19**, 426–429.

Solursh, M., Vaerewyck, S. A. & Reiter, R. S. (1974). Depression by hyaluronic acid of glycosaminoglycan synthesis by chick cultured embryo chondrocytes. *Devl Biol.* **41**, 233–244.

SRIVASTAVA, V. M. L., MALEMUD, C. J. & SOKOLOFF, L. (1974). Chondroid expression by lapine articular chondrocytes in spinner culture following monolayer growth. *Conn. Tiss. Res.* **2**, 127–136.

TOOLE, B. P. (1973*a*). Hyaluronate and hyaluronidase in morphogenesis and differentiation. *Am. Zool.* **13**, 1061–1064.

TOOLE, B. P. (1973*b*). Hyaluronate inhibition of chondrogenesis: antagonism of thyroxine, growth hormone and calcitonin. *Science, N.Y.* **180**, 302–303.

TOOLE, B. P., JACKSON, G. & GROSS, J. (1972). Hyaluronate in morphogenesis: Inhibition of chondrogenesis *in vitro*. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1384–1386.

WHEATON, R. M. & BAUMANN, W. C. (1953). Non-ionic separations with ion exchange resins. *Ann. N.Y. Acad. Sci.* **57**, 159–176.

WIEBKIN, O. W. & MUIR, H. (1973*a*). The inhibition of sulphate incorporation in isolated adult chondrocytes by hyaluronic acid. *FEBS Letters, Amsterdam* **37**, 42–46.

WIEBKIN, O. W. & MUIR, H. (1973*b*). Factors affecting the biosynthesis of sulphated glycosaminoglycans by chondrocytes in short term maintenance culture isolated from adult tissue. In *Biology of Fibroblasts* (ed. E. Kulonen & J. Pikkarainen), pp. 231–236. New York:Academic Press.

WIEBKIN, O. W. & MUIR, H. (1975*a*). Influence of the cells on the pericellular environment. The effect of hyaluronic acid on proteoglycan synthesis and secretion by chondrocytes of adult cartilage. *Phil. Trans. R. Soc. Ser. B* **271**, 283–291.

WIEBKIN, O. W. & MUIR, H. (1975*b*). Hyaluronic acid-proteoglycan interaction and the influence of hyaluronic acid on proteoglycan synthesis by chondrocytes from adult cartilage. In *Extracellular Matrix Influences on Gene Expression* (ed. H. Slavkin & R. Greulich), pp. 209–223. New York: Academic Press.

WIEBKIN, O. W., HARDINGHAM, T. E. & MUIR, H. (1975). The interaction of proteoglycans and hyaluronic acid and the effect of hyaluronic acid on proteoglycan synthesis by chondrocytes of adult cartilage. In *Dynamics of Connective Tissue Macromolecules* (ed. P. M. C. Burleigh & A. R. Poole), pp. 81–104. Amsterdam: North-Holland Publishing.

WIEBKIN, O. W. & MUIR, H. (1977). Synthesis of cartilage specific proteoglycan by suspension cultures of adult chondrocytes. *Biochem. J.* **164**, 269–272.

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Bartold, P.M., Wiebkin, O.W., and Thonard, J.C., (1981) Glycosaminoglycans of human gingival epithelium and connective tissue.
Connective Tissue Research, v. 9 (2), pp. 99-106.