

GLYCOSAMINOGLYCAN SYNTHESIS BY NORMAL HUMAN MAMMARY  
EPITHELIAL CELLS IN PRIMARY CULTURE.

by

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## ABSTRACT

The extracellular matrix (ECM) influences cell growth and differentiation. Glycosaminoglycans (GAGs), alone or complexed with protein (proteoglycans), are a major component of the ECM affecting cell behavior. GAG synthesis has been studied extensively in animal models and malignant cells. This research centres on studying GAG production by normal human mammary epithelial cells in culture. Mammary tissue obtained from reduction mammoplasties were dissociated to single cells. The epithelial cell population was seeded onto hydrated collagen gels at  $2-2.5 \times 10^5$  cells/cm<sup>2</sup> in medium containing 5% FCS and 5 µg/ml of insulin. Ultrastructural studies confirmed the epithelial nature of the cultures. To measure GAG synthesis, cultures were incubated with <sup>3</sup>H-glucosamine for 24 hours at 3 time points; days 3-4, 9-11 and 17-18. The cultures were proliferating at the early time point and had reached a stationary phase at the later time points. Cell, ECM and medium fractions were analyzed for GAGs as identified by enzyme degradation and cellulose acetate electrophoresis. At day 4, when cells were actively growing, the majority of GAGs produced were released into the medium fraction (75-80%). The predominant GAG was the nonsulfated GAG, hyaluronic acid (HA). Of the sulfated GAGs chondroitin sulfate (CS) 4 and 6 comprised only 18% of total GAGs; dermatan sulfate (DS) synthesis was negligible. At the later time periods, when cultures had ceased growing a higher percentage of total GAG was incorporated into an ECM (50-65%). The sulfated GAGs were preferentially incorporated into the ECM, CS 4 and 6 comprising 70% and DS comprising 30%. The marked difference in type and location of GAGs produced was not merely a function of time in culture. Cultures seeded at high densities ( $5 \times 10^5$  cells/cm<sup>2</sup>) were not proliferating when terminated at day 4. Their GAG profile was similar to that of lower density cultures at day

10. This data provides a baseline from which we can determine if cell-synthesized GAGs, play a role in maintaining differentiated and malignant phenotypes.

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## ABBREVIATIONS

ECM - extracellular matrix  
PG - proteoglycan  
GAG - glycosaminoglycan  
HA - hyaluronic acid (hyaluronate)  
CS - chondroitin sulphate  
HS - heparan sulphate  
DS - dermatan sulphate  
BM - basement membrane

## INTRODUCTION

Historically, the extracellular matrix (ECM) was considered to be an inert substrate providing support for cells and not interacting in any significant manner with these cells. Over the last two decades, however, information has accumulated indicating that this was an over-simplification of its function. The interactions between cells and the ECM are regarded as significant and imperative in such important areas of cell functioning as growth (Gey et al., 1974), migration (Greenberg et al., 1981), differentiation (Emerman and Pitelka, 1977, Emerman et al., 1977, 1981; Kleinman et al., 1981; Parry et al., 1982) and determination of cell shape (Emerman et al., 1979, Gospodarowicz et al., 1978). The ECM is composed of macromolecules from four major classes - collagen, proteoglycans, glycoproteins and elastin (Hay, 1981). Together these components form a structurally stable material that lies under epithelia and surrounds connective tissue cells.

### The Basement Membrane

The term basement membrane refers to an organized complex of ECM components which include collagen, proteoglycans and glycoproteins, that are associated with the basal surfaces of epithelial cells whenever they contact connective tissue (Vracko, 1974). To further classify the basement membrane, it can be described according to its morphological appearance which include 3 or 4 major zones (Martin et al., 1982): 1) the lamina lucida externa (or lamina rara) which is an electron lucent region 20 to 40 nm wide found just below the epithelial basal cell surface, 2) the lamina densa (or basal lamina) is a middle layer 20 to 100 nm wide and contains a meshwork of fine filaments giving it an electron dense appearance, 3) the

lamina lucida interna which is an electron lucent region of variable wide and found between the lamina densa and the underlying connective tissue (Figure 1) and the reticular lamina. The reticular lamina is a meshwork of fine collagen fibers between the basal lamina and connective tissue.

The major collagen component present in the ECM is Type IV collagen (Kleinman, 1982). This has been demonstrated using various techniques including autoradiography and immunolocalization (Sano et al., 1981). Type IV collagen was first identified by Kefalides in 1966. He was able to solubilize a unique collagen protein after pepsin digestion from canine glomerular basement membranes. While it was initially thought that Type IV contained a single type of chain it has been subsequently proven that this collagen has two distinct chains designated pro  $\alpha$  1 (IV) and pro  $\alpha$  2 (IV) (Crouch et al., 1980; Glanville et al., 1979). Unlike Types I, II and III, Type IV collagen does not arise biosynthetically from precursor molecules called procollagens. Instead, the pro  $\alpha$  I (IV) and pro  $\alpha$  2 (IV) chains are incorporated into the basement membrane as such (Heathcote et al., 1976; Karakashian et al., 1982; Risteli et al., 1981). Type IV collagen has been localized to the lamina densa portion of the basement membrane by several studies (Yaoita et al., 1978; Laurie et al., 1980; Roll et al., 1980). The actual function of Type IV collagen is not known, however, its non-fibrillar structure may be lending both elasticity and stability to the basement membrane. It has been suggested that like ends of the collagen type IV molecules interact with each other forming a continuous network (Timpl et al., 1981). Other models have also been proposed and will be discussed later in the Introduction.

Another collagen present in basement membranes, as well as other areas, Type V collagen, was originally isolated from placenta (Bailey et al.,

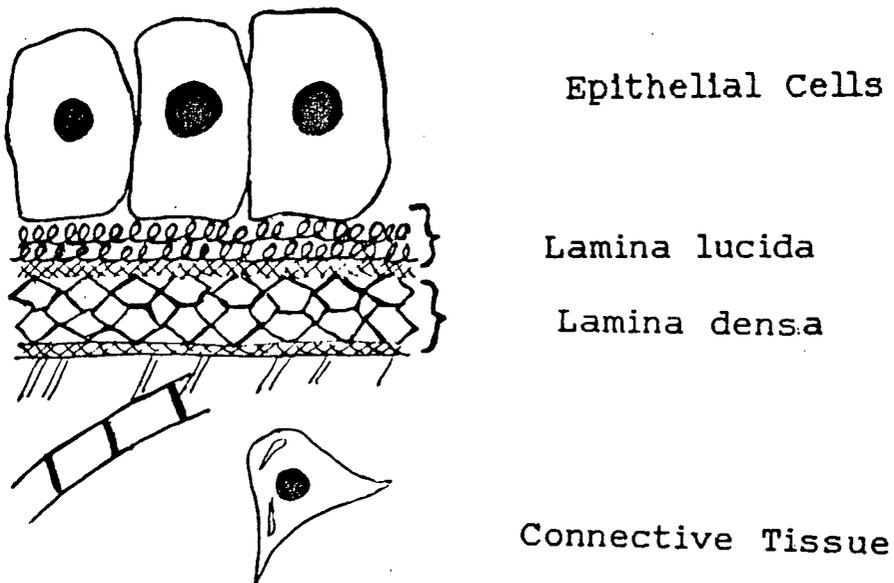


Fig. 1 Schematic representation of the basement membrane. (Martin et al., 1982.)

1979; Burgeson et al., 1976; Chung et al., 1976). It also contains two distinct chains,  $\alpha 1$  (V) and  $\alpha 2$  (V). The role of this collagen in basement membranes is unclear. Immunolocalization studies indicate its presence in the lamina densa (Roll et al., 1980) as well as emanating from there to the underlying connective tissue (Martinez-Hernandez et al., 1982). This latter finding has led Martinez-Hernandez et al. to suggest that Type V collagen may act by anchoring dissimilar tissue types together.

The glycoproteins found in the basement membrane include fibronectin (Vaheri et al., 1978), laminin (Timpl et al., 1981) and entactin (Carlin et al., 1981). Fibronectin is a large glycoprotein (MW 440,000) made up of 2 identical 220,000 Dalton chains linked by disulfide bonds and found in serum, on cell surfaces and in the ECM of connective tissues (Ruoslahti et al., 1981). Its function in the ECM has been related to its ability to aggregate and bind to a number of other molecules (Vaheri et al., 1978). It has been localized by immunoelectron microscopy to the lamina lucida as well as throughout the basement membrane (Foidart et al., 1980). This glycoprotein binds various types of cells to collagens Type I, II, III & IV. A few of the cell types studied include primary fibroblasts (Murray et al., 1978), rat hepatocytes (Hooper et al., 1976), myoblasts (Ketley et al., 1976) and established cell lines such as CHO and 3T3 (Grinnell, 1978). From the studies to date it appears reasonable to conclude that fibronectin is the glycoprotein used as an attachment mediator between other ECM components and cells of mesenchymal origin (Kleinman, 1982). Its place in the structured basement membrane is not known for certain but it has been shown to bind to numerous cell types as well as to collagens (Type I to V) and proteoglycans (Woodley et al., 1984).

Laminin is a very large glycoprotein (MW  $10^6$ ) first isolated from the Engelbreth-Holm-Swarm (EHS) tumor (Timpl et al., 1979) and found in all basement membranes (Timpl et al., 1980). It is composed of two types of chains (200,000 and 400,000 Daltons) that are linked by disulfide bonds in a cross formation (Liotta, 1983), with 3 chains being 200,000 Daltons (short arms) and 1 chain being 400,000 Daltons (long arm). Laminin has been implicated in the adhesion mechanisms between ECM components, particularly Type IV collagen and PGs, in the several cell types including breast epithelial cells (Terranova et al., 1980) guinea pig epidermal cells, bovine lens epithelial cells and monkey pigmented epithelial cells. Laminin binds preferentially to Type IV collagen and promotes the adhesion of epithelial and endothelial cells (Terranova et al., 1980). It has also been shown that laminin binds to proteoglycans with the highest affinity being to heparin and heparan sulfate (Del Rosso et al., 1981). Certain cells such as metastatic T241 fibrosarcoma cells (Murray et al., 1980) will adhere to Type I collagen via laminin. Cells that adhere via laminin can synthesize their own laminin (Terranova et al., 1980) and the same is probably true of cells requiring fibronectin for adhesion (Dessau et al., 1978). Cells that are capable of synthesizing both proteins are likely capable of utilizing both for adhesion (Foidart et al., 1980).

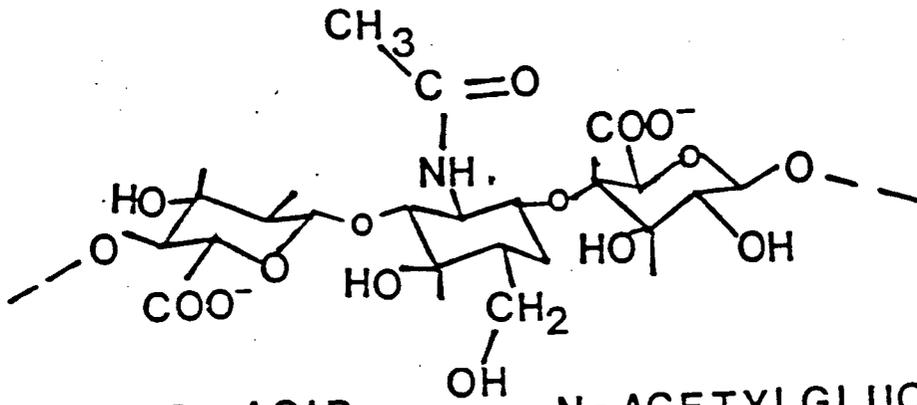
Entactin is the most recently discovered glycoprotein and also appears to be a component of many basement membranes (Bender et al., 1981; Carlin et al., 1981). In an ultrastructural study of the basal surfaces of epithelial cells after exposing them to antibodies to the protein, Bender et al. were able to localize entactin in close association with these surfaces. Little is known as yet regarding the function of the glycoprotein in the basement membrane.

## Proteoglycans

Proteoglycans are another class of molecules abundant in the BM as well as the ECM in general (Kleinman, 1982). Proteoglycans are long chain polymers of repeating disaccharides with either carboxyl or sulfate groups (Toole, 1982). The disaccharide unit is made up of either a glucuronic or iduronic acid residue, with either N-acetyl-D-glucosamine or galactosamine. The number of sugar residues can vary from 300, which is a common amount with sulfated proteoglycans, to 2,000 to 3,000 residues seen in the average HA molecule. The proteoglycan is distinct from glycoproteins because of the high percentage (90-95%) of carbohydrate. Glycoproteins typically have less than 60% carbohydrate. Structurally identified proteoglycans include hyaluronic acid, chondroitin sulfate 4, chondroitin sulfate 6, dermatan sulfate, heparin and heparan sulfate. (Keratan sulfate is also an identified proteoglycan but, because it is related almost solely to cartilage and cornea, it will not be discussed further). A representation of two proteoglycans is presented in Figure 2.

Proteoglycans differ from each other in several ways. All proteoglycans in their native state are linked to a protein core with the exception of hyaluronic acid. An average protein core may contain 1900-2000 amino acid residues which are generally serine-rich. More recent research indicates that the protein core can vary markedly in size and amino acid content (Rapaeger et al., 1985). This difference in protein core may in part be responsible for the final location of synthesized proteoglycans (Rapaeger et al., 1985; Chang et al., 1985). This will be discussed further in another section of the thesis. Attached to the protein core are from 100-150 side chains of a particular carbohydrate sequence as shown in Figure 2. The protein core is attached covalently to the carbohydrate side

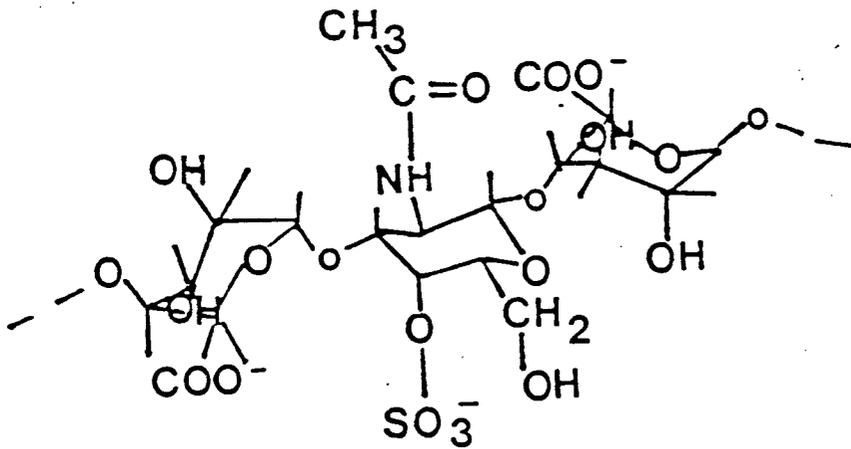
HA



GLUCURONIC ACID

N-ACETYLGLUCOSAMINE

DS



IDURONIC ACID

N-ACETYL GALACTOSAMINE  
(4-SULFATE RESIDUE)

Fig. 2 Chemical structures of HA(non-sulfated) and DS(sulfated).

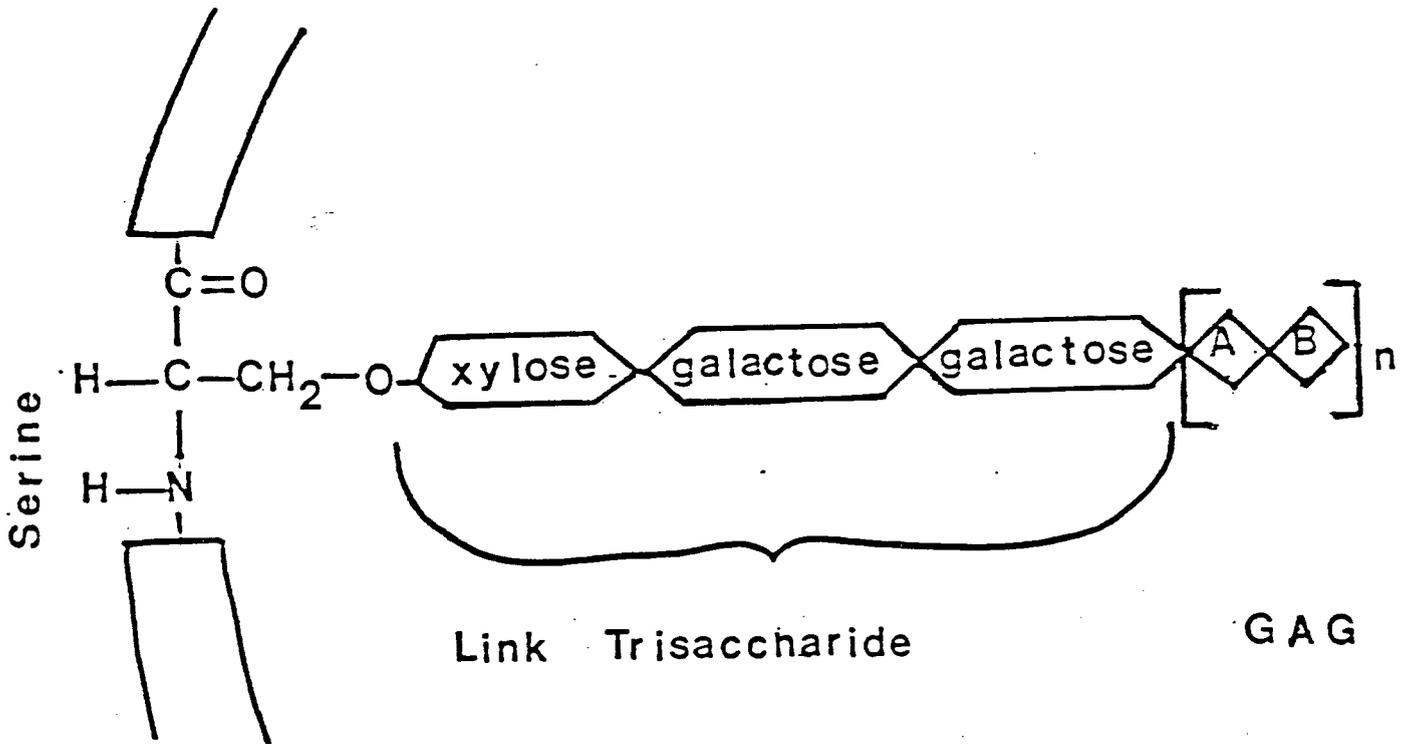


Fig. 3 The common link trisaccharide between the GAG component and the protein core of PG.

chain via a xylose-serine linkage (Figure 3). Historically, the proteoglycans were named according to their carbohydrate side chains and no account was taken at that time as to the nature of the protein core (Kraemer, 1979).

The proteoglycans differ from each other in the type of monosaccharide present in the repeating disaccharide unit. It is on the basis of this difference that several of the degradative enzymes differentiate between proteoglycans (Yamagata et al., 1968). HA, CS 4 and CS 6 all have glucuronic acid as one monosaccharide whereas dermatan sulfate and heparan sulfate have either glucuronic or iduronic acid. CS 4, CS 6 and dermatan sulfate all have N-acetyl-galactosamine as the second monosaccharide while HA and HS have N-acetyl-glucosamine.

The third area where proteoglycans differ is in their degree of sulfation. The following are listed according to degree of sulfation from least to greatest: CS 4, CS 6, DS HS. CS 4 is sulfated on the carbon 4 and CS 6 on carbon 6 of the N-acetyl-galactosamine residue (Kraemer, 1979). DS is similar to CS 4 but has epimerized  $\beta$ -D-glucuronic acid to  $\beta$ -L-iduronic acid (Kraemer, 1979). However, it appears that a large amount of heterogeneity exists in the CS and DS proteoglycans such that copolymers of both have been observed (Kraemer, 1979). They may contain sequences of iduronic acid interspersed with sequences of CS 4 or CS 6 either non-sulfated or disulfated on the N-acetyl-galactosamine residue. Heparan sulfate is unique in that it possesses an acid-labile sulfate group linked to the amine group of the hexosamine residue. HA is a non-sulfated proteoglycan. A summary of the proteoglycans can be seen in Table I.

Proteoglycans have had several functions attributed to them in their role as ECM components. They are generally believed to be involved in

TABLE 1: PROTEOGLYCANS<sup>1</sup>

Glycosaminoglycan	Molecular Weight	Repeating Disaccharide A	Repeating Disaccharide B	Sulfates/ Disaccharide Unit	Protein Core
Hyaluronic acid	4,000 - 8,000,000	D-glucuronic acid	N-acetyl-D-glucosamine	0	NO
Chondroitin Sulfate-4	500 - 50,000	D-glucuronic acid	N-acetylgalactosamine	0.2-1.0	YES
Chondroitin Sulfate-6	500 - 50,000	D-glucuronic acid	N-acetylgalactosamine	0.2-2.3	YES
Dermatan Sulfate	15,000 - 40,000	D-glucuronic OR L-iduronic acid	N-acetylgalactosamine	1.0-2.0	YES
Heparan Sulfate	5,000 - 12,000	D-glucuronic OR L-iduronic acid	N-acetylglucosamine	0.2-3.0	YES
Heparin	6,000 - 25,000	D-glucuronic OR L-iduronic acid	N-acetylglucosamine	2.0-3.0	YES

<sup>1</sup> Albert et al., 1983.

cell-substrate adhesions (Toole, 1982; Culp, 1976; Culp et al., 1979). Both HA and HS are present on the surfaces of cells (Culp et al., 1979; Rapraeger et al., 1985). These proteoglycans have also been shown to bind to fibronectin (Yamada et al., 1980) and laminin (Kleinman et al., 1981). From this information it would appear that proteoglycans not only help link cell surface to basement membrane (cell-associated proteoglycan) but also help to stabilize basement membrane components (BM proteoglycan) within this structurally defined area.

How do these molecules discussed thus far interact to form the structurally stable basement membrane? To summarize briefly, in some manner the glycoprotein present, with the help of proteoglycans, enhances or enables the cell to adhere to a particular type of collagen. There are numerous theories on the actual structure and several are shown in Figure 4. They mainly differ in the arrangement of the components within the basement membrane although they all demonstrate one or more receptors for ECM components on the plasmalemma. More recent studies (Laurie et al., 1985) using rotary shadowing electron microscopy, found laminin mainly bound to Type IV collagen 81 nm from the carboxyl terminus and large heparan sulfate bound to Type IV collagen 206 nm from the carboxyl terminus. Laurie et al. postulate a model whereby heparan sulfate binds to the collagen molecule in such a way as to allow interaction between the free long arm of laminin and the cell. However, they have also suggested other possible models from their data and this area of interaction of ECM components in the BM is by no means conclusive.

I will now discuss proteoglycans in depth as they are the basis of the thesis work.

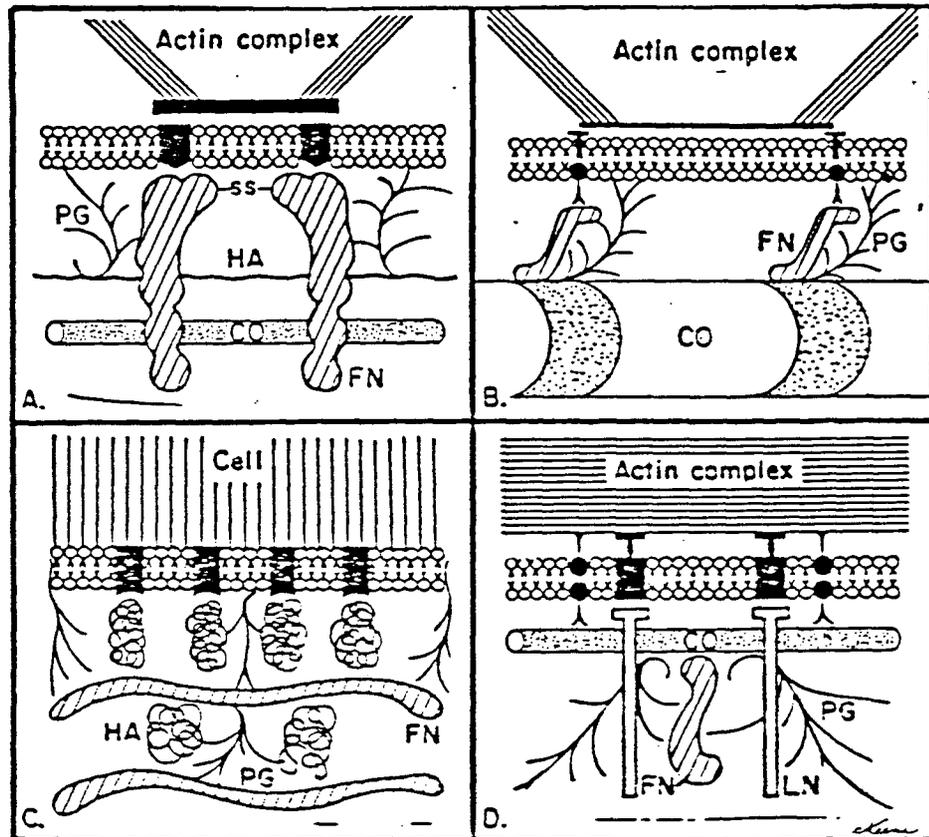


Fig. 4. Schematic representations of postulated ECM interactions.

- A. Based on Hynes(1981)
- B. Kleinman et al(1981)
- C. Toole(1981)
- D. Sugrue and Hay(1982)

HA= Hyaluronic acid  
PG= Proteoglycan  
FN= Fibronectin  
CO= Collagen  
LN= Laminin

Although they show different interactions between the ECM components, all models envision receptors at the cell surface for one or more of these components.

### Hyaluronic Acid

HA has been implicated in motility and growth of developing tissue (Toole, 1977). Toole et al. (1971) observed that the major proteoglycan being synthesized during chick embryo corneal migration is HA. They also observed that the onset of HA synthesis actually occurs before the onset of migration. HA has also been shown to promote detachment of a variety of cells including neural crest cells and dog kidney cells (Turley, 1984; Abatangelo et al., 1982). HA has been implicated in the ability of tissue to reach high degrees of hydration (McCabe, 1972). Hydrated tissues exert a hydrostatic pressure capable of opening pathways through the tissue to allow for migration of cells. Coupled with this function are the weak adhesions or bonds formed by HA (Toole, 1977). HA has been noted in footpads of motile fibroblast cells (Culp, 1976; Latterra et al., 1982) and radiolabelled and fluorescent hyaluronate occurs in retraction fibers (Turley, 1984). Its presence here may be related to the ability of HA to "lubricate" an area (Culp, 1976) and allow the cell to slide along unimpeded by strong cell-substrate attachments.

HA has also been implicated in the prevention of precocious differentiation (Toole et al., 1972). Small amounts of hyaluronate added to high density cultures of stage 26 chick embryo somite cells inhibit the formation of cartilage-like nodules that otherwise develop. Coinciding with this finding are the results of studies on chick embryo sclerotomal cells and chick embryo limb mesoderm hyaluronidase activity (Toole, 1972). These cells, as well as the corneal cells, show a large increase in hyaluronidase activity at the end of the active cell migration stage. This enzyme removes HA and signifies the onset of differentiation. The effects of HA on growth as opposed to migration are not well documented. In

studies involving embryonic tissue, growth and migration are occurring simultaneously and it is therefore difficult to separate the effect of HA on each.

It has been suggested that with regard to certain cell types, HA does not promote locomotion. Neural crest cells (Erickson et al., 1983) show little or no locomotion when seeded on a hyaluronate-coated substratum. The adhesion ability of HA also now appears more cell specific than originally thought. Although, as stated earlier, HA is associated with weak cell-substratum and cell-cell interactions, for certain cells HA does act as an adhesion mediator. For example, HA is involved in the attachment of SV40-3T3 cells to a sulfated proteoglycan substratum (Toole, 1982) and the attachment of chondrocarcinoma cells to tissue culture surfaces (Mikuni-Takagaki et al., 1980). The type of involvement of HA in adhesions may be related not only to the cell type but also the type of substrate the cell is adhering to, the time in culture and the developmental sequence (Turley, 1984). This may relate to the ability of a cell to synthesize HA binding sites, the availability of HA or the number of binding sites available to interact (Underhill et al., 1981). In general, HA has mainly been found to mediate adhesion to non-fibronectin substrates (Schubert et al., 1982; Brennan et al., 1983) as opposed to other proteoglycans, which mediate adhesion to both fibronectin and non-fibronectin substrates (Schubert et al., 1982).

A final point about HA is in regard to its ability to aggregate other proteoglycans. There is a binding site on the protein core of some proteoglycans containing CS 4, CS 6 or DS for HA (Hascall et al., 1981; Oegema et al., 1981). As a result, HA is able to form large aggregates of these molecules. It may be via this mechanism that cell-associated HA

(Turley, 1984) may attach to proteoglycans present in the basement membrane thereby anchoring the cell to the substratum. The role of HA as a binding molecule is not fully understood and is currently under investigation (McCarthy et al., 1985; Lacey et al., 1985; Marks et al., 1985). McCarthy et al. (1985) examined the mechanisms by which extracellular aggregates of PGs are maintained at the chondrocyte cell surface. They were able to determine in both normal chick and Swarm rat sarcoma chondrocytes that 50-60% of the aggregated proteoglycans are held at the cell surface via interaction with hyaluronate, which is susceptible to streptomyces hyaluronidase. In preliminary studies they have been able to identify hyaluronate-binding sites at the cell surface and believe it may be via these receptors that the HA-aggregates attach. HA binding sites are also being identified on a number of other cells including 3T3 cells (Lacey et al., 1985), adult chick brain (Marks et al., 1985) and chick neural crest cells (Turley, 1984). As research continues in this area the role of HA will become clearer, however, at this point it would be reasonable to assume that HA is not simply a proteoglycan that hydrates tissue and allows for ready detachment and motility of cells, although assistance with cell motility is almost certainly a function of proteoglycans (HA and others) as treatment with DON will stop cell movement in vitro (Turley, 1980). DON acts by inhibiting the synthesis of glycosaminoglycans via inhibition of the formation of glucosamine, a precursor for GAG. Here again the results of experiments on different cell types reveals that the response to HA appears to be cell-type specific. For example, chick heart fibroblasts will migrate into collagen gels (Bernanke et al., 1979) and move over a plastic culture dish when hyaluronate is added to the medium (Turley, 1984). Conversely, its addition to 3T3 cell cultures (Turley, 1984)

leucocytes (Forrester et al., 1981) and neural crest cells (Erickson et al., 1983; Newgreen et al., 1982) either does not affect motility, as in the case of the 3T3 cells, or inhibits motility, as in the case of the latter two cell types. Again, is it a question of availability of HA, HA receptors or both or do these cells type not respond to HA under any circumstances; rather, do they utilize another molecule or mechanism for motility? It is also possible that HA may bind competitively with other proteoglycans causing inhibition of motility.

To summarize the functions of HA in the ECM and specifically the basement membrane it appears in some instances to aid in cell detachment and facilitate motility and in others to act as an adhesion molecule to maintain cell-substrate proximity. In some cells HA is found in cell lamellae in others it is dispersed over the entire cell surface (Turley, 1984). It has been reported to be in close association with the actin component of the cytoskeleton of 3T3 cells (Lacey et al., 1985).

#### Sulfated Proteoglycans

Sulfated proteoglycans have been linked with cytodifferentiation (Trelstad et al., 1974; Praus et al., 1971). In their studies on chick embryo corneal development, Trelstad and co-workers found that, coinciding with the onset of differentiation, the major proteoglycan present changes from HA to sulfated proteoglycan (they did not identify specific proteoglycans). Toole et al. (1977) also analysed 4 and 8-day chick embryo limb chondrocytes for chondroitin sulfate. They determined experimentally that the CS formed at the earlier day is less reactive to collagen (decreased binding) and less sulfated and of a smaller molecular weight than the CS from the later day when the cells are further differentiated. However, as with HA, the effects of CS on various cell types are

dissimilar. For example, when added to cultures of fibroblasts, yolk sac or neural crest cells it inhibits spreading and causes detachment of cells from the substrate. Due to the poor spreading ability and rapidity of movement associated with CS (Turley et al., 1979; Erickson, 1984), this proteoglycan is thought to have weakly adhesive properties. In some cells, such as human melanoma cells (Reisfeld, 1984), an antibody specific to CS stops the initial spreading of these cells over basement membrane components.

CS has been found at the cell surface of mammalian skin fibroblasts where it interacts with the cell membrane via its carbohydrate chains (Saito et al., 1972) or via its protein core, which does not appear to be intercalated with the cell membrane but held by another "attachment" molecule (Glossyl et al., 1983). It is not known if there is a specific binding site for this proteoglycan but sites have been isolated which appear to have a high affinity for CS and CS hybrid or dermatan sulfate molecules (Glossyl et al., 1983; Truppe et al., 1978).

Heparan sulfate, unlike HA and CS, is known for its ability to form adhesions between ECM components (Hook et al., 1982; Keller et al., 1982). HS binds to both fibronectin and laminin (Yamada et al., 1980; Hynes et al., 1982; Woodley et al., 1984) and is present both on the surfaces of cells and in the ECM deposited by cells (Culp et al., 1979). Reports show that for some cell types, including neural crest (Erickson et al., 1983) 3T3 cell lines (Turley, 1984) and neuronal cells (Stamatoglou et al., 1983), the addition of HS to the culture medium supports both attachment and spreading. Conversely, heparan reduces attachment of CHO cells (Klebe et al., 1982) and M3A cells to fibronectin-containing substrates. One reason for this discrepancy may be in the method of analysing attachment

of cells. Laterra et al. (1983) find that if they looked at attachment rather than cell-removal assays, the addition of heparan to their cultures of fibroblasts does not inhibit adhesion to fibronectin and treatment with heparinase does not detach the cells. Woodley et al. (1984) find that HS binds preferentially to laminin over fibronectin and to Type IV collagen over Types I to III. They did not find that the addition of HS to either a Type IV collagen or laminin premixed coated culture dish increases the binding between the two (collagen and laminin). Others have reported that HS does enhance the binding between fibronectin and collagen (Johansson et al., 1980). It would appear, however, that HS is, in some way, related to adhesion as it is often located in footpads and adhesion sites (Lark et al., 1984). HS has been shown to bind to rat liver cells via its carbohydrate chains (Kjellen et al., 1977) and to mouse mammary cells via direct intercalation of the protein core into the plasma membrane (Rapraeger et al., 1985). Although it has been shown, as stated, that HS does enhance binding of glycoproteins and collagen to the cell surface it has not been clearly demonstrated that it acts as a cell receptor for these molecules (Culp et al., 1982). It is known that, unlike CS, which can be aggregated by HA via a binding site on the CS protein, HS does not have such a binding site or, at least, does not aggregate around an HA molecule (Culp et al., 1982).

HS has also been implicated in kidney function where it is found to be cell membrane associated and also present in the glomerular basement membrane (Kanwar et al., 1979). It is believed to play an important role in providing a selective barrier to molecules trying to pass into the kidney tubules. A HS molecule (MW 750,000) was isolated from the basement membrane of kidney tumor tissue (Hassell et al., 1980) and an antibody to

this molecule (to the protein core) demonstrated its presence in EHS tumor basement membrane and in normal kidney tissue basement membranes (Kanwar et al., 1979).

To summarize, the sulfated proteoglycans appear to play a major role in cell-substrate adhesion, organization and continuing stability of the basement membrane and are present in greater amounts when cells are beginning to differentiate or at least to have stopped growing and / or migrating. The same could be said of the proteoglycan HA. It appears that proteoglycans involved in functions such as adhesion for some cell types are not involved in the same function in other cell types: ie., what one type of proteoglycan does for one cell type another proteoglycan does for another cell type or, some functions may not be required by all cells. It would then seem important to analyse the functions of proteoglycans within a specific cell type and not assume that what one proteoglycan does in one instance will hold true for all cells.

#### Mammary Epithelial Cells

In the area of mammary epithelial cell research it has been reported by Silberstein and Daniels (1982) that growing mouse mammary epithelial cells synthesize predominantly HA in vivo and it is found in the basement membrane. The non-growing epithelial cells synthesize predominantly sulfated GAG, which is further identified by enzyme degradation to be CS. Parry et al. (1985) analysed proteoglycan synthesis by mouse mammary epithelial cells in vitro. The cultures, all nongrowing, show variations in PG synthesis depending on the type of substrate on which they are maintained. They produce HA, CS 4, CS 6, DS and HS in varying amounts and in different locations (cell, ECM and medium). Chandrasekaran et al. (1979) find that the normal human mammary cell line, HBL-100, maintained on

a plastic substrate, synthesize mainly HA and it is present in the medium. It was not stated, however, whether these cells are growing or at some level of differentiation, therefore it is difficult to compare results of this work with the previous two. This exemplifies the problems encountered when comparisons are made between experiments. Furthermore, Silberstein and Daniels (1982) looked at virgin mouse mammary gland that had not been stimulated to differentiate as were the mouse mammary cells in Parry et al.'s work. Whereas Silberstein and Daniels and Parry et al. report proteoglycans in an ECM, Chandrasekaran et al. did not analyse an ECM fraction. Parry et al. find that their mouse mammary epithelial cells synthesize almost equal amounts of HS and CS and these are found in an ECM while Silberstein and Daniels find mainly CS in the BM of the so-called "stabilized" non-growing cells. However, Parry et al.'s cells were exposed to hormones designed to facilitate differentiation (insulin, cortisol and prolactin) while Silberstein and Daniels' mouse mammary glands were not. Another difference in experimental design that may account for discrepancy in like cell types and proteoglycan synthesis is whether the cells are observed in vitro or in vivo. It has been demonstrated that cells in culture synthesize greater amounts of proteoglycan and matrix components when compared to their counterparts in vivo (Muir, 1977; Nevo et al., 1984). A variation as subtle as changing the feeding schedule from daily to every other day has been shown to alter the ECM components synthesized by chick chondrocytes (Katagiri et al., 1981). So standardizing experimental procedure is very important.

#### Proteoglycans and Malignancy

If proteoglycans play an important role in normal cell functioning, as the many studies cited so far indicate, might malignant cells show altered

proteoglycan synthesis and composition? Studies began on proteoglycan synthesis and malignancy in the early 1970's. Since then, results have accumulated indicating that malignant cells do indeed have altered proteoglycan synthesis compared to normal counterparts, both in the type and amount produced. The results, however, are extremely varied. In their study on human thyroid tissue, Shishiba et al. (1984) compared normal to adenoma and adenocarcinoma with respect to proteoglycan synthesis. Normal tissue contains mainly HS (Shishiba et al., 1983) while malignant tissue contains either a mixture of HS (60%) and CS or DS (40%) or mainly CS and DS (90%). As well, in the two malignant tissue samples examined one contains a 2-3 fold greater amount of proteoglycans and the other 6-15 fold greater when compared to the normal. Iozzo et al. (1982) found in normal human colon tissue a large HS proteoglycan and a smaller DS proteoglycan in about equal amounts. In colon carcinoma cells (Iozzo, 1984) there is less HS synthesized and the predominant proteoglycan is a small CS. The HS found in the malignant cells is either cell-associated or released into the medium and has distinct structural differences from the normal tissue HS, in that the malignant HS is of a large hydrodynamic size, higher bouyant density and has shorter GAG side chains than the normal synthesized HS. These differences caused Iozzo to hypothesize that these HS macromolecules may be responsible for altered surface properties seen on neoplastic cells (Iozzo, 1984). The list of altered proteoglycan synthesis goes on - an increase in CS in human lung carcinomas (Hatal et al., 1977) and human hepatoma (Kojima et al., 1975) has been found. The addition of CS to in vitro and in vivo human mammary carcinoma cells (Takeuchi, 1965; Ozzello et al., 1960) show growth-stimulating results. The addition of chondroitinases (to degrade CS) can retard the growth of these cells as

well (Takeuchi et al., 1972).

Toole et al. (1979) find that rabbit V<sub>2</sub> carcinoma cells grown in rabbits have 3 to 4 times as much HA synthesized at the interface between tumor mass and connective tissue than do the same cells injected into nude mice. In rabbits these V<sub>2</sub> carcinoma cells are invasive while in nude mice they are not. These same findings occur regardless of the site chosen for injection of the cells. They conclude that HA provides an environment conducive to invasion of the carcinoma cells into surrounding tissues. They propose that the HA enables cells to migrate by exerting force via increased swelling in tissues to open pathways along cell-collagen layers. Chandrasekaran et al. (1979) analyzed proteoglycan synthesis by two human breast carcinoma cell lines, MCF-7 and MDA-MB-231, and its location either in the medium or cell (intracellular or cell-associated). They reported that the predominant proteoglycans synthesized are CS and HS with HA being a very minor (0-12%) component and the majority of it (HA) present in the medium. This is opposite to the findings they reported for the normal cell line (HBL-100) under the same conditions (Chandrasekaran et al., 1979). In that case, HA is the predominant GAG (90%) with very little detectable HS (3%). They did not find a difference in the amount of proteoglycan synthesized per cell between normal (HBL-100) and the malignant (MDA-MB-231) cells. This is contrary to the other reports (Shishiba et al., 1984; Angello et al., 1982) that do find an increase above normal in proteoglycan synthesis in malignant tissue. The MCF-7 cell line actually synthesizes much less than the two other lines (MDA-MB-231 and HBL-100).

Angello et al. (1982) looked at two sub-populations of a mouse mammary cell line. The -SA cells (no growth in soft agar) are non-aggressive in vivo (slow growth) while +SA cells (growth in soft agar) are aggressive and

grow rapidly in vivo with a short tumor latency period. Both appear morphologically similar. It is found that +SA cells in culture incorporate 8 times more labelled glucosamine than do the -SA cells and the major identified proteoglycan is HA. Although both sub-populations have approximately the same percentage of total PG located in the 3 fractions analysed (medium- 12 to 15%, cell surface - 50-58%, cell - 30-34%) the +SA synthesizes 57.7% HA and only 29.9% HS while -SA synthesizes 40% HA and 46.3% HS. They conclude that epithelial cells are able to condition their environment and that the conditioning with increased HA encourages proliferation. Other studies have also shown increased HA in mammary adenocarcinomas (Palmer et al., 1979; Takeuchi et al., 1976). Takeuchi et al. (1976) examined the proteoglycan content of eleven human breast tumors. They find that in six of the cases HA is the predominant GAG (between 25 and 70  $\mu\text{g}/\text{mg}$  dry wt.) and HS is the least (less than 2  $\mu\text{g}/\text{mg}$  dry wt.). The chondroitin sulfates are next in amount followed by DS. In the other 5 cases HA, CS, and DS are relatively equal and lower (under 10  $\mu\text{g}/\text{mg}$  dry wt.). Again HS is lowest. (less than 2  $\mu\text{g}/\text{mg}$  dry wt.). Angello et al. (1979) suggest that sub-populations in heterogenous tumors may establish a matrix that not only promotes their growth but also the growth of other sub-populations.

As with normal cells, the discrepancies seen in the literature regarding PG synthesis and malignancy may be related to several factors. Some results are reported from experiments carried out on whole tissue recently removed from a patient (Shishiba et al., 1983; Takeuchi et al., 1976). Others are based on cells in culture and the majority of these are cell lines (Iozzo 1984; Angello et al., 1982; Chandrasekaran et al., 1979). As reported earlier in the Introduction, normal proteoglycan synthesis appears

to be specific to cell type. The same may be true for malignant cells. The type of proteoglycan produced by a malignant cell may also be related to its degree of differentiation and invasiveness or metastatic potential. For example, malignant cells in the process of rapid growth may synthesize one major proteoglycan (Toole et al., 1979) while cells that have "travelled" to a new site may wish to adhere and "settle down" and may synthesize a different proteoglycan (Iozzo, 1984). Numerous researchers (Iozzo, 1984; Liotta et al., 1979; Bauer et al., 1979) have pointed out that it may be the stromal tissue surrounding the tumor cells that is stimulated to synthesize various altered matrix components. Interestingly, the small CS molecule associated with the colon carcinoma cell was discovered by autoradiographic techniques not to be produced by the colon cells but rather synthesized by the mesenchymal cells in the surrounding CT stroma (Iozzo et al., 1982). Alternately, these stromal cells may be synthesizing a factor designed to increase or decrease synthesis of matrix components by the tumor cells. David et al. (1981, 1982) discovered that the reason the transformed NMuMG cells (mouse mammary epithelial cell line) they studied does not accumulate a proteoglycan-rich BL on collagen substrates is because they are unable to decrease proteoglycan degradation. The normal NMuMG cells incorporate proteoglycan into a distinct basal lamina and have the same synthesis rate as the transformed cells. However, when these same normal cells are cultured on a plastic substrate, they become unable to decrease PG degradation.

An obvious problem associated with studies of malignant cell types and proteoglycan synthesis is the lack of data on normal tissue to be used as a comparison, coupled with the fact that proteoglycans synthesized by different cells do not appear to influence the same functions in all cells.

### Thesis Problem Formulation

Due to the obvious influence of PGs on cell function, it seemed appropriate to determine the type and location of proteoglycans synthesized by normal human mammary epithelial cells in culture during the exponential and stationary phases of growth and how they relate to normal cell function. This is not only important in understanding the normal cell-proteoglycan relationship but also necessary for establishing if alterations occur in the malignant cell.

Normal human mammary epithelial cells were obtained from reduction mammoplasties. The tissue was processed to select for epithelial cells and the cells were grown on collagen gels, as it has been demonstrated that epithelial cells grow better on a substrate derived from components of basal lamina or stromal tissue than on a plastic substrate (Emerman and Pitelka, 1977; Hay, 1981; Kleinman et al., 1981; Michalopoulos et al., 1975; Richards et al., 1982; Wicha et al. 1979, 1982; Yang et al., 1979). The proteoglycans were labelled with  $^3\text{H}$ -glucosamine added to the medium 24 hours prior to termination. Cultures were terminated at growing and nongrowing stages as determined by DNA analysis.

Analysis of proteoglycans was accomplished by identification of the glycosaminoglycan side chains. The proteoglycans were treated with a non-specific protease to cleave the carbohydrate side chains from the protein core and the latter was discarded. The GAG portion was identified by enzymes specific to each and by cellulose acetate electrophoresis (Parry et al., 1985; Angello et al., 1982; Kanwar et al., 1982; Crawford et al., 1984; Iozzo, 1984; Shishiba et al., 1984).

The information gained from these experiments on normal human mammary epithelial cells in culture provides a baseline with which to compare the

effects of various factors on GAG synthesis and function. Such factors include a) hormones b) substrates c) differentiation d) receptors and e) malignancy. Of particular interest is the effects of various hormones controlling mammary growth and differentiation on proteoglycan synthesis. Very little information pertaining to this area can be found in the literature (Kidwell et al., 1982). The addition of various proteoglycans (GAGs) either to the medium or incorporated into a substrate would render information regarding the effects of specific GAGs on cell function. For example, would creating a substrate rich in the proteoglycan found in non-growing cells cause growing cells to stop growing or would growth be stimulated in quiescent cells if they were on a substrate rich in the proteoglycan found synthesized by growing cells? Would adding the proteoglycan (GAG) to the medium be as effective as its presence in a substrate? Another large area open for investigation is that of GAG receptors. The presence of GAG is defined in this thesis as being medium, ECM (includes cell-associated) or cell (intracellular) located. It would help in the understanding of GAG function to know which GAGs are cell-associated and how (via receptors, intercalated by the protein core) and which are basement membrane associated. Finally the results of this study could be used as a baseline with which to compare results of malignant human mammary cells grown under the same conditions. It would enable some conclusions to be drawn regarding the effects of altered GAG synthesis on the growth and maintenance of the altered phenotype.

## MATERIALS AND METHODS

### Dissociation Procedure

Tissue was obtained from reduction mammoplasties. A box was delivered to the operating room containing 3 to 4 250 ml cups each containing 100 ml of sterile transport medium (see Appendix 1) on ice. Samples were brought back to the culture room where, under sterile conditions, excess fat was removed from the glandular portions using a scalpel and scissors. The fat was discarded and the glandular portions were minced finely using 2 scalpel blades then placed in a 250 ml flask containing 50 ml of dissociation medium (Appendix 2). The flask was then placed in a 37°C incubator and kept stirring for approximately 22 h. Of the three samples used for this thesis, one was dissociated after 18 h, one at 21 h and one at 22 h. The dissociation was considered complete when only small aggregates of cells remained. The solution was then divided equally in 4 15 ml centrifuge tubes and centrifuged in a clinical centrifuge (Fisher Scientific) for 4 min at 800 rpm (80xg). This centrifugation speed was designed to preferentially pellet the epithelial cells present in the sample. The supernatant was discarded and the pellets were combined and resuspended in 100 ml of DME. The tubes were centrifuged again using the clinical centrifuge for 4 min at 1000 rpm (100xg). The supernatants were removed and the pellets were washed a second time in DME. The washes removed any remaining collagenase. After a final centrifugation as above, the supernatant was discarded, and the pellet was resuspended in 5 ml of growth medium (Appendix 3) and put through a 150µm Nitex filter to decrease clumping of cells. For cell counting, 0.1 ml of the cell suspension was removed and placed in a 2 ml tube. The remaining cell suspension was placed in a 37°C water bath while counting took place. A minute drop of

trypan blue (pH 7.2) was added to the 0.1ml solution to distinguish viable cells from dead cells. Counting was done using a hemocytometer .

If the total number of cells was greater than that required for the experiment, the extra cells were pelleted and resuspended in freezing medium (Appendix 4) at a concentration of approximately  $1 \times 10^7$  cells / ml. They were quick frozen and immediately transferred and stored in a cryogenic tank at  $-70^{\circ}\text{C}$  (Union Carbide).

#### Preparation of Collagen Gels

The collagen solution was prepared from rat tails in the following manner. The tails were placed in 95% alcohol for 15 min. The tendons were dissected out and teased apart using scalpel blades and forceps, weighed and placed in a 60 mm Petri dish containing sterile distilled water and exposed to the ultraviolet light in the tissue culture hood for 24 h. The fibers were then suspended in a dilute acetic acid solution (1:1000) and stirred at  $4^{\circ}\text{C}$  for 48 h. They were left to sit for another 24 h at  $4^{\circ}\text{C}$ . Finally, the solution was transferred into 50 ml centrifuge tubes and centrifuged in a Sorvall centrifuge at  $10,000\text{Xg}$  for 30 min. The supernatant, the collagen solution, was bottled and stored at  $4^{\circ}\text{C}$ .

To prepare the gels, a solution of medium 199 (10X concentration) was combined with 0.34N NaOH in a ratio of 2:1 to make a total of 0.4 ml of solution. This was added to 1.6 ml of the collagen solution and stirred together in a tube on ice to prevent premature gelling. The solution was then transferred to a 35 mm Petri dish. When 16 mm wells were used, the gels were prepared by combining 0.1 ml solution of the Medium 199 and 0.34N NaOH (2:1) and 0.4 ml of the collagen solution. The culture dishes were placed in an incubator (National, Inc.) at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  and 95% air for one

h to allow complete gelling of the solution. After that time, 2 ml of experimental medium was added to the 35 mm Petri dishes and 0.5 ml to the wells for 24 h prior to the experiment to allow equilibration.

#### Cell Culture Procedure

Initial cultures were seeded at of  $2-2.5 \times 10^5$  cells/cm<sup>2</sup> onto the hydrated collagen gels in 35 mm Petri dishes on wells. Identical cultures for DNA determination were grown concurrently with the cultures destined for glycosaminoglycan analysis. The cultures were incubated at 37°C, 5% CO<sub>2</sub> and 95% air and observed daily. The medium was changed every second day at the same time during the day. With daily observation under the phase contrast microscope, it was determined at what point the cultures were approximately 50% confluent and 100% confluent. The former were identified as growing and the latter as stationary cultures by DNA analysis. The first stage was attained between day 3 and 4, the second between days 9-11, depending on the growth rate of each particular culture. A third stage, between days 17-18, was identified as late stationary. Morphologically, the cultures appeared 85% epithelial. At these times, the cultures was labelled with <sup>3</sup>H-glucosamine in growth medium (S.A.=30-60 μCi/mmol; NEN, DuPont Canada, Inc.). The growth medium contained only insulin (5 μg/ml) and 5% FCS (necessary for growth). No hormones were added to stimulate differentiation.

Cultures were also seeded at a higher density of  $5.0 \times 10^5$  cells/cm<sup>2</sup>. These cultures were identified as stationary at day 4 by DNA analysis.

#### Radiolabelling Procedures

All experiments were labelled with <sup>3</sup>H-glucosamine at 100 μCi/ml in 1.5ml of growth medium. Two experiments were labelled with <sup>35</sup>S-sulphate

at 100  $\mu$ Ci/ml (NEN, carrier-free) instead of  $^3\text{H}$ -glucosamine. This was done as an alternate means of identifying synthesized sulphated GAG. All labelling was done for a 24 h time period.

#### Resolution into Fractions - Medium, Cell and Extracellular Matrix

After labelling the medium was removed from the culture and stored on ice in a 15 ml centrifuge tube. The gel was placed in a 1.5 ml Eppendorf tube (VWR Scientific, Inc.) and centrifuged in a microcentrifuge (Western Scientific Services) at 13,000xg for 5 min at 4<sup>o</sup>C. This was done to squeeze any medium out of the interstitial spaces. This medium was then removed and added to the original medium. The gel was then rinsed in approximately 1 ml of Tris-saline solution, pH 7.4 (Tris 10mM, 0.85% NaCl) for 30 min to remove additional trapped medium. This tube was also kept on ice during the time period and vortexed every few minutes. The gel was then spun down at 13,000xg for 15 min in the microcentrifuge at 4<sup>o</sup>C. The supernatant was added to the original medium tube.

To lyse the cells, the gels were extracted on ice with 1 ml of detergent solution (Appendix 5) and vortexed occasionally over a 30 min period. The gels were then centrifuged a final time at 13,000xg for 15 min at 4<sup>o</sup>C. The supernatant constituted the soluble cell fraction while the pellet was called the extracellular matrix fraction. They were separated and placed into 15 ml centrifuge tubes and stored on ice.

#### Extraction of Glycosaminoglycans

To each of these three fractions was added a nonspecific protease solution (Sigma, type XIV, Streptomyces griseus) at a concentration of 10mg/ml in 10mM Tris, pH 7.4. One ml of protease solution was added for

every 2 ml of fraction sample. A drop of sodium azide (0.01% w/v) was added to each tube to prevent bacterial growth. All fractions were then incubated in a water bath at 38<sup>o</sup>-41<sup>o</sup>C for 48 h. Fresh protease was added after 24 h of incubation. Some samples were left for 60-72 h. These were compared to those that were incubated for the 48 h time period to ensure no further enzyme action took place after that time.

Digests were precipitated with 10% trichloroacetic acid (Sigma) for 1 h at 4<sup>o</sup>C. They were then centrifuged using a tabletop centrifuge (Western Scientific Services) at 200-250 Xg for 10 minutes. The supernatants were saved and the pellets discarded. The supernatants were divided into 1.5 ml Eppendorf tubes (VWR Scientific) with each tube receiving 0.3 ml of the solution. The tubes were clearly marked so as not to mix the 3 fractions (medium, cell and ECM). A GAG mixture was prepared containing 1mg /ml of each of the following GAGs: hyaluronic acid (Sigma, umbilical cord), chondroitin sulfate type A (Sigma, whale cartilage), type B (Sigma, shark cartilage) and heparin (Sigma, porcine intestinal mucosa). Twenty-five  $\mu$ l of this mix was added to each Eppendorf tube. This acted as a cold carrier to help "bring down" labelled GAG. Each tube was diluted with 3 volumes of 90% ethanol/1.4% potassium acetate to give a final volume of 1.5 ml. The ethanol/potassium acetate solution acted to help precipitate labelled GAG. Each tube was vortexed and allowed to sit for 24 h at -20<sup>o</sup>C.

Precipitated GAGs were collected by centrifuging all Eppendorf tubes for 30 minutes in the microcentrifuge at 4<sup>o</sup>C and 13,000xg. The supernatants were discarded and the pellets resuspended in 0.3 ml of glucosamine (0.1 mM) in water using a Pasteur pipet. The tubes were then allowed to sit for 1 h at 4<sup>o</sup>C to ensure that the pellet completely dissolved. Afterwards

another 3 volumes of 90% ethanol/1.4% potassium acetate was added to each tube to bring the total volume to 1.5 ml. The procedure described above was repeated, that is, all tubes were vortexed and stored for 24 h at  $-20^{\circ}\text{C}$ . The whole precipitation procedure was carried out 3 times in total. After the final centrifugation, the pellets from one fraction (up to 12 Eppendorf tubes may contain pellets from one fraction) were pooled and the total sample of GAG was dissolved in 1.5ml of distilled water. The samples were marked MEDIUM, CELL or ECM. All were stored for at least 24 h at  $-20^{\circ}\text{C}$  before the identification of GAG procedure began.

#### Analysis of GAG by Enzyme Digestion

##### (1) Hyaluronic Acid Identification

*Streptomyces hyaluronidase* (500 units/ml, Sigma) was dissolved in 1 ml of 50 mM sodium acetate at pH 5.0. The assay was carried out in 1.5 ml Eppendorf tubes by adding 100  $\mu\text{l}$  of sample, 30  $\mu\text{l}$  of enzyme and 15  $\mu\text{l}$  of NaCl (1.5 M). All samples were run in triplicate. The samples plus enzyme were incubated at  $40^{\circ}\text{C}$  for 4 h. The enzyme was then inactivated by boiling in water at  $100^{\circ}\text{C}$  for 15 min. The samples were allowed to cool completely. A 25  $\mu\text{l}$  aliquot of the GAG mix (1mg/ml of each GAG) was added to each sample followed by 1 ml of 90% EtOH/1.4% KAC. Each tube was then vortexed and allowed to sit overnight at  $-20^{\circ}\text{C}$ . All tubes were then centrifuged (microcentrifuge, 13,000xg, 30 min,  $4^{\circ}\text{C}$ ) and the supernatants and pellets separated and counted (to be described under scintillation counting). One tube containing the pellet from each sample (MED., CELL, ECM) was kept for the next assay.

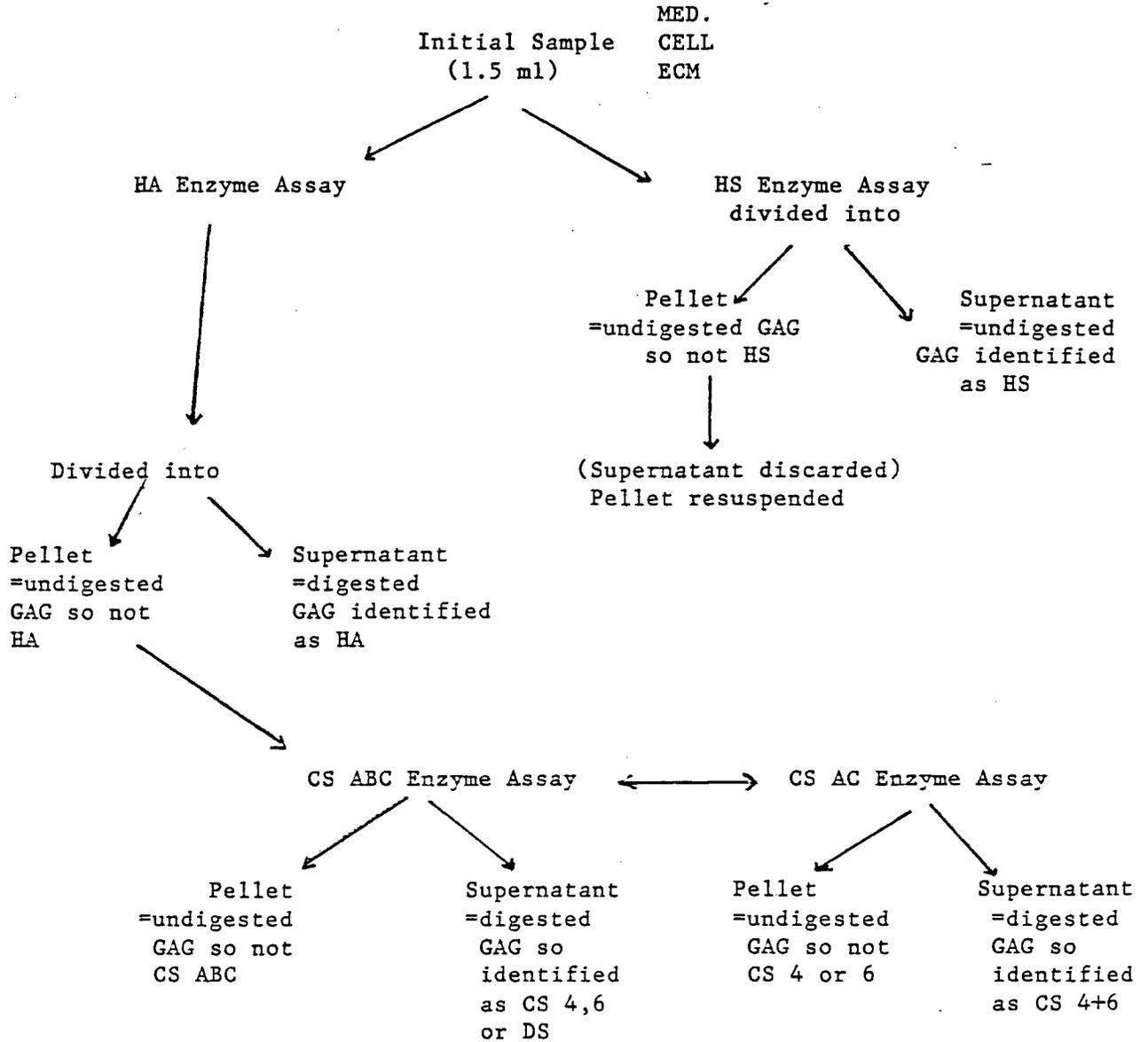


Fig. 5 Flow Chart of Identification of GAG by Enzymes

(2) Chondroitin Sulfate Identification

Two enzymes were used to identify chondroitin sulfate (Yamagata et al., 1968):

- (i) chondroitinase ABC (Sigma, 10 units/ml) - degrades all CS
- (ii) chondroitinase AC (Sigma, 10 units/ml) - degrades CS 4 and 6  
(not DS)

Because chondroitinase ABC will also digest HA, the samples used for these assays must have HA removed first. Therefore, the pellet from one of the triplicate tubes from the HA enzyme assay for each fraction was resuspended in 400  $\mu$ l of distilled water. These were used for the CS assays.

Both enzymes (CS ABC, CS AC) were dissolved separately in distilled water (Sigma, 10 units/ml). The assay was carried out in 1.5 ml Eppendorf tubes by adding 60  $\mu$ l of the resuspended pellet from the HA samples (MED., CELL, ECM), 60  $\mu$ l of the enzyme and 20  $\mu$ l of "enriched" Tris (Appendix 6) at pH 8.0. All samples were done in duplicate.

All samples were incubated at 37<sup>o</sup>C for 4 h after which time they were boiled in water at 100<sup>o</sup>C for 15 min to inactivate the enzyme. After cooling completely, 25  $\mu$ l of GAG mix (1mg/ml of each GAG) was added to each tube followed by 1 ml of 90% EtOH/1.4% KAC. All tubes were vortexed and allowed to sit overnight at -20<sup>o</sup>C. The next day all tubes were centrifuged in the microcentrifuge at 13,000xg for 30 min at 4<sup>o</sup>C and the supernatants and pellets separated and counted (to be described under scintillation counting).

(3) Heparan Sulfate Identification

One hundred  $\mu$ l of nitrous acid (prepared by adding 0.3 M sodium nitrate

and 2.9 M acetic acid) was added to 50  $\mu$ l of the initial sample of MED., CELL and ECM fractions in 1.5 ml Eppendorf tubes. All samples were done in duplicate. After incubating the tubes at room temperature for 80 min, the reaction was stopped by the addition of 50  $\mu$ l of ammonium sulfamate (2 M) to each tube and incubated at room temperature for another 30 min. One ml of 90% EtOH/1.4% KAC was added to each of the tubes which were then vortexed and stored overnight at  $-20^{\circ}\text{C}$ . The tubes were then centrifuged (microcentrifuge, 13,000xg, 30 min at  $4^{\circ}\text{C}$ ) and the supernatant and pellets separated and counted.

#### Scintillation Counting

Supernatants were placed in 20 ml glass scintillation vials (NEN Products) and 15 ml of scintillation fluid (Aquasol, NEN) was added. Pellets were dissolved in 200  $\mu$ l of distilled water and placed in 6 ml plastic scintillation vials (NEN Products) to which 5.8 ml of scintillation fluid was added (Aquasol, NEN). All vials were counted in a Mark II Scintillation Counter for 4 min. A quench curve was constructed from six known samples containing  $^3\text{H}$ -solutions with various amounts of quench. These samples were commercially purchased (Amersham/Searle, Ltd. and NEN, DuPont Canada, Inc.). A control containing only scintillation fluid was read with every set of vials to indicate background radioactivity. The scintillation counter delivered counts only in CPM (counts per minute) and as the amount of quenching was variable in the samples, the CPMs were converted to DPMs (disintegrations per minute) to remove the variable quench as a factor in the results.  $^{35}\text{S}$  results were read using a  $^{14}\text{C}$  quench curve and standards commercially purchased (Amersham/Searle, Ltd.).

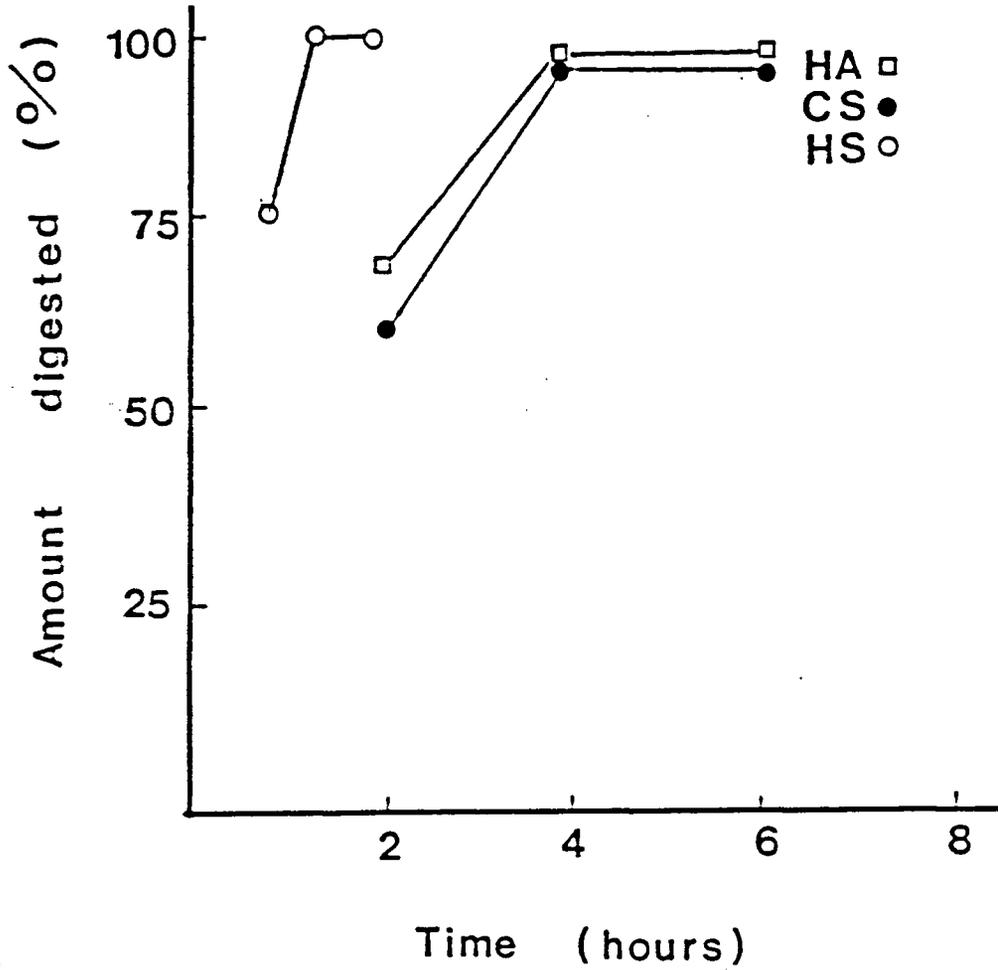


Fig. 6 Enzyme digestion over time. All enzymes were incubated with a known sample and the optimum time for maximum degradation of the substrate was chosen for subsequent assays.

### Calculation of Assay Results

The supernatant contained the amount of GAG which was degraded by the particular enzyme used and the pellet contained the amount of GAG which was not degraded by the enzyme. The ratio of the degraded to non-degraded was calculated as a percentage of the total and identified as the GAG attacked by that particular enzyme. All ratios were compared to standard samples which were run concurrently with all samples and contained all additives except the enzyme, which was substituted by an equal amount of distilled water.

Initially, enzyme assays were carried out over various incubation times (Fig. 6 ) and the times chosen for subsequent assays were those that permitted maximum degradation. To ensure that the enzymes were degrading the GAG in question, a sample of each GAG at 1mg/ml was spotted on a Whatman filter paper and stained with Alcian blue (1%) in water, pH 2.5. Enzyme was then added to each known sample and the incubation times carried out as described. Afterwards a second spot was placed on a filter paper and stained with Alcian blue. A positive result was obtained when the second spot did not stain, indicating the degradation of that GAG had occurred. This procedure was also carried out between all known standards and all enzymes to determine the specificity of each enzyme. It was noted that streptomyces hyaluronidase was specific to HA, chondroitinase ABC degraded HA, CS 4+6 and DS, chondroitinase AC degraded CS 4+6 and HA (slight decrease in staining after enzyme) and nitrous acid degraded only HS.

All enzyme assays were carried out in duplicates or triplicates.

### Identification of GAG Using Electrophoresis

The GAG present in each sample was also identified by cellulose acetate electrophoresis. Cellulose acetate strips (Gelman Sciences, Inc.) were soaked for 20 min in 100 ml of 0.15 M zinc acetate, pH 5.8, blotted dry and marked at one end as "standard" or "sample". They were then placed in an electrophoretic unit (Gelman Sciences, Inc.) containing 1 liter of 0.15 M zinc acetate, pH 5.8, so that both ends were in contact with the buffer. At the midpoint of the strip, 5  $\mu$ l of sample and 3  $\mu$ l of standard solution containing 0.5 mg/ml of each GAG were applied widthwise across the strip with a few centimeters separating the two. The output was from a constant power supply apparatus (LKB) and was set at 2 mA/strip and allowed to run for 3 h. After the allotted time, the strips were removed and placed in a solution containing 500 ml of 1% Alcian blue, 5% acetic acid and 10% EtOH for 0.5 h. The strips were then rinsed overnight in 5% acetic acid and water and dried flat. All standards were determined by running known quantities of each separate GAG and staining as outlined. The stained areas, corresponding to the known standards, were carefully cut out and placed in a 20 ml scintillation vial with 10 ml of Aquasol (NEN, DuPont Canada, Ltd.) and counted in a MARK II Scintillation Counter.

### Film Developing for Autoradiography

Several samples of each fraction (MED., CELL, ECM) separated and identified using the electrophoretic and staining procedures described above were further identified by autoradiography. In the darkroom, strips and x-ray film were secured by tape and held flat between two pieces of glass. These were then covered in aluminum foil and stored in a light-resistant bag and allowed to sit in a cool dark place for up to 6 wk

undisturbed. The strips from the nongrowing cultures were developed after 2 wk. Leaving these longer (up to 4 wk) did not improve the autoradiographs. However, the strips from growing cultures could not be developed before 6 wk. Even at 6 wk, the autoradiographs were faint. The stationary culture strips were found to contain 5-fold higher counts in each band representing a GAG than growing culture strips. A minimum of 1000 counts per 5  $\mu$ l sample applied was necessary to affect the  $^3\text{H}$ -sensitive film.

The film was developed in developer (Picker International) in distilled water (1:4) for 4 min, stopped in 5% acetic acid in water and fixed (Kodak Rapid Fix) for 10 min. The film was then rinsed for 30 min in running water and allowed to dry overnight.

#### DNA Determination

Cultures for DNA assay were frozen prior to DNA analysis to facilitate membrane lysing. Thawed gels were sonicated in 1 ml of  $\text{Na}_2\text{HPO}_4$  buffer, pH 7.4 (Appendix 7) for 3 min. Afterwards, a 300  $\mu$ l aliquot was removed, combined with 150  $\mu$ l of a Horchst dye solution (1 mg/ml of water) and vortexed to mix thoroughly (Labarca et al., 1980). All assays were done in triplicate.

The solutions were read immediately in a fluorospectrophotometer (American Instrumentation Co.) with an activating wavelength of 356 nm and a fluorescence wavelength of 458 nm. The results were compared with standards ranging from 10 - 100  $\mu$ g of DNA using a calf thymus DNA stock (Sigma) at 100  $\mu$ g/ml. Calculations of cell number were based on the fact that 7pg = 1 mammary epithelial cell (Kraenbuhl et al., 1981).

### Electron Microscopy

Cultures for electron microscopy were prepared by removing the medium and washing each gel 3 times with Karnovsky's solution (Appendix 8) then left for 1 h in this solution. After this, the gels were washed 3 times in 0.1 M sodium cacodylate, pH 7.4, and stored in this solution at 4°C until embedding. The embedding, sectioning and photography were carried out by Mrs. Hella Prochaska.

## RESULTS

### Growth

Normal human mammary epithelial cells were seeded onto collagen gel-coated 35 mm Petri dishes at  $2-2.5 \times 10^5$  cells /cm<sup>2</sup>. They were maintained in culture for 17-18 days. Early in culture, days 3 - 4, these cells formed sub-confluent monolayers with little evidence of cell stratification. Later in culture, at days 9 - 11, the cells were confluent and patches of stratified cells were evident. By days 17 - 18, the collagen gels had retracted from the culture dish and contracted to approximately 10-15% of their original size and the cells could not be visualized by phase-contrast microscopy.

Growth studies (Figure 7 ) show that days 3 - 4 cultures were in an active phase of growth. Cultures terminated on days 9 - 11 were in a stationary growth phase, indicated by those terminated on days 17 -18. Cultures varied by 2 - 3 days in the time it took them to reach sub-confluent (50%) and confluent states, as all cultures did not grow at identical rates.

To determine if high density inhibited growth in culture, several cultures were seeded at  $5 \times 10^5$  cells/cm<sup>2</sup>. No growth had occurred in these cultures by day 4 and the cell number was comparable to that of low density cultures terminated at day 9 - 11. It was concluded that cultures seeded at high density appeared to be in a stationary growth phase.

### Total Glycosaminoglycans Synthesis

Cultures of human mammary epithelial cells incubated with <sup>3</sup>H-glucosamine were terminated at 3 time points. Table 2 shows the

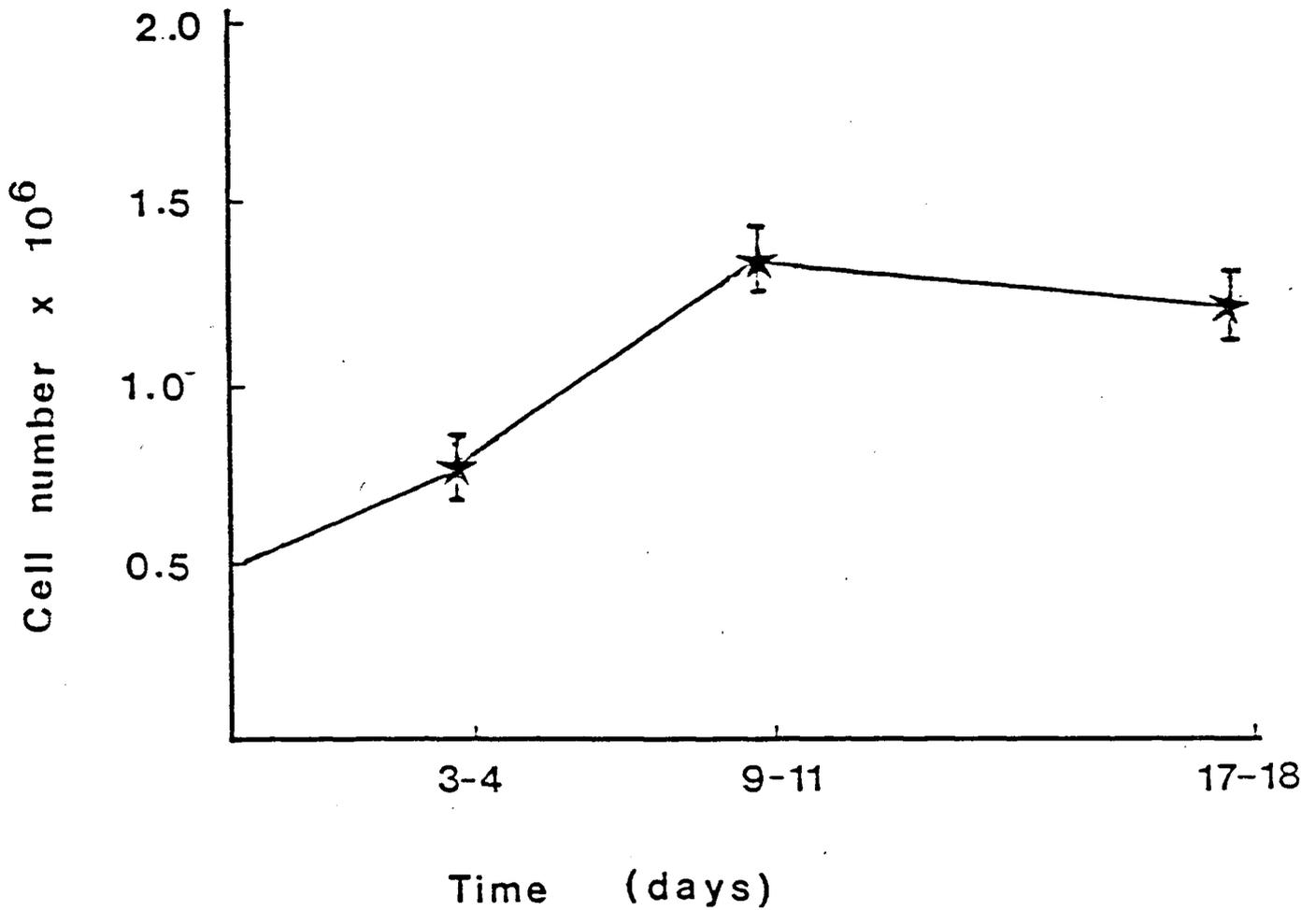


Fig. 7 Growth study of duplicate cultures of normal human mammary epithelial cells seeded at low density. Day 3-4 was considered growing; day 9-11 and 17-18 were considered stationary. Cell number was determined by DNA assay. Errors bars indicate SEM. (N = 3)

percentages of total GAG synthesized present in the medium, cell and ECM fractions during these time points. While the cells were proliferating (days 3 - 4) the majority of synthesized GAG was found in the medium fraction. The ECM contained approximately 1/5 of the total GAG synthesized at this stage of growth. When the cultures reached a confluent state and were no longer growing ( Table 2 - early stationary - days 9 - 11 in culture and late stationary - days 17 - 18 in culture ), the ECM contained approximately 1/2 of all the synthesized GAG.

To determine if the differences seen in localization and type of glycosaminoglycan synthesized by cultures of human mammary epithelial cells were due to their growth status and not a phenomenon of time in culture, two cultures were seeded at high density ( $5 \times 10^5$  cells/cm<sup>2</sup>) and terminated on day 4. The cultures, which were not growing on day 4, showed a pattern of GAG distribution similar to that of the nongrowing cultures (days 9 - 11 and later) seeded initially at low density (Table 2). This suggests that GAG localization was dependent on growth phase rather than time in culture.

When total GAG synthesized in each fraction is expressed per cell (Figure 8 ), the growing cultures showed the cells were releasing the majority of the GAG into the medium, a 4 - fold increase over the amount found in the ECM. By the early stationary growth phase both medium and ECM showed greater amounts of synthesized GAG / cell indicating that more overall GAG was synthesized at this stage. The GAG / cell found in the medium was less than 1-fold greater than that found in the medium of the growing culture. In contrast, synthesized GAG / cell in the ECM in early stationary cultures was 8 - fold greater than that of growing cultures. Also by the stationary phase the ECM had more synthesized GAG / cell than did the medium. The late stationary cultures (days 17 - 18) showed the

TABLE 2. Distribution of <sup>3</sup>H-glucosamine labelled glycosaminoglycans in normal human mammary epithelial cells in culture.

Culture Conditions	Medium	Cell	ECM
	% incorporation		
LOW DENSITY: <sup>2</sup>			
Growing	81 ± 1.50 <sup>1</sup>	3 ± 0.54	16 ± 1.59
Early stationary	39 ± 0.76	9 ± 1.22	51 ± 0.96
Late stationary	44 ± 2.26	6 ± 0.91	46 ± 2.25
High density:			
Stationary	28 ± 0.70	4 ± 0.70	68 ± 1.00

1. Mean ± S.E.M.

2. Refers to seeding

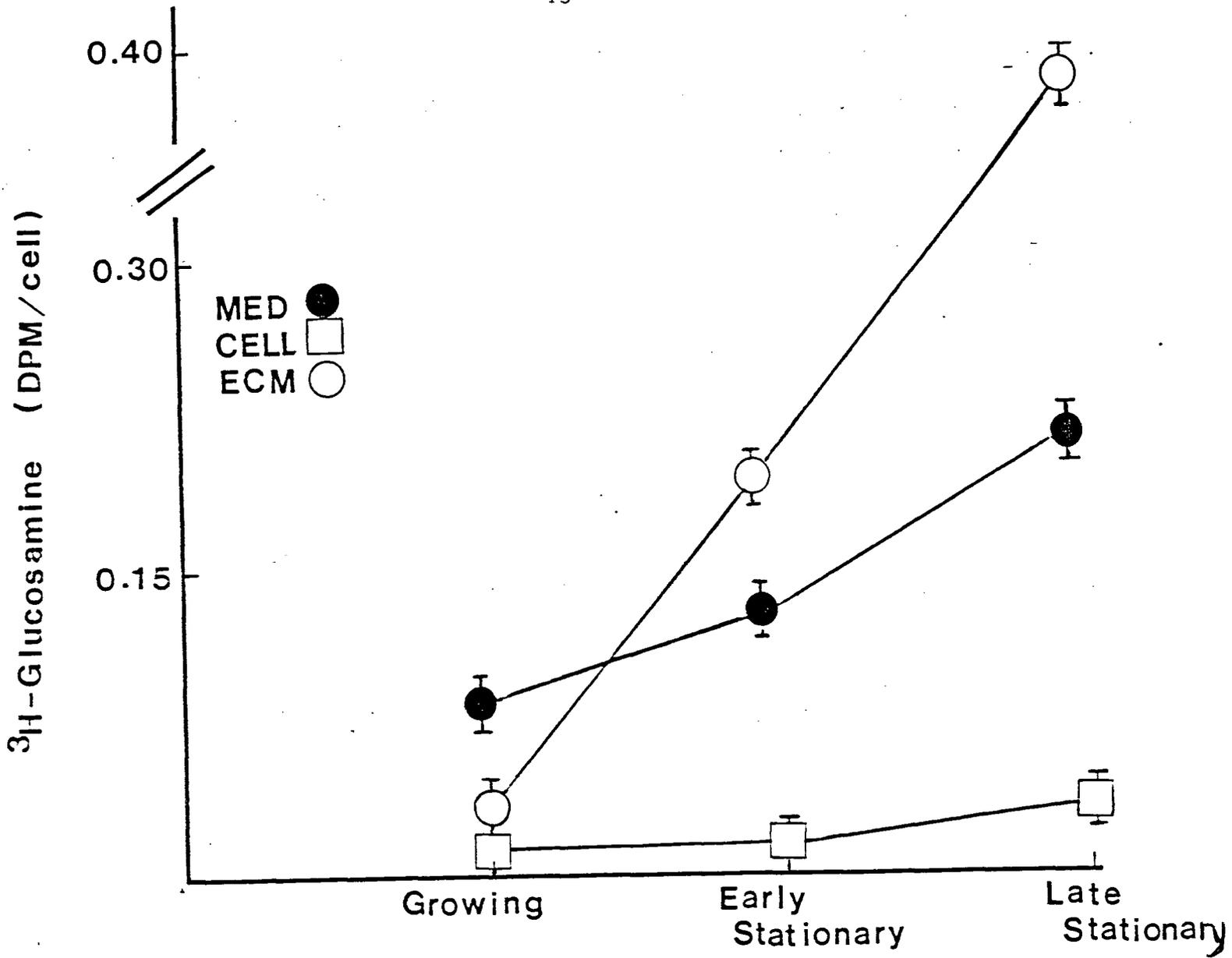


Fig. 8 Total  $^3\text{H}$ -glucosamine incorporation into glycosaminoglycans in cultures of normal human mammary epithelial cells. The medium, cell and ECM fractions were analysed at growing (days 3-4), early stationary (days 9-11) and late stationary (days 17-18) phases. Error bars indicate S.E.M. of three determinations of one experiment.

greatest variation in GAG synthesis. Of the 3 experiments, one synthesized a greater amount of GAG, one a comparable amount and one less than the amount of GAG synthesized by the early stationary cultures. Figures 8, 9 and 11 are representative of one experiment. Possible explanations for the variations in the late stationary cultures can be found in the Discussion.

The cultures that were seeded at high density and terminated day 4 closely resemble the early stationary cultures in both overall localization of synthesized GAG ( Table 2 ) and synthesized GAG / cell ( Fig. 11a and b ) indicating that these patterns were related to growth status and not time in culture.

#### Hyaluronic Acid Synthesis

Hyaluronic acid (HA) was the most predominant GAG present in the medium regardless of the growth state of the culture. In growing cultures the medium had more than a 7 - fold greater percentage of HA than sulphated GAG. In the stationary cultures the medium contained a 4 - fold greater percentage of HA ( Table 3 ). The percentage of total GAG identified as HA changed very little from the growing to confluent periods in the medium fraction and in the ECM. However, the percentage of HA in the medium was 20%-30% higher than the percentage found in the ECM at all times .

Although the total percentage of GAG identified as HA in each fraction did not change greatly between growing and confluent stages, the actual amount synthesized / cell did vary ( Figure 9 ). During growth the amount of HA was 5 - fold higher in the medium than in the ECM on a per cell basis. Once the stationary growth phase had been reached, while all fractions showed an increase in the amount of HA synthesized / cell, there was 4-5 times as much of the newly synthesized HA in the ECM as there was

in the ECM during growth while the medium showed less than a 1-fold increase. This brought the HA / cell in the medium and the ECM closer to a 1:1 ratio (Figure 9, 11a and b). In the growing phase, cells synthesized twice as much HA / cell when compared to the synthesis of sulphated GAGs. By stationary growth, the ECM had approximately a 1:1 ratio of HA to sulphated GAGs. This pattern was quite distinct from GAG synthesis into the medium which showed predominately synthesized HA, and had a ratio of nonsulphated to sulphated GAGs closer to 3:1 throughout all stages of growth.

In the cultures seeded at high density, where no growth occurred, the amount of HA synthesized / cell was very similar to the low density stationary cultures (Figure 11a and b). In the medium, the high density cultures had a 3:1 ratio of HA to sulphated GAGs ; in the ECM the HA to sulphated GAG ratio was 1:1.

#### Chondroitin Sulphate Synthesis

The total percentage of GAG identified as CS was less than that identified as HA in all fractions regardless of growth state ( Table 3 ). This difference was between 1.5 and 8 fold. When comparing the percentage of GAG identified as CS among the fractions analysed, the ECM had the greatest percentage of CS regardless of the growth state, between 1.5 - 3 fold higher than that found in the medium. This is in contrast to synthesized HA localization. The greatest percentage of HA was found in the medium regardless of growth state. Table 3 also shows the percentages of identified GAG synthesized by high density cultures. The percentage of CS in the 2 fractions of high density cultures were similar to that of the stationary cultures.

When the amount of synthesized CS was expressed / cell (Figure 10) the growing cultures showed that all fractions contained relatively small but similar amounts. By the stationary growth phase, while the medium and cell fractions showed some increase in the CS synthesized / cell (1.5 - 2 fold) the ECM fraction had the greatest increase, 10 - 12 fold. By the time growth ceased, the ECM also contained a 3 - fold greater amount of CS than did the medium. The percentage of synthesized CS in the ECM fractions of growing and early stationary phases showed only a 7% increase (Table 3). This was due to the large increase in GAGs synthesized in the ECM fraction by the early stationary cultures. The actual amount of CS synthesized / cell however, was 4 - fold greater in stationary versus growing cultures.

In the high density cultures terminated at day 4 ,the percentage of CS (Table 3) and the amount of CS synthesized / cell (Figure 11a and b) were similar, in both medium and ECM fractions, to the low density stationary cultures. This indicates again that the type and localization of synthesized GAG are functions of growth status and not a time-in-culture phenomenon.

The ratio of nonsulfated to sulfated GAGs has been described under the HA Synthesis section of the Results.

#### Dermatan Sulfate Synthesis

In all cultures, regardless of growth status, the ECM contained the highest percentages of synthesized DS. This amount ranged from 4 - 7 fold higher than that found in the medium of any cultures (Table 3). DS accounted for 19 - 20 percent of the sulfated GAGs in the ECM regardless of growth phase while only accounting for 8 - 10 percent of the sulfated GAG in the medium, a difference of approximately 2 fold. The high density cultures showed percentages of DS similar to the stationary cultures for

TABLE 3. <sup>3</sup>H-glucosamine incorporation into individual glycosaminoglycans in normal human mammary epithelial cells in culture.

Culture Conditions	Fraction <sup>1</sup>	Hyaluronic Acid	Chondroitin Sulfate	Dermatan Sulfate	Heparan Sulfate
		% incorporation			
Low density: <sup>3</sup>					
Growing	Medium	88±0.83 <sup>2</sup>	10±0.84	1±0.00	1±0.43
	ECM	60±1.97	26±0.68	7±1.59	3±1.37
Early stationary	Medium	80±1.42	17±1.42	2±0.68	1±0.57
	ECM	50±1.53	33±1.06	9±1.22	3±0.77
Late stationary	Medium	78±1.53	18±1.33	1±0.00	2±0.57
	ECM	59±1.65	30±1.44	6±0.89	3±0.61
High density:					
Stationary	Medium	76±0.92	20±0.57	1±0.00	1±0.00
	ECM	46±0.79	32±0.53	12±0.42	4±0.61

1. Cultures were resolved into 3 fractions: Medium, Cell and ECM. The distribution of glycosaminoglycan did not differ in the cell fraction (HA, 76-78%; CS, 15-19%; DS, 1-3%; HS, 4-5%) regardless of growth conditions so the data from this fraction is not shown.

2. Mean ± S.E.M.

3. Refers to seeding.

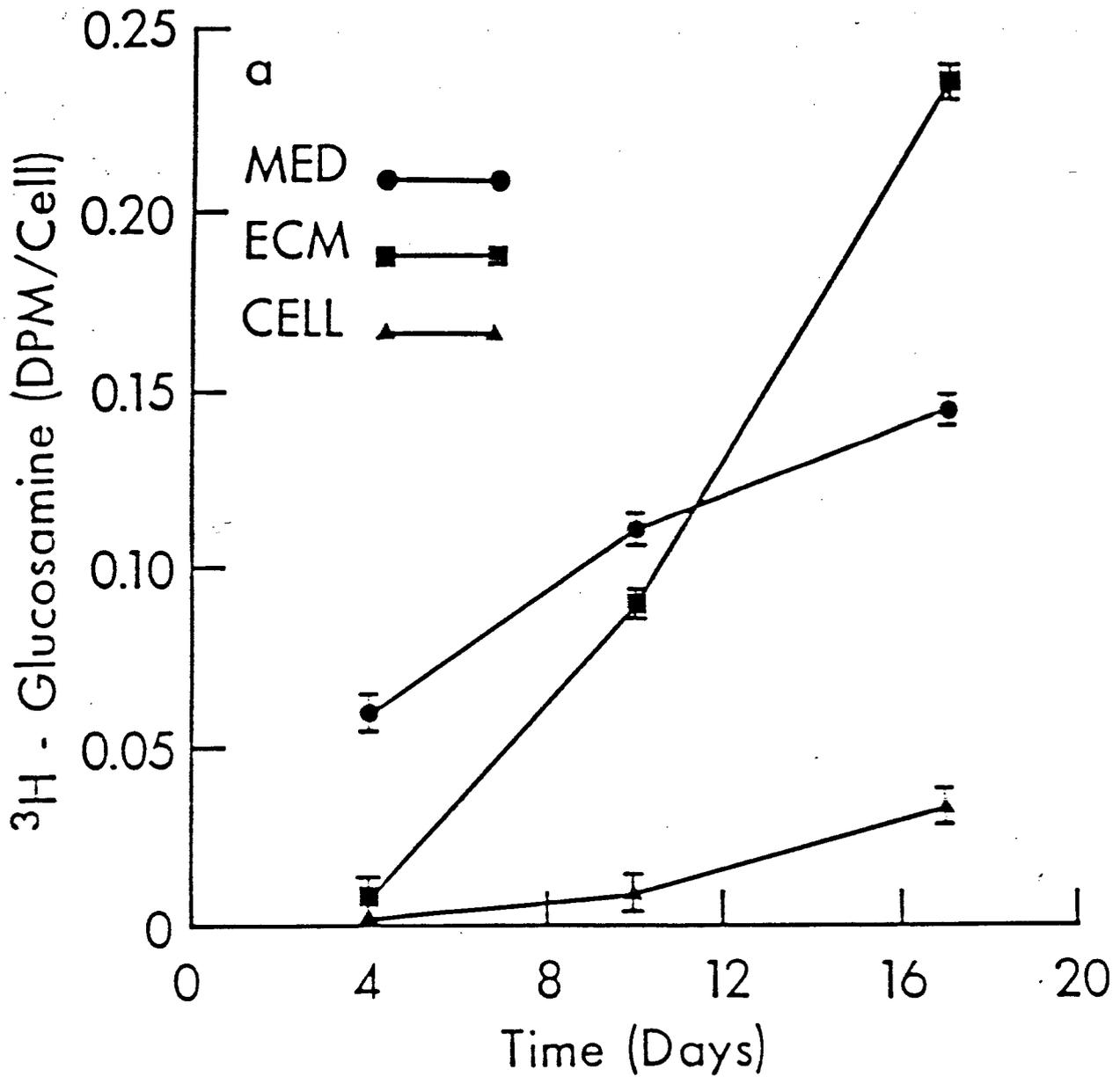


Fig. 9  $^3\text{H}$ -glucosamine incorporation into HA/ cell. The medium, cell and ECM fractions were analysed in growing (day 4), early stationary (day 10) and late stationary (day 17) cultures of normal human mammary epithelial cells. Error bars indicate S.E.M. of three determinations of one experiment.

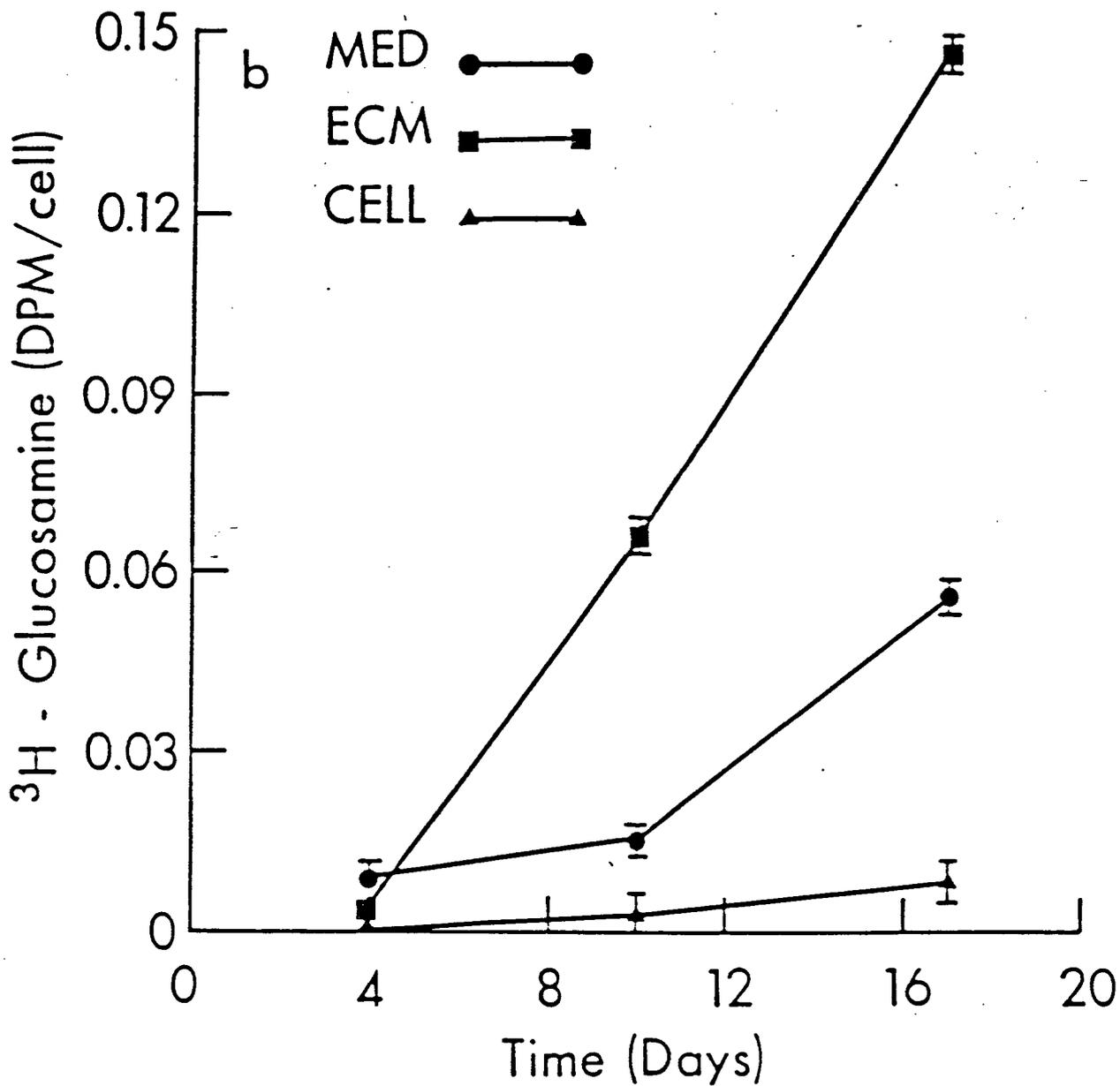


Fig. 10  $^3\text{H}$ -glucosamine incorporation into CS/ cell. The medium, cell and ECM fractions were analysed in growing (day 4), early stationary (day 10) and late stationary (day 17) cultures of normal human mammary epithelial cells. Error bars indicate S.E.M. of three determinations of one experiment.

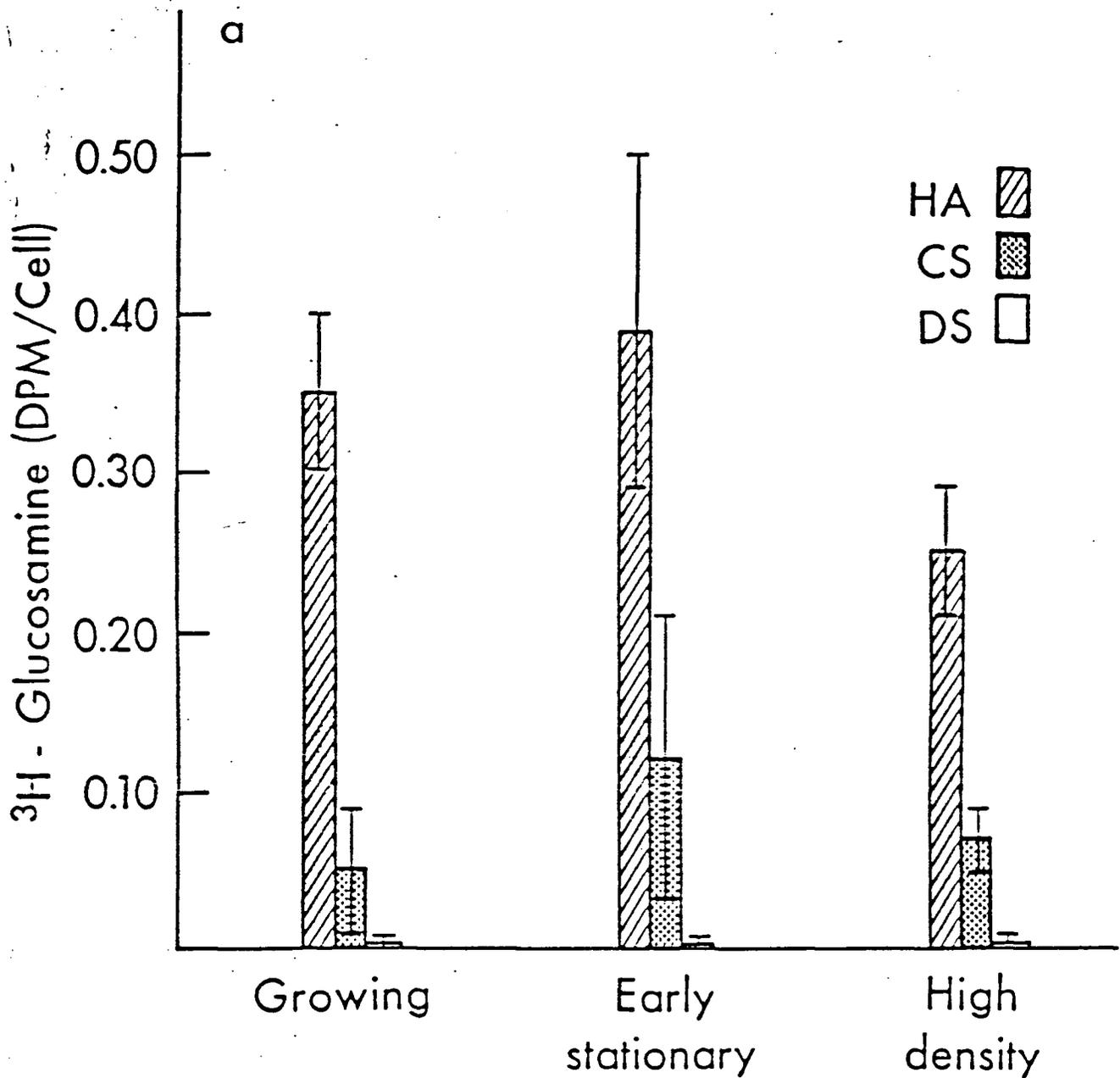


Fig. 11 a) <sup>3</sup>H-glucosamine incorporation into HA, CS, and DS /cell in the medium fraction of triplicate cultures of normal human mammary epithelial cells at growing, early stationary and high density (stationary) phases. Error bars indicate S.E.M. (N = 3)

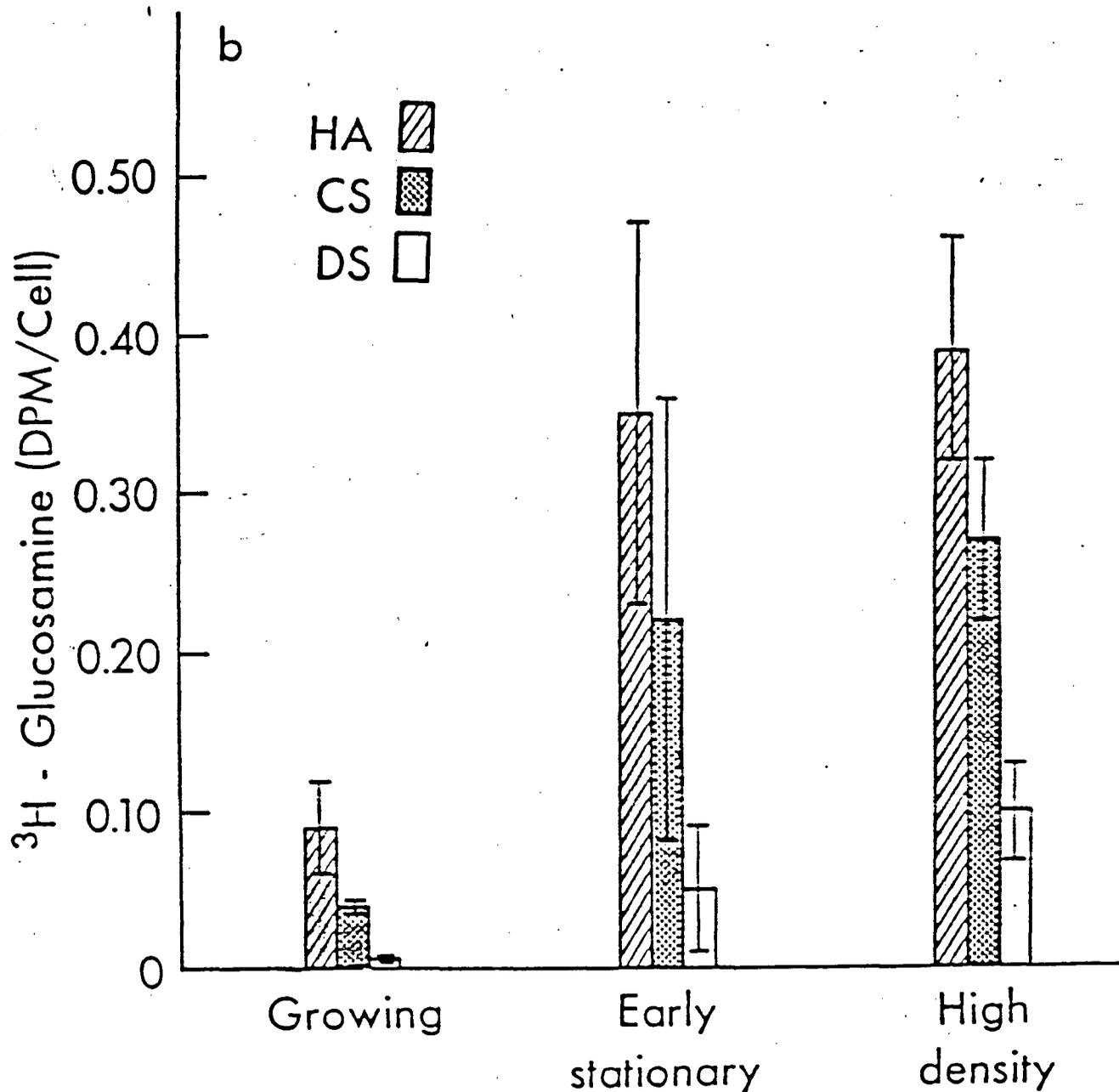


Fig. 11 b)  $^3\text{H}$ -glucosamine incorporation in the ECM fraction of triplicate cultures of normal human mammary epithelial cells at growing, early stationary and high density (stationary) phases. Error bars indicate S.E.M. (N = 3)

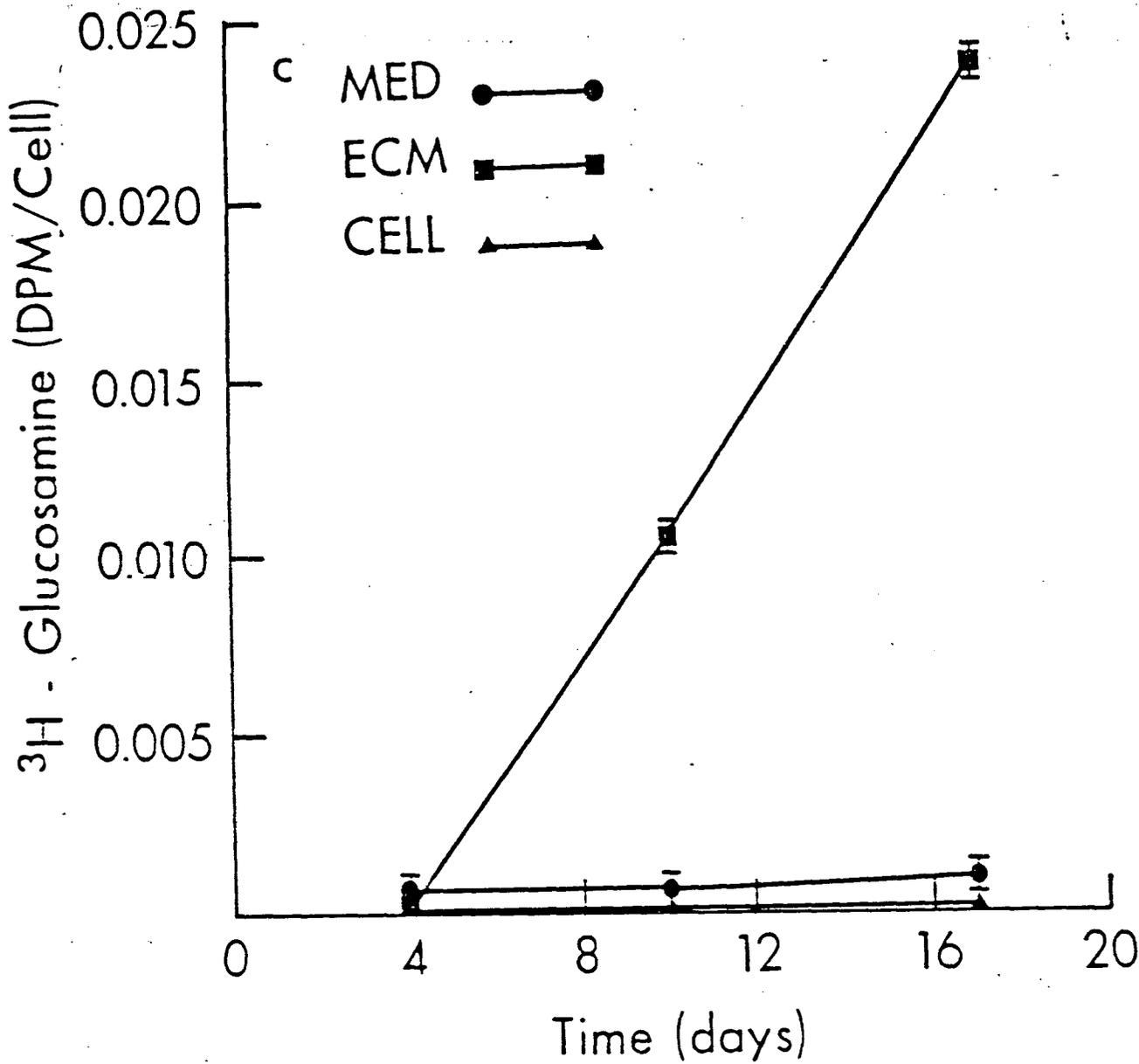


Fig. 12 <sup>3</sup>H-glucosamine incorporation into DS/ cell. The medium, cell and ECM fractions were analysed in growing (day 4), early stationary (day 10) and late stationary (day 17) cultures of normal human mammary epithelial cells. Error bars indicate S.E.M. of three determinations of one experiment.

both the medium and ECM (Table 3). DS was 25 percent of the total sulphated GAG in the ECM and only 5 of that percent in the medium.

When DS was expressed / cell, all fractions showed equally low amounts in the growing cultures (Figure 11a and b, 12). When growth ceased, DS had increased by 10 fold in the ECM fraction while the medium fraction remained low, increasing by only 1 - 2 fold.

#### Heparan Sulfate Synthesis

The percentage of total GAG identified as HS does not exceed 5 percent in any fraction at any time point (Table 3). However, the actual amount of HS synthesized is higher in the ECM fraction than the medium fraction of stationary cultures. This is not the case in the growing cultures where all fractions had only a small amount of synthesized HS (Table 3).

The high density cultures showed similar results (Table 3); that is, uniformly low percentages in all fractions.

#### Electrophoresis Results

GAGs in all fractions were identified by cellulose acetate electrophoresis (Table 4). The amount of <sup>3</sup>H-glucosamine labelled material applied to the cellulose acetate strip was up to 20 fold higher for the stationary cultures compared to growing cultures due to the greater amount of total GAG synthesized by confluent cultures. This may account for some discrepancy between the electrophoretic results and the enzyme assay results, especially in the growth stage. Since the more labelled material applied to the strip the more accurate the results, any strip having less than 1000 DPM was not useable. Therefore, very low counts of

TABLE 4: Electrophoresis Identification of GAG (% of Total)

Culture Conditions	Sample	%HA	%HS/DS	%CS
Low Density: <sup>1</sup> Growing	MED	100 $\pm$ 0.00	ND	ND
	CELL	100 $\pm$ 0.00	ND	ND
	ECM	79 $\pm$ 2.64	4 $\pm$ 0.96	17 $\pm$ 1.80
Low Density: Early Stationary	MED	89 $\pm$ 1.36	3 $\pm$ 1.07	8 $\pm$ 1.17
	CELL	86 $\pm$ 1.79	3 $\pm$ 0.77	11 $\pm$ 1.24
	ECM	58 $\pm$ 1.84	9 $\pm$ 1.08	33 $\pm$ 1.55
Low Density: Late Stationary	MED	89 $\pm$ 1.04	2 $\pm$ 0.51	9 $\pm$ 1.22
	CELL	90 $\pm$ 1.36	1 $\pm$ 0.43	9 $\pm$ 1.40
	ECM	71 $\pm$ 1.98	8 $\pm$ 1.11	21 $\pm$ 1.67
High Density: Stationary	MED	85 $\pm$ 1.54	1 $\pm$ 0.23	14 $\pm$ 0.83
	CELL	81 $\pm$ 1.21	ND	19 $\pm$ 0.85
	ECM	56 $\pm$ 2.01	16 $\pm$ 0.54	28 $\pm$ 1.07

ND = level is not detectable

Mean of 3  $\pm$  SEM

1 - refers to seeding

labelled material, such as in the case of sulfated GAGs in the medium of growing cultures, resulted in non-detectable quantities. However, the majority of the electrophoresis results compared within 10 - 15 percent of the enzyme assay results (Table 4). The distribution of each GAG in the three fractions compared favourably between the two methods for growing and stationary cultures initially seeded at low density as well as in the high density cultures. The medium showed the largest percentage of HA regardless of growth status. The ECM showed the largest percentage of CS regardless of the growth status. The percentage of HS and DS were combined together as they co-migrate on the strip. The medium and cell fractions contained only small percentages of HS and DS while the ECM fraction contained the largest percent in all cases. Presumably the increased percentage of HS and DS in the ECM was due to the increased percent of DS as was seen with the enzyme assay results. The high density cultures compared with the stationary cultures in all GAG percentages as was seen in the enzyme assay results.

#### Cultures Labelled with $^{35}\text{S}$ - Sulfate

Normal human mammary epithelial cells seeded at  $5 \times 10^5$  cells/cm<sup>2</sup> (high density) and  $2 \times 10^5$  cells/cm<sup>2</sup> (low density) were labelled with  $^{35}\text{S}$ -sulfate and terminated on day 4. DNA analysis of these cultures indicated that cultures seeded at high density had not grown over the four day period while cultures seeded at low density had increased from  $2 \times 10^5$  cells/cm<sup>2</sup> to  $4 \times 10^5$  cells/cm<sup>2</sup> by day 4. These cultures provided information on the synthesis of sulfated GAGs - CS, DS and HS. Again, only the medium and ECM fractions are significant as the cell results varied only slightly between the two densities and were negligible

TABLE 5: Percentage of Sulfated GAG in the Medium and ECM of High and Low Density Cultures labelled with  $^{35}\text{S}$ -Sulfate.

Experiment	Fraction	% CS	% DS	% HS
High Density	Med.	$71 \pm 0.54^1$	$8 \pm 0.23$	$2 \pm 0.21$
	ECM	$72 \pm 0.59$	$18 \pm 0.67$	$2 \pm 0.37$
Low Density	Med.	$77 \pm 0.57$	$7 \pm 0.57$	--
	ECM	$65 \pm 0.96$	$17 \pm 0.59$	--

<sup>1</sup> Mean of 3  $\pm$  SEM.

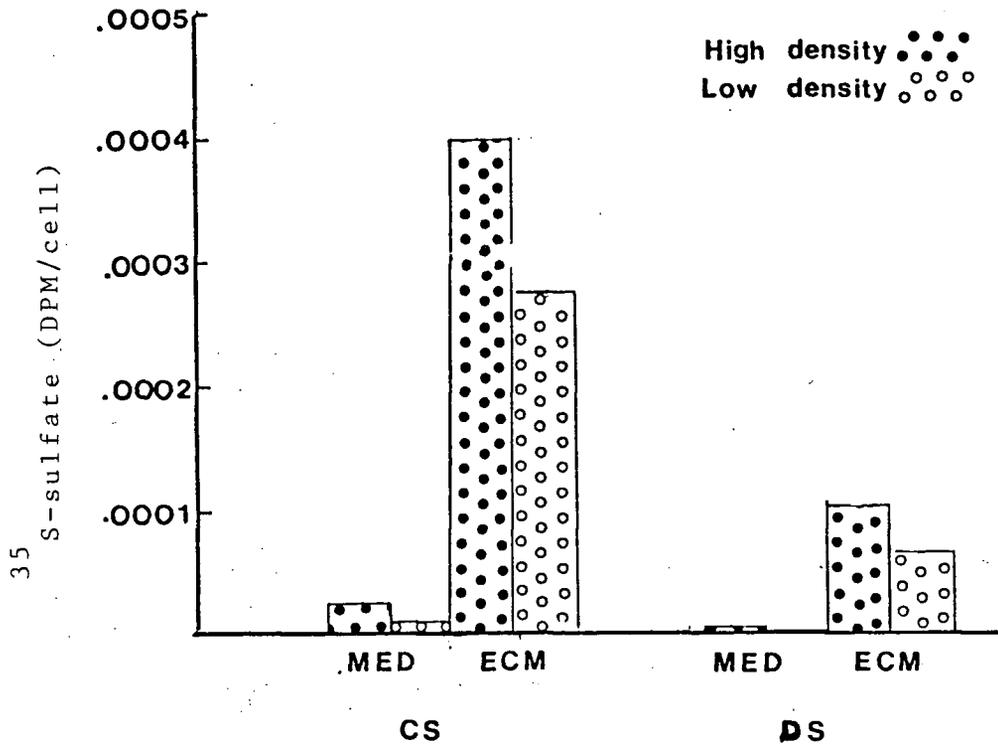


Fig. 13  $^{35}\text{S}$ -sulfate incorporation into CS and DS/cell in the medium and ECM fractions of duplicate cultures of normal human mammary epithelial cells at high ( $5 \times 10^5$  cells/cm<sup>2</sup>) and low ( $2 \times 10^5$  cells/cm<sup>2</sup>).

in amounts (Table 5).  $S^{35}$ -sulfate labelled GAGs demonstrated similar patterns to the  $^3H$ -glucosamine labelled sulfated GAGs in growing and stationary cultures. In the  $^{35}S$ -sulfate labelled high density culture, CS was 9 - fold greater than DS in the medium fraction and 4 - fold greater than DS in the ECM fraction. This was comparable to an 8 - fold and 4 - fold greater amount of CS over DS in the medium and ECM respectively in the  $^3H$ -glucosamine low density stationary cultures. In the low density  $^{35}S$ -sulfate labelled culture, CS was 10 - fold higher than DS in the medium fraction and 4 - fold higher than DS in the ECM fraction. This again compared favourably with the  $^3H$ -glucosamine labelled low density growing cultures which had a 10 and 4 fold greater amount of CS over DS in the medium and ECM fractions respectively.

As in the  $^3H$ -glucosamine labelled cultures, the predominant sulfated GAG / cell in both  $^{35}S$ -sulfate labelled cultures was CS followed by DS (Figure 13). HS was very low in the high density culture and not detectable in the low density culture. Also comparable was that the amounts of CS and DS / cell were always greater in the ECM fraction than in the medium fraction regardless of growth status. The medium fraction contained more sulfated GAG in the high density culture than in the low density, however this was not a great amount when compared to that in the ECM (20 fold greater in the ECM).

#### Autoradiography

GAGs separated by cellulose acetate electrophoresis were identified by autoradiography (Figure 14). The stationary cultures showed the greatest concentration of  $^3H$  - glucosamine labelled GAGs in the ECM fraction. DS

was not detectable using this technique. The growing cultures showed only the medium fraction and the greatest concentration of labelled material there was identified as HA.

#### Electron Microscopy

Electron microscopy revealed human mammary cells with epithelial characteristics (Fig. 15) including tight junctions and microvilli at the apical surfaces. These were typical findings in the predominant cell type throughout the cultures, both growing and stationary. The ratio of epithelial to fibroblast cells was approximately 4 to 1.

MEDIUM

CELL

ECM



Fig. 14 Autoradiograph of  $^3\text{H}$ -glucosamine incorporated GAG in the medium, cell and ECM fractions on an early stationary culture of normal human mammary epithelial cells.

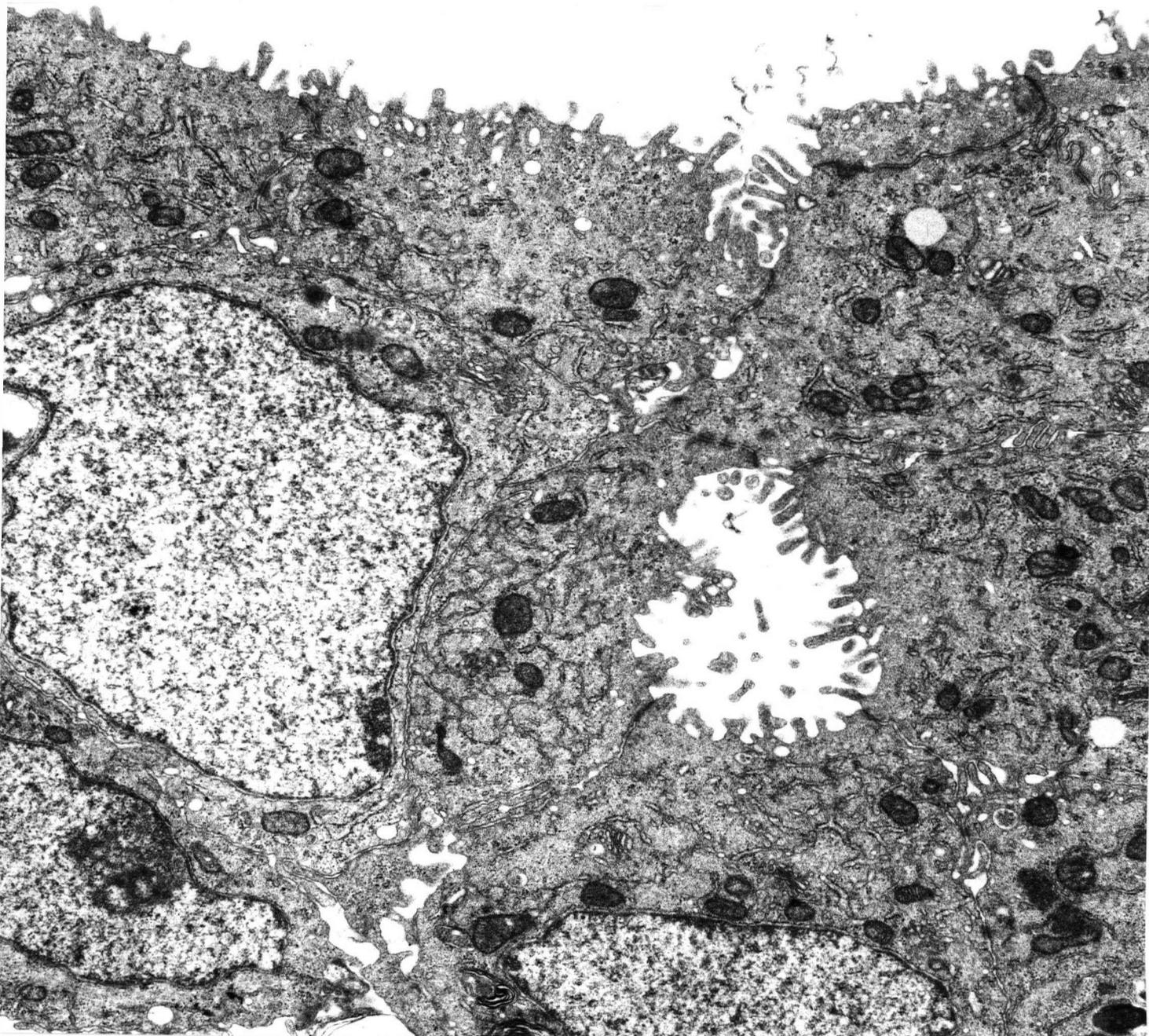


Fig. 15 Electronmicrograph of stationary normal human mammary cells in culture demonstrating an epithelial nature.

N = nucleus  
TJ = tight junction  
MV = micro villi

## DISCUSSION

The type, amount and localization of glycosaminoglycans synthesized by normal human mammary epithelial cells in culture were shown to vary with the growth status of the culture. The results will be discussed under the following headings:

- 1) culture status
- 2) distribution of synthesized GAGs
- 3) type of synthesized GAG
- 4) overall amount of synthesized GAG

The discussion will be completed with a brief outline of questions generated by the research presented in this thesis and the direction of future work.

### 1) Culture Status

The cultures of normal human mammary epithelial cells described in this thesis are growing or nongrowing (stationary). Briefly, growing cultures are 3 - 4 day old cultures seeded at low density ( $2.0-2.5 \times 10^5$  cells/cm<sup>2</sup>). Stationary cultures are 9 -11 day old cultures seeded at low density ( $2.0-2.5 \times 10^5$  cells/cm<sup>2</sup>) and 4 day old cultures seeded at high density ( $5.0 \times 10^5$  cells/cm<sup>2</sup>). The GAG profile of stationary cultures is the same regardless of time in culture suggesting that the type, amount and localization of synthesized GAG depends on the growth status of a culture and not time spent in culture. This finding supports the work of Cohn et al. (1976).

The 17 - 18 day old cultures initially seeded at low density ( $2.0-2.5 \times 10^5$ ) (late stationary cultures) have varied GAG patterns and will be

discussed separately from the other nongrowing cultures.

## 2) Distribution of Synthesized GAGs

The cultures were separated into 3 fractions, the medium, the cell (soluble cell fraction only) and the ECM (including the cell membrane) as previously described by Parry et al. (1985) and Nevo et al. (1984). However, it should be noted that cell surface and ECM GAG are not equivalent. Recent studies have shown that certain types of GAGs are cell surface specific and are not found in the ECM and vice versa (Rapraeger and Bernfield, 1985). Many studies do not define an ECM component (Cohn et al., 1979; Angello et al., 1982; Chandrasekaran et al., 1979) but instead use one, two, or all of the following - cell, cell surface and medium. Most of the synthesized GAGs in this study were either in the medium or the ECM fraction with very little in the cell (Table 2) indicating that these molecules were not accumulating there during any stage of growth. Parry et al. (1984) also found little accumulation of synthesized GAG in mouse mammary epithelial cells cultured on collagen gels. Conversely, Cohn et al. (1976) found a large amount (up to 90 percent) of synthesized GAG present in their 3T3 cells cultured on plastic and Parry et al. (1985) found equal amounts of GAG in the cell and medium synthesized by mouse mammary epithelial cells cultured on plastic.

In growing tissue in vivo, it has been clearly demonstrated that cells migrate toward or create their own suitable extracellular environment conducive to continued growth. In embryonic tissues release of GAGs allow migratory pathways to form (Toole et al., 1971). Growing cells have been observed moving along these pathways until their final destination is reached (Hay, 1982). No further release of the migration-stimulating GAG

occurs and the cells begin to differentiate and accumulate an ECM. In culture, the cells may also be releasing synthesized GAG into the medium to create a suitable environment for growth. During growth the cultures accumulated most of the synthesized GAG in the medium (Table 2). With cessation of growth, the localization of GAG changed so that the ECM contained a substantial increase in the percentage of synthesized GAG. There still remained a portion of the synthesized GAG in the medium, however this was half of what it was during growth.

Nevo et al. (1984) suggest in their study on bovine corneal endothelial cells in culture that the ECM does not play a role in early growth. They grew cells on plastic and on ECM and noted that the growth rate for both is the same over the first 3 days in culture. However, by the fifth day, the final cell count for cultures on the ECM is 18 percent higher than cultures on plastic. They conclude that the major function of the ECM occurs in a confluent culture. An ECM may permit higher cell densities, however, as a confluent state is not attained until day 4 and greater growth was noted for the cultures on the ECM than on plastic for the 24 hours prior to the confluent state. Therefore, an ECM may not be necessary for growth and cells in culture need not incorporate synthesized GAG into an ECM during this stage.

From their experiments on a normal human breast cell line (HBL-100) and two malignant human breast cell lines (MDA-MB-231 and MCF-7), Chandrasekaran et al. (1979) show that the majority of synthesized GAG is found in the medium. Their cultures were labelled when 80 percent confluent and terminated 48 h later. Although it is not stated in their paper, the cultures appear to be growing at the time of labelling. These results compare favourably with the results of growing human mammary

epithelial cells described in this thesis..

Cohn et al. (1976) found that the medium contains less than 20 percent of the synthesized GAGs when their mouse 3T3 cells are  $3.5 \times 10^4$  cells/cm<sup>2</sup> (not confluent) and a higher percentage (35 percent) when the cells are  $7.0 \times 10^4$  cells/cm<sup>2</sup> (confluent). Although the increase in percentage of GAGs in the medium is not large, they conclude that the inhibition of the growth of cells at higher density may be related to the increased presence of GAG in the medium. Although their methodology and results differ from those reported in this thesis, it seems possible that cell density and density-dependent growth inhibition alter the type and distribution of synthesized GAGs in their work as it appeared to do in this work.

A strong relationship exists between cell density and growth such that the density changes as growth occurs. Because of this, it is difficult to assess which of the factors plays the major role in altering GAG synthesis. Cohn et al. (1976) relates many of the altered synthesis patterns seen in the cultured 3T3 cells to differences in cell density. It would indeed seem reasonable to assume that density does play a role in GAG synthesis. High cell density in cultures studied for this research is compatible with nongrowth. Nongrowing cultures show an altered GAG synthesis compared to growing. The growing cultures have a much lower cell density.

Although low and high density cultures are either growing or nongrowing, there are other explanations for changes in GAG localization due to density besides growth. Cells that are growing are generally migrating across a surface. Therefore the location of GAGs may differ between migrating and non migrating cells. Cells in contact with other cells alter their shape

resulting in altered receptor shape or distribution. Both of these factors may affect synthesis and distribution of different GAGs.

Factors in addition to growth and density may affect the location of synthesized GAGs. For example, Parry et al. (1985) looked at confluent cultures of mouse mammary epithelial cells on three different substrates and found that the type of substrate influences localization of newly synthesized GAG. Cells on plastic release the majority of their GAGs into the medium while the cells on collagen gels release the majority of their GAGs into the ECM. Cells grown on plastic may not be able to incorporate synthesized GAGs into an ECM despite the fact that they are no longer growing. The human mammary epithelial cells cultured on collagen gels incorporate GAG into an ECM at a stationary growth state (Table 2), just as mouse mammary cells cultured on collagen gels do.

In the late stationary cultures of normal human mammary epithelial cells (cultures seeded at low density and terminated day 17-18) the distribution of GAGs do not differ from the earlier stationary cultures. However, the total GAG synthesis varies from the early stationary and between the 3 late stationary cultures, such that 1 culture has a greater amount of GAG, 1 has an equal amount and 1 culture has less than the amount of GAG found in early stationary cultures (data not shown). Several factors could account for this difference. A possible explanation is that the cells are in a crowded condition; in some cases they contract the collagen gel to approximately 10 percent of its original size. This may have made it difficult for normal nutrient and gas exchange to occur resulting in cell dying and death. Varied GAG synthesis appears to be related somehow to the physical state of the substrate.

## Types of Synthesized GAGs

### Hyaluronic Acid

As discussed in the Introduction, various GAGs are associated with certain biological functions on the basis of where they are found before, during and after such functions and by the types of cell responsible for their synthesis (ie. growing cells). The term "growth" in this thesis refers to an increase in cell number not an increase in individual cell size. Normal human mammary epithelial cells in culture synthesize predominantly HA during growth and release it to the medium (Table 3). It is possible that these cells are synthesizing HA and releasing it into the medium because they are growing or migrating. In embryological growth and migration of tissue, HA is the most abundant GAG being synthesized by both the epithelial cells in the process of migrating and the surrounding non-migrating tissue (Toole, 1982; 1979). HA can regulate cell motility in a number of ways: a) by promoting cell surface protrusions responsible for locomotion, as seen in rapidly moving fibroblasts where it is concentrated in the extending lamella and retraction fibers (Turley et al., 1984), b) by maintaining only weak adhesions allowing cells to detach from underlying substrates easily, as seen in a variety of cells which detach readily in the presence of HA (Abatangelo et al., 1982), c) by hydrating large areas of tissue, creating open spaces which cells can move through, as seen in neural crest cells (Tosney, 1978) and d) by preventing early differentiation (Toole, 1977). Toole (1972) shows that addition of HA into chick embryo somite cells beginning to differentiate stop further cartilage formation. HA synthesis is not only associated with embryological growth. Silberstein and Daniels (1982, 1984) demonstrate by histochemical techniques that mouse mammary gland in vivo synthesize HA. This HA is

mainly associated with the cap region of the growing gland where cells are shown to be actively proliferating and penetrating tissue. They also show that this specific GAG synthesis and deposition is present in serially aged glands and is thus related to growth status and not tissue age.

In vitro, the addition of HA promotes locomotion of various cell types (SV40-3T3, chick heart fibroblasts) across and into various substrates, including collagen and fibronectin (Turley, 1984; Bernanke et al., 1979). Other cells do not respond to the addition of HA, such as 3T3 cells (Turley, 1984), leucocytes (Forrester et al., 1981) and neural crest cells (Newgreen et al., 1982). This has led to the conclusion that the response to HA is cell type specific and/or cells must have available receptors to HA (Turley, 1984; Underhill et al., 1981). HA binding receptors may only be present at certain times during a cell's development and addition of HA during inappropriate times may account for the lack of response in these cells. Normal human mammary epithelial cells synthesize their own HA and presumably responded to its presence by growing and / or migrating.

HA has little affinity for laminin, a glycoprotein found in the ECM (Del Rosso et al., 1981). Laminin, as discussed in the Introduction, is known to have a major role, by binding with collagen and proteoglycans, in securing cells to a basement membrane. The lack of affinity between HA and laminin creates an environment conducive for detachment for cells associated with excess HA thereby facilitating growth and / or motility. For growing cells in tissue it would appear beneficial, if not obligatory, to have an abundance of HA present. This would ensure growth and / or motility up to the point where a final destination is reached and prevent any possibility of early adhesion and precocious differentiation. It is

likely that normal human mammary epithelial cells are able to create an environment conducive to their growth, ie. HA synthesis (Table 3).

Stationary cultures, however, also synthesize HA which is found both in the medium and the ECM. There are several explanations for this. First, once the cultures are stationary there is most likely an ongoing cycle of cell death and proliferation that may continually be causing the synthesis of HA and its release into the medium. Secondly, it appears from recent studies (discussed in the Introduction) that HA has several functions. What HA may be doing for the growing cell may be quite different from its function in a stationary or differentiated cell, particularly if it is found in a different location, ie. HA present in the ECM of stationary cultures (Figure 9) may have a very different function from the HA present in the medium of growing cultures. Growing cultures of normal human mammary epithelial cells have only approximately 20 percent of total synthesized HA in the ECM while the stationary cultures have 50 percent of total HA incorporated there. Thirdly, synthesis of HA and its release into the medium may be a culture phenomenon such that some HA will be found there regardless of the growth status of the culture. It is interesting to note that HA is the only GAG of any abundance in the medium of all growing and nongrowing cultures, 3 to 5 times the amount of CS, the next most abundant GAG. Parry et al. (1985) demonstrate that for confluent mouse mammary epithelial cells cultured on plastic the medium is rich in HA. This is also true of the cells on attached collagen gels but these cultures show approximately equal amounts of HA in the medium and ECM. This finding is identical to the findings of the stationary normal human mammary epithelial cultures on attached collagen gels. Other studies also show a predominance of HA in the medium, including studies of cloned pigmented

retinal epithelial cells maintained on plastic substrates (Crawford and Crawford, 1984) and the normal human breast cell line (HBL-100) maintained on plastic (Chandresekaran and Davidson, 1979). Cohn et al. (1976) find that regardless of cell density and growth status of their 3T3 cells on plastic, the medium always has mainly HA ( 60-70 percent ). They find that the percentage of HA in the medium actually increases with a high density or nongrowth situation.

It appears that cells in culture preferentially release HA into the medium over other GAGs being synthesized. This phenomenon appears in cultures of various cell types both growing and nongrowing. Cohn et al. (1976) find that although the medium contains mainly HA in high (stationary) and low (growing) density cultures, the overall amount of synthesized GAG (particularly HA) is less in the stationary cultures so, although the percentage of HA in the medium is higher, the actual amount is less. Parry et al. (1985) also find that the mouse mammary epithelial cells synthesize less HA in the medium when they are grown on collagen gels than on plastic and are approximately equal to the sum of the other GAGs present in the medium (CS, HS). When the collagen gels are allowed to float early in culture they synthesize even less HA into the medium and ECM when compared to the cells on attached gels. Parry et al. (1985) point out that cells on floating gels are more differentiated than cells on either plastic or attached collagen gels, as analysed by milk protein synthesis. It may be that cells that are fully differentiated do not require as much HA to function or as much of any GAG, resulting in less synthesis and turnover of GAGs. The normal human mammary epithelial cells studied in this research are not differentiated although they do stop growing when they reached a confluent state.

The cell fractions of the cultures described in this thesis do not vary with growth and stationary phases with regard to the type and amount of GAG (Table 2). This suggested that most of the GAG synthesized is either released to the medium, incorporated into an ECM or rapidly degraded. Parry et al. (1985) also find that the cell fraction contains the least amount of GAG in confluent mouse mammary epithelial cultures on collagen gels. This is 2 - and 5 - fold less than the amount of GAG in the medium and ECM respectively. The work reported here and other work (Parry et al., 1984; Nevo et al., 1984) also indicates that the presence of a collagen based substrate prevents intracellular GAG build-up. Both Cohn et al. (1976), looking at 3T3 cells cultured on plastic, and Parry et al. (1985), looking at mouse mammary cells on plastic, find greater or equal amounts of total synthesized GAG in the cell compared to that in the medium regardless of their growth status. The reasons for these differences may be related to the components of the substrate, the physical nature of the substrate and cell-cell interactions.

The ECM of growing human mammary epithelial cell cultures contain little of the total amount of synthesized HA, most of which is found in the medium. As the cultures become stationary the amount of HA increases in the ECM by almost 4 - fold, resulting in approximately equal amounts of HA in the ECM and medium (Figure 11a and b). The major function of HA seems to be associated with growth and / or migration (Toole, 1977; 1979; 1982; Underhill, 1982). Therefore, why is the HA found in the ECM and why is HA found in cultures of nongrowing cells? In light of recent developments on binding sites for various GAGs, HA may play a much more varied role in cell function than was initially appreciated (Turley, 1984). HA is known to aggregate other GAGs (except heparan sulfate) and has binding sites on cell

surfaces such as fibroblasts (Underhill, 1982). HA has also been shown to bind to fibronectin (Yamanda et al., 1980). Specific proteins which are thought to represent HA binding sites have been isolated (Turley, 1982). The binding properties of HA may relate to available receptor sites, the dependency of the particular cell type on HA mediated adhesion, the nature of the growth surface and the stage of adhesion to the underlying substrate (Turley, 1984). Turley suggests that HA may be involved in early adhesion formation and be replaced over time by other GAGs or possibly glycoproteins.

In the normal human mammary epithelial cells cultured for this study, very little HS is synthesized. As pointed out in the Introduction, HS is associated with strong cell-matrix adhesion properties (Hook et al., 1982). It may very well be that HA, acting as an aggregate or intermediate binding molecule, holds the collagen-bound sulfated GAGs (mainly CS and DS) and cell surfaces together. The mouse mammary epithelial cell line NMuMG has recently been shown to have distinct cell-associated HS acting as an anchoring mechanism between the cell and underlying ECM (Rapraeger et al., 1985). It may be that, in the absence of HS, cell-associated HA (found in the ECM fraction) may perform the same service for the cell. During growth, as opposed to nongrowth, cells require the ability to move because of the close association between growth and motility. Thus, they may lack substantial amounts of HA in the ECM at that time. Parry et al. (1985) find the same amount of HA present in the medium and ECM of stationary mouse mammary epithelial cells on attached collagen gels, although on floating collagen gels these same cells, which are more differentiated, have a third of the amount of synthesized HA in the ECM (6 percent as compared to 18 percent in the attached gels) and 30 percent more sulfated

GAG. Cohn et al. (1976) find in their 3T3 cell cultures that HA is the major cell-surface GAG in both the growing and stationary cultures although it does decrease by 20 percent when the culture stop growing. Gordon and Bernfield (1979), while studying midpregnant mouse mammary epithelial cells, find that 60 percent of the GAG in the ECM (including cell membrane) is HA. Crawford and Crawford (1984) find that for cloned pigmented chick retinal epithelial cells in culture HA actually increases relative to sulfated GAGs during differentiation indicating that the function of HA may be different in different cell types.

#### Sulfated GAGs

The small amounts of sulfated GAG compared to HA (Figure 11a) released into the medium has been observed by others (Cohn et al., 1976; Chandrasekaran et al., 1979; Crawford et al., 1984). In contrast, Parry et al. (1985) find that confluent cultures of mouse mammary epithelial cells cultured on plastic release equal amounts of HA and CS into the medium, although Cohn et al. (1976) find mainly nonsulfated GAG in the medium of mouse 3T3 cells. When these cells enter a stationary growth phase they release an undersulfated CS that accounted for approximately 40 percent of the GAG found in the medium. They also find that less than 5 percent of the GAG in the medium is HS regardless of growth status. This is very similar to the findings of this study (Table 3).

Although the functions of HA are still not widely understood, even less is known of the functions of CS. It has been shown that their adhesive properties are greater than those of HA for certain cell types such as neural crest cells and leucocytes (Hook et al., 1982; Turley, 1984), although they do not appear as strong as the more highly sulfated DS and

HS. CS has also been shown to have a stronger affinity than HA for the ECM glycoprotein laminin but not as strong as the affinity of DS and HS (DeL Rosso, et al., 1981). It would appear that CS may act as an intermediate GAG between the functions of HA and the more highly sulfated GAGs.

When monocytes change morphologically and express more macrophage-like characteristics (a process equivalent to differentiation) the transition is also accompanied by a switch in synthesis of CS-4 to an over-sulfated galactosamine (Kolset et al., 1983, 1984). Silberstein and Daniels (1982) find that the ECM of mouse mammary epithelial cells in the flank region of developing mammary gland incorporates synthesized CS as the major GAG. The cells in these region are nongrowing and considered "stabilized" tissue. The nongrowing and differentiated mouse mammary epithelial cells in cultures studied by Parry et al. (1985) have predominantly S-GAG in the ECM, ranging from a 4 - fold greater amount than HA for cells on plastic to a 12 - fold greater amount than HA for cells on floating collagen gels. Of the amount of S-GAG, approximately 55 percent is CS (includes DS) and 45 percent is HS. The cells on the attached collagen gel are similar to those on the floating collagen gel although they do not synthesize as much HS. It appears that mouse mammary epithelial cells synthesize mainly CS when they are in a stationary growth state (but not differentiated) and CS and HS when they are differentiated. These results compare favourably with the finding of this work. Normal human mammary epithelial cells in culture that are nongrowing have a substantial amount of CS incorporated into the ECM, almost 5 - fold higher than the amount of CS in the ECM of growing cultures (Figure 10).

Also interesting to note is the virtual lack of DS in any fraction but the ECM (Figure 11) in this study. Parry et al. (1985) also find that DS

is only present in appreciable amounts in the ECM of the mouse mammary epithelial cells. They find that the more differentiated the cultures are the greater is the amount of DS in the ECM. This amount ranges from 1 to 21 percent of the total CS pool in the cells on plastic to 42 percent for the cells on floating collagen gels (most differentiated) with the attached collagen gel cultures being between the two. In the other fractions, medium and cell, the amount is much lower (between 5 and 8 percent of total CS pool) and in several cases is too low to be detected. The same pattern emerges in the human mammary epithelial cells cultured in this study. The medium and cell fractions often have nondetectable levels while the ECM always has most of the DS (Figure 10). DS is most pronounced in the ECM of nongrowing cultures, 4 - fold greater than the amount of DS in the ECM of growing cultures (Figure 11). DS contains predominately L-iduronic acid residues which are epimers of the carbon-5-glucuronic acid. The presence of iduronic acid has been linked with the function of the orderly arranging of collagen fibrils (Iozzo, 1985). Its presence in the ECM may be essential for proper construction or orientation of the matrix components.

Heparan sulfate is associated with having strong binding properties to cell surfaces and other ECM components (Hook et al., 1982). Increasingly, research is demonstrating that different species of heparan sulfate exist and are either cell surface or ECM associated (Rapraeger et al., 1985). Parry et al. (1985), as mentioned, find almost equal amounts of HS and CS in the ECM of confluent mouse mammary cell cultures on collagen gels. All cultures in their study have a hormonal milieu designed to stimulate differentiation. Nevo et al. (1984) also find 50 percent of the synthesized GAG in the ECM of their differentiated bovine endothelial cells

to be HS. An interesting finding of both Parry et al. (1984) and Cohn et al. (1976) is that the cell fraction of their cells grown on plastic have a significant amount of HS, much higher than the other fractions contain. Presumably the cells are able to synthesize HS but are unable to release it to the medium or incorporate it into an ECM. Only small percentages of HS are detected in the human mammary epithelial cell cultures studied for this thesis (Table 3). One explanation for this may be that, if HS is related to the process or maintenance of differentiation, the cells described are not differentiated and therefore are not able to synthesize or do not require HS. They are obviously able to adhere to the substrate either by the use of other proteoglycans or glycoproteins or possibly by utilizing any HS present in the collagen gel.

Although the evidence for the importance of HS in cellular differentiation is not conclusive, several studies have shown that it is not conducive to growth. Clark et al. (1975) find that the addition of dextran sulfate (an artificial S-GAG) to cultures of BHK cells in doses as low as 1 µg/ml caused a nontoxic G<sub>1</sub> arrest. It was discovered that this surrogate GAG binds to cell surfaces and is able to change the morphology of the cells as well as causing growth changes (Goto et al., 1973). Majacek and Bornstein (1984) conclude that components of an ECM can control the biosynthetic activity of cells. They observed that addition of exogenous soluble HS or DS can increase the production of 2 noncollagenous proteins in cultured rat smooth muscle cells. Other proteoglycans do not have this effect. They postulate that these two proteins played a role in growth inhibition. The mode by which the HS molecule acts upon the cell may include any or all of the following: 1) alteration of the cell shape via the cytoskeleton, in turn altering biosynthetic abilities, 2) via

surface receptor and a response evoked by a "second messenger" system or 3) endocytosis of the proteoglycan and direct delivery to its site of action (Majacek et al., 1984). The first method stated would appear to be the most logical way for HS incorporated into an ECM, including the cell surface, to exert its effect on the cell although the second method would work equally well.

To summarize, human mammary epithelial cells do not appear to require the presence of synthesized S-GAG or an ECM to grow. On the other hand, in nongrowing cultures an ECM is produced and approximately one half of the synthesized GAG found there is sulfated with the majority of that being CS (Figure 11b). DS is also present in the ECM of nongrowing cultures (Figure 10).

### 3) Overall Amount of Synthesized GAG

The overall amount of GAG synthesized by cells seems related to several factors including 1) growth and differentiation, 2) in vitro vs. in vivo conditions, 3) normal vs. malignant, 4) substrate and 5) age of cells. Parry et al. (1984) find that mouse mammary epithelial cells on plastic, which are neither growing nor differentiated, synthesize much larger quantities of GAG than those cultures on collagen gels (a 4 - fold greater amount). Cohn et al. (1976) also find that their mouse 3T3 cells, in a nongrowing phase, synthesize less GAG when compared to the growing cultures, a decrease of approximately 20 percent. The results in this study show an increase in the amount of GAG synthesized as the cells go from a growing to nongrowing state (Figure 7). However, preliminary studies underway in this laboratory indicate that these cells, when stimulated to differentiate via addition of hormones, decrease their

overall GAG synthesis, a phenomenon found by other researchers (Parry et al., 1984).

It has been stated that in vitro cells synthesize excessive amounts of matrix when compared to their in vivo counterparts (Muir, 1977). Nevo et al. (1984) find this to be true in their study on cultured bovine endothelial cells. They compared their results with reports on proteoglycan content of basement membranes isolated from other endothelial tissue and discovered that cultured cells are synthesizing greater amounts. In this study no analysis of GAG from normal breast tissue is done as a comparison and there are no reports of this information in the literature. It would be interesting to see if this tissue, like others, showed increase GAG synthesis in culture.

Much work has been done in the area of malignancy and GAG synthesis. As this was discussed at length in the Introduction only differences in total GAG synthesis will be included here. Generally, it has been found that malignant cells, when they are compared to their nonmalignant counterparts, synthesize greater amounts of GAG (Shishiba et al., 1984; Angello et al., 1982). In some instances these studies compared malignant and normal cells of the same cell type. Shishiba et al. (1984) looked at normal human thyroid tissue and human thyroid adenocarcinoma tissue and compared them for total GAG synthesis. They find a 6 to 15 fold greater amount of GAG in the adenocarcinoma tissue. However, some studies compared malignant to nonmalignant cells with the assumption that nonmalignant and normal are synonymous. For example, Angello et al. (1982) compared two subpopulations of a mouse mammary tumor cell line (WAX-2T), one which is fast growing and can grow in soft agar and one which is slow growing and does not grow in soft agar. They find that the more aggressive tumor subpopulation

synthesizes 8 times more GAG than the less aggressive one. Chandrasekaran et al. (1979), in studying GAG synthesis in cell lines, compared the normal human breast cell line (HBL-100) to two human breast carcinoma cell lines (MDA-MB-231 and MCF-7). They find that the HBL-100 and MDA-MB-231 synthesize equal amount of GAG and the MCF-7 considerably less. Cell lines, especially so-called "normal" ones, should be compared with normal primary cells with some reservation.

The cells used in the experiments presented in this thesis are from three different women (details in Appendix 9). Two of the experiments produced approximately equal amounts of total GAG at all stages of growth. One experiment produced greater amounts of GAG, up to 5 - fold higher. However, the percentages of GAG in each fraction and the ratio of one GAG to another are very similar. Why did this occur? When dealing with normal human mammary epithelial cells several factors must be taken into account. The age of the donor may be significant. The two experiments with less overall GAG synthesis were from two women in their mid to late thirties. The third experiment is from a 19 year old woman. It may be that cells from younger women are more metabolically active. The overall potential for growth (cell proliferation and GAG synthesis) may be decreasing in older women. Silberstein and Daniels (1984) looked at serially aged mouse mammary ducts and compared GAG synthesis and localization using autoradiographic and histochemical techniques. Interestingly, they find that GAG type and localization is similar between young and old ducts but that older ducts appear to synthesize less overall GAG, identified by their decreased ability to concentrate radiolabelled material.

### Future Research

The results of this study provide the framework for two major areas of research. First, experiments designed to achieve a differentiated phenotype in the normal human mammary epithelial cell in vitro would permit analysis of GAG synthesis under this condition. A change in GAG synthesis in differentiation as opposed to growth/nongrowth would allow for further postulation on the function of various GAGs as they relate to cellular development. Second, malignant mammary epithelial cells can now be compared to a normal baseline in culture regarding GAG synthesis and localization.

Expanding further on the overall function of GAGs it would appear, given the structural heterogeneity of GAGs and proteoglycans, that they are not limited to providing hydration or adhesion molecules to the ECM. Enhancing the substrate with various amounts and types of GAG may alter the functioning of the cells on that substrate and aid in elucidating the role of GAGs in cell behaviour. For example, growing cells may stop growth and differentiate if placed on a substrate designed to stimulate differentiation. This should increase understanding of cell-matrix interactions.

Indirectly related but closely tied to GAG research is the role of the protein core in a proteoglycan. Recent research indicates that the protein core may in part be responsible for the final destination of a proteoglycan (Nevo et al. 1984). Addition of a B-xyloside to cultures changes the pattern of GAG synthesis. B-xyloside acts as a "pseudo" core protein which the cell uses to link synthesized GAG side chains. The study by Nevo et al. (1984) demonstrates that the addition of B-xyloside causes the cells to release most of their synthesized PG into the medium whereas the same

cells without B-xyloside incorporated the PG into an ECM. The cultures treated with B-xyloside have 72 percent of the final cell count of the cultures without B-xyloside. The importance of the protein core is just beginning to be examined and the system used in this study would lend itself to studies aimed at examining protein core function. A corollary to this would be to examine the GAGs that are cell-associated as opposed to those that are definite ECM constituents. It may be, as discussed in the HA section, that hyaluronic acid is mainly cell membrane associated. At the present time it is not clear how these molecules are attached to the plasma membrane and what their function is there as opposed to the ECM. The system used in this study would have to be modified to separate the ECM into a cell membrane fraction and an ECM fraction.

The techniques and information presented should lend themselves to further interesting and valuable experiments in the continuing search for cell-ECM interactions as they relate to cell functions.

## SUMMARY

This thesis research was designed to investigate the synthesis and distribution of GAGs by normal human mammary epithelial cells in tissue culture. The medium fraction of all cultures contained HA regardless of the growth status. The ECM fraction varied in the type and amount of GAG depending on growth status - the growing cultures had only a small percentage of total synthesized GAG while the stationary cultures had approximately 50-60 percent. Of the percentage of GAG in the ECM of stationary cultures, 50 percent were sulfated; the sulfated GAG included CS (70%) and DS (30%). HS did not comprise more than 5-6 percent in any culture.

To remove the length of time spent in culture as a possible explanation for the changes seen in GAG synthesis between growing and stationary cultures, normal human mammary epithelial cells were seeded at high density and terminated on day 4. No growth occurred. The GAG profile of these cultures closely resembled the stationary cultures seeded at low density and terminated at days 9 - 11.

The type and location of synthesized GAG was found to be dependent on the growth status of the culture. Sulfated GAGs appeared to be more related to stationary growth and were located in the ECM. HA appeared to be present in all stages of growth and were the predominant GAG in the medium.

Appendix 1

Transport Medium

Fl2:DME - (1:1)  
Hepes buffer - 10mM  
Calf serum - 5%  
Insulin - 5 µg/ml

DMe - Delbecco's Modified Eagles Medium

Appendix 2

Dissociation Medium

Fl2 - (1:1)  
Hepes buffer - 10mM  
BSA - 2%  
Insulin - 5 µg/l  
Collagenase - 300U/ml  
Hyaluronidase - 100U/ml

Appendix 3

Growth Medium

Fl2:DME - (1:1)  
Hepes buffer - 10mM  
Fetal Calf Serum - 5%  
Insulin - 5 µg/ml

Appendix 4

Freezing Medium

DME - 50%  
DMSO - 6% (dimethylsulfoxide)  
CS - 44%

Appendix 5

Detergent Solution

Tris pH 7.2 - 10mM  
Triton X-100 - 1%  
Deoxycholate - 1%

Appendix 6

Enriched Tris

Tris base - 3.0g  
Na Acetate - 2.4g  
NaCl - 1.46g  
BSA - 50µg  
HCl - 0.13M  
In 100 ml of distilled water.  
pH to 8.0

Appendix 7

a) DNA buffer

Na<sub>2</sub>HPO<sub>4</sub> - 50mM  
NaCl - 2mM  
EDTA - 2mM (tetrasodium salt)  
adjst to pH 7.4

b) Hoechst Dye (Calbiochem)

stock = 20 µg/ml in H<sub>2</sub>O

c) DNA (calf thymus)

stock = 100 µg/ml in H<sub>2</sub>PO<sub>4</sub> buffer

Appendix 8

Karnovsky's solution

0.5 g paraformaldehyde  
7.5 ml distilled water  
1 to 2 drops NaOH  
2.5 ml 25% glutaraldehyde  
12.5 ml 0.2M Na-cacodylate  
pH to 7.3

Appendix 9

<u>Patient</u>	<u>Age</u>	<u>Reason for Surgery</u>
1) M.A.S.	34	accessory breast tissue
2) J.K.	37	reduction mammoplasty
3) J.H.	19	reduction mammoplasty

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