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Title: The Effects of Extracellular Matrix Glycosaminoglycans on Adhesion and Histogenesis of Neural Retina Cells

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Glycosaminoglycans, highly anionic long chains of repeating disaccharides, are present in the extracellular matrix of almost all vertebrate tissues. In addition to their structural role in adult tissues, these molecules have been implicated as mediators of important developmental processes such as cell migration, cell-cell recognition and adhesion, and cytodifferentiation. The mechanism of their action in affecting these processes probably involves an interaction between the glycosaminoglycan and the cell surface. The work reported here examines the nature and degree of specificity of the interaction which occurs between cells of the developing neural retina and the glycosaminoglycans found in retina extracellular matrix.

A study of retina cell behavior on collagen gels containing glycosaminoglycan is presented in an appendix to this thesis, as are studies related to the physical rather than biological nature of the glycosaminoglycans. In the body of this thesis experiments are described wherein neural retina cells from 10-day chick embryos were tested for their ability to attach to Sepharose 4B beads which had been derivatized with either chondroitin-6-sulfate or hyaluronic acid.

The cells were isolated by trypsin dissociation and were tested directly or after preincubation in medium with or without either glycosaminoglycan. Attachment of cells was inhibited when they had been previously exposed to 10 mg/ml chondroitin sulfate or 2.5 mg/ml hyaluronic acid, regardless of the type of bead included in the culture. By using freshly-dissociated cells to which chondroitin sulfate was added simultaneously with the addition of either chondroitin sulfate-derivatized or nonderivatized beads, cell attachment was found to be inhibited at certain threshold concentrations which differed for the two types of bead, being lower for the cells combined with the chondroitin sulfate-derivatized beads. These results support a model of cell-glycosaminoglycan interactions in which a close association of exogenous glycosaminoglycan with the cell surface blocks cell attachment to beads either by steric effects of the bulky sugars or by masking cell adhesion sites.

The Effects of Extracellular Matrix Glycosaminoglycans on Adhesion and Histogenesis of Neural Retina Cells

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Introduction

The second half of this century has seen intense scientific and popular interest in genetics, resulting in a rapid expansion of our knowledge of how the genome directs development. With this increased knowledge, though, has also come an increased appreciation of the importance of "non-genetic" factors in development. Over the past fifteen years or so attention has been drawn to the role of the extracellular matrix in developmental processes such as cell migration, cell-cell recognition and aggregation, and cell differentiation. Of particular relevance to this thesis are glycosaminoglycans (GAGs), long chain polysaccharides found in the matrix either free or, if covalently bound to proteins, as proteoglycans. These molecules, most notable of which are hyaluronic acid (HA), heparan sulfate (HS) and the chondroitin sulfates (ChS), have been postulated to act as mediators in several systems of some or even all of the four processes mentioned above.

Hyaluronic acid, a single chain unbranched polysaccharide composed of repeating β(1-4)D-glucuronic acid-β(1-3)D-D-acetyl glucosamine units (Laurent, 1970), has been the subject of numerous studies in both adult and embryonic tissues. Singh and Bachhawat (1965) and Margolis et al. (1975) each found that in rat brains the concentration of hyaluronate reaches a peak when the rat is seven days old, after which time the amount of water-extractable HA declines quite rapidly (50% in

3 days) and remains at adult levels. Singh and Bachhawat have noted that the period of decline in HA corresponds with a period of active myelination in the still differentiating rat brain. That hyaluronic acid may somehow be involved in the myelin sheath is further supported by their findings that white matter of the adult sheep brain is higher in content of HA than the gray matter.

Pessac and Defendi (1972) found that mammalian cells and chick embryonic neural retina and liver cells release aggregation factors which, although insensitive to ribonucleases and proteases, are destroyed by periodate treatment and inactivated by exposure to testicular hyaluronidase. They concluded that hyaluronate (or a very similar molecule) was acting as a cell ligand, binding with receptors that were found to be protease susceptible. In another study with similar implications, Wasteson et al. (1973) showed that cat lymphoma cells aggregate when hyaluronic acid is present although under similar conditions no aggregation was noted if cells had been trypsinized.

Several more recent investigations have also shown that HA binds directly to the cell surface and promotes aggregation of several mouse cell lines such as SV-3T3 cells (Underhill & Dorfman,1978; Underhill & Toole,1979). Aggregation of SV-3T3 cells occurs by two mechanisms--one dependent on the presence of divalent cations and the other independent of their presence. Underhill and Dorfman (1978) found that exogenous HA inhibits that latter mechanism as do enzymes which cleave HA; they consequently proposed that this form of aggregation involves an interaction between the endogenous HA of one cell and HA receptors on adjacent cells. Furthering this work, and using standard affinity vs. inhibition binding assays, Underhill and Toole (1979) have

demonstrated that HA does indeed bind to the surface of SV-3T3 cells. Finally, they have most recently shown that variations in the ability of other tumor/parent cells to bind HA can partially account for the differences between them with respect to their ability to aggregate (Underhill & Toole,1981).

In other systems, HA seems to work to prevent aggregation of cells and rather is correlated with cellular migration or tissue remodeling. Toole (1973) has stated that cartilage-like aggregate formation by stage 26 chick somite cells is blocked by hyaluronic acid in vitro. In a related study of chick embryo limb chondrocytes, Solursh et al. (1980) have shown that exogenous HA (decasaccharide units) rapidly cause displacement of newly synthesized proteoglycans from the cell layer into the culture medium while HA tetrasaccharides, after a 12 hour latency period, will depress incorporation of sulfate into GAG.

Polansky, Toole and Gross (1974) have reported that high levels of both hyaluronic acid and hyaluronidase exist in chick brain during its embryonic formation but that these levels decline soon after hatching, at which time most if not all neuronal migrations have stopped. Solursh et al. (1979) have also correlated the presence of HA with cellular migration, in this case during the formation of the sclerotome. Sclerotomal cells produce an extracellular matrix rich in HA that is highly hydrated and which thus facilitates the movement of the sclerotomal mass towards the notochord. Embryonic chick skin presents a somewhat similar case: HA and ChS turnover are needed for remodeling of tissue during days 14-16 of development although from day 20 on an increasing deposition of HA in the matrix is correlated with an increasing amount of water bound in the tissue space (Nakamura

& Nagai,1980). Hyaluronate turnover is also associated with morphogenetic events in the regenerating newt limb and in chick embryo limb and cornea; Toole suggests (1973) that in these systems HA turnover could regulate such events as cell migrations by interfering with cell interactions leading to aggregation and thereby permitting a requisite accumulation of cells prior to the onset of histogenesis.

This interference in cell interactions whereby cell migration is promoted and aggregation is inhibited could take place by several different mechanisms. One possibility is that the HA, by virtue of its great attraction for water molecules and consequent large solvent domain, is simply providing an optimal medium (one that is highly hydrated) through which cells may migrate. Another possibility is that, due to its high viscosity in solution, HA in slightly higher concentrations in the matrix may act as a glue, trapping or immobilizing cells in a mesh and thus essentially isolating them from other cells. On the other hand, Morris has recently proposed (1979) that glycosaminoglycans in chick retina matrix enhance aggregation mainly due to their steric exclusion of the cells into a smaller space wherein they are more likely to interact. He has presented data showing that aggregation rate is directly related to polymer viscosity, molecular weight, and concentration and is not dependent on polymer charge. This mechanism differs from others proposed in that it involves nonspecific influences rather than specific binding of the GAGs to proteins associated with cell membranes. It must be noted though, that none of these proposed mechanisms of action are mutually exclusive as they could operate to differing degrees in different systems.

That hyaluronic acid does bind to specific proteins in the extracellular matrix has been shown in (adult) bovine nasal cartilage where it forms aggregates with proteoglycans (Hardingham & Muir,1973; Hascall & Heinegard,1974a,b,c). Aggregate size is determined by the length of the HA molecule involved (Hascall & Heinegard,1974a). When proteoglycans (chondroitin sulfate and keratan sulfate) are digested off the aggregate, 25% of the total protein remains bound to the hyaluronic acid; this protein has been shown to be in part a portion of the protein found in the proteoglycan molecule, and in part derived from low molecular weight link proteins in the proteoglycan aggregates (Heinegard & Hascall,1974c). Hardingham and Muir (1973) have found good evidence to suggest that each proteoglycan possesses only one hyaluronic acid binding site which is approximately a decasaccharide unit in length.

Sulfated glycosaminoglycans have also been implicated as mediators of developmental processes. Chondroitin sulfate, whose repeating disaccharide unit is composed of D-glucuronic acid and galactosamine, is sulfated at either the 4 or 6 position of the galactosamine. The extent to which chondroitin sulfate is actually sulfated varies, as does the number of these polysaccharide chains attached to a protein core in proteoglycan arrangements (Roden,1970). The relative proportions of chondroitin-4- to -6-sulfate and of these two chondroitins to other sulfated GAGs (heparan and dermatan sulfate) have been shown to vary depending on the developmental stage of several organisms (Dietrich,et al.,1977; Morris,1976). Although the significance of such a patterned synthesis is yet unclear, Ch6S has been implicated as

a promoter of cell division, perhaps acting via its disruption of cellular recognition sites through competition with Ca^{++} and other sulfated GAGs on the outer cell coat (Dietrich et al.,1977).

Meier and Hay (1974) showed that the synthesis of chondroitin sulfates and heparan sulfate by (developing) corneal epithelium may be regulated by the adjacent extracellular matrix. Chondroitin sulfate, both in free glycosaminoglycan form and in proteoglycan aggregates, heparin and heparan sulfate all were shown to enhance epithelial synthesis of ChS and heparan sulfate-like compounds although hyaluronic acid and dermatan sulfate exhibited no such effect. Keratan sulfate, on the other hand, inhibited glycosaminoglycan synthesis. Nevo and Dorfman (1972) showed that purified chondromucoprotein, as well as several other polyanionic molecules, stimulates the rate of synthesis de novo of chondromucoprotein in embryonic cartilage cells. It did not appear that any of the exogenous chondromucoprotein entered the chondrocytes; further, when chondrocytes were incubated with trypsin, synthesis decreased unless the cells were incubated for a "recovery" period. Nevo and Dorfman therefore proposed that the rate of chondromucoprotein synthesis, indicative of a chondrocyte's differentiated state, is controlled at least in part by cell surface receptors.

There is also evidence to suggest that ChS interacts with collagen. Speziale et al. (1980) have shown that of the two types of proteoglycan subunits in bovine cornea, the proteochondroitin sulfate one is responsible for the proteoglycan's interaction with collagen. The ChS portion of this subunit binds to collagen-derivatized Sepharose 4B columns; digestion of ChS results in no interaction between the collagen and proteoglycan.

Chondroitin sulfate as well as heparan sulfate and HA will associate with cell surface fibronectin, a large glycoprotein that has recently received a lot of attention because of its effect on both cell migration and adhesion and the fact that addition of fibronectin to cultured transformed cell lines restores a more normal cell morphology and behavior (Culp et al.,1980; Perkins et al.,1979; Yamada et al.,1980; Yamada & Olden,1978). Yamada has reported that the fibronectin molecule contains distinct binding sites for HA and heparan sulfate (1980). Binding of either of these two carbohydrates is not blocked by EDTA or other GAGs. Perkins and coworkers have been able to crosslink fibronectin to cell surface sulfated proteoglycans (1979). Related to this is a study by Culp et al. (1980) showing that cell surface proteoglycans, specifically those left behind migrating cells as so-called substrate-attached material (SAM), can reversibly form two types of "supramolecular complexes". One such type contains mainly heparan sulfate and seems to be the one directly involved in cell adhesion. The other type of complex contains HA and ChS; it links newly synthesized fibronectin to SAM at the edge of adhesion sites, labilizing the fibronectin and consequently mobilizing the cells. In further work, Culp (1980) has shown that adhesion sites of neuroblastoma and glioma cells differ in their composition, those of the latter being more enriched in HA and ChS which corresponds with this cell line's greater motility. Thus GAGs may also influence cells when organized as proteoglycans associated with other large molecules.

Morris, Hopwood, and Dorfman (1977) have studied the biosynthesis of glycosaminoglycans in the developing chick retina. This is especially interesting with respect to the sulfated GAGs since the

pattern of their synthesis changes over development. Retina development may be divided into three major phases: one of high mitotic activity and cellular migration during days 2-8 of incubation, a second period of cellular rearrangement involving cell-cell recognition and reaggregation during days 8-10, and, thirdly, from day 10 extending through hatching, a period of final cell differentiation wherein cells form intercellular junctions and otherwise take on characteristics of the adult retina (Kahn,1974). Between days 5 and 14 of chick retina development exogenous precursors are increasingly incorporated into Ch4S as opposed to Ch6S, with chondroitin sulfate in general being the most rapidly accumulating species of glycosaminoglycan. Heparan sulfate synthesis decreases during this time interval (Morris et al.,1977). Work by A. Millemann (unpublished) suggests that a higher percentage of total tissue GAG is found associated with the cell surface at 14 days than at 7 days development. This surface-associated GAG is most likely organized as proteoglycans (Morris & Ting,1981).

Sheffield and Moscona (1970) have also studied neural retina cells from 7 to 14 days development. They found that dissociated retina cells from different ages formed different patterns of aggregation which reflected (a) the state of differentiation and cellular composition of the donor tissue and (b) the types of intercellular connections which were prevalent at the time of dissociation. It is notable that the type of junction prevalent between 7-day retina cells, the zonula adhaerens or intermediate junction, is one wherein the two adjacent cells' membranes come into close contact at certain points which do not include any intercellular materials (extracellular matrix) (De Robertis et al.,1975). The macula adhaerens type of cell junction, or

desmosome, is found between retina cells of 14-day chicks (Sheffield & Moscona,1970); in this type adjacent cells are 30-50 nm apart. In the intercellular gap are glycosaminoglycans and proteins that appear as a discontinuous middle line; desmosomes are thus susceptible to trypsin, collagenase and hyaluronidase and are also sensitive to calcium chelarors. Most importantly, it has been found that the maintenance of cellular adhesion in this type of junction is dependent on the extracellular material (DeRobertis et al.,1975).

The role of calcium in cell adhesion has long been recognized, but only recently have several laboratories paid particular attention to it as regards the adhesion between embryonic retina cells or between Chinese hamster fibroblasts (V79 cells) (Brackenbury et al.,1981; Grunwald et al.,1981; Magnani et al.,1981; Urushihara,1979). In both these systems, cell-cell adhesion may occur by two functionally distinct mechanisms--one is calcium-independent while the other depends on the presence of calcium to remain functional. Low level trypsinization of cells destroys the latter mechanism yet leaves the former intact; cells adhering via the calcium-independent mechanism are also obtained using chymotrypsin (Grunwald et al.,1980). If calcium is included during exposure of cells to normal or high levels of trypsin the calcium-dependent mechanism remains active.

Takeichi et al. (1979) have shown that antibodies to cell surface inhibit aggregation via the calcium-independent adhesion mechanism. They have now identified a 125,000 MW glycoprotein from V79 cells that is of central importance to this mechanism (Urushihara & Takeichi,1980). Brackenbury and his group also find evidence that the calcium-independent mechanism is disturbed by antibodies to cell adhesion

molecules (1977, 1981); their cellular adhesion molecule (CAM) is a 140,000 MW (as determined by SDS-PAGE) polypeptide (Thiery et al., 1977). They further suggest that calcium-independent adhesion is the mechanism by which neurite bundles fasciculate and by which cell layers become organized in the retina (Brackenbury et al.,1981).

Also of developmental significance in the retina, Grunwald et al. (1980, 1981) have determined that the calcium-dependent mechanism of adhesion is developmentally regulated whereas the calcium-independent mechanism of adhesion is not. The former system operates maximally between days 7 and 10, sharply decreasing its activity days 10-13 and disappearing after day 16 of development (1981). Thus the calcium-dependent mechanism of cell adhesion operates when retina cells are rearranging themselves initially in the retina, and the calcium-independent mechanism is used as older cells establish more stable permanent connections.

Such cell adhesion molecules in the retina may be the analogues of fibronectin in other cells. (So far no one has reported the existence of fibronectin in the retina). Further characterization will reveal whether CAM are associated with glycosaminoglycans; it would not be surprising if they are, for GAGs do have a marked influence on the kinetics of cell aggregation (personal observation). Morris has reported (1976) that ChS transiently promotes nonspecific adhesion of 10-day embryonic chick neural retina cells to the substrate at the expense of cell-cell associations. Furthermore, ChS interferes with cell type recognition, retarding histotypic sorting out of mixed embryonic liver and retina cells. These data, in conjunction with the synthesis pattern of ChS during retinal development, suggest that

though retina chondroitin sulfate promotes cell attachment non-specifically, it does so primarily when the cells are already in place. Viewed in this way, it also seems logical that ChS may be associated with the calcium-independent mechanism of adhesion.

Rationale

The work presented here represents several different attempts to further elucidate the way in which certain glycosaminoglycans interact with chick neural retina cells. Based on Morris' finding (1976) that while HA as well as ChS promote cell attachment heparan sulfate has no such effect, the former two GAGs were used in experiments. I began my research by isolating hyaluronic acid from human umbilical cords (Appendix A) and from this project proceeded to study the effects of different concentrations of HA and ChS on cultured retina cells. Numerous attempts were made to establish GAG gradients as substrata for cells in culture; these trials are documented in Appendix B. Because the effects of GAGs on cells may be due in part to the greatly increased viscosity of the culture medium in which they are included, the viscosities of different concentrations of GAG as single solutes or mixtures in medium were measured. These data were also considered with respect to the possibility that GAGs in solution may self-associate through their carbohydrate chains; Appendix C presents these results. Finally, the main part of my thesis is a manuscript prepared for publication concerning the effects of GAGs on retina cells as measured by culturing cells with Sepharose beads derivatized with different glycosaminoglycans.

Interactions between Glycosaminoglycans and Aggregating Retina Cells

Summary

Neural retina cells from 10-day chick embryos were tested for their ability to attach to Sepharose 4B beads which had been derivatized with either chondroitin-6-sulfate or hyaluronic acid. The cells were isolated by trypsin dissociation and were tested directly or after preincubation in medium with or without either glycosaminoglycan. Attachment of cells was inhibited when they had been previously exposed to 10 mg/ml chondroitin sulfate or 2.5 mg/ml hyaluronic acid, regardless of the type of bead included in the culture. By using freshly-dissociated cells to which chondroitin sulfate was added simultaneously with the addition of either chondroitin sulfate-derivatized or underivatized beads, cell attachment was found to be inhibited at certain threshold concentrations which differed for the two types of bead, being lower for the cells combined with the chondroitin sulfate-derivatized beads. These results support a model of cell-glycosaminoglycan interactions in which a close association of exogenous glycosaminoglycan with the cell surface

blocks cell attachment to beads either by steric effects of the bulky sugars or by masking cell adhesion sites.

The effects of extracellular matrix glycosaminoglycans (GAG) on cells during development have been well documented for a number of cell types although the mechanism(s) of their action have yet to be determined. While the sulfated GAGs (the chondroitins and heparan) are known to promote cell aggregation, adhesion, and/or differentiation (Culp, et al.,1980; Culp et al.,1978; Dietrich et al.,1977; Morris, 1976; Morris & Dorfman,1976), the presence of hyaluronic acid seems to be correlated with periods of cell migration and rearrangement as well (Pessac & Defendi,1972; Polansky & Toole,1974; Solursh et al., 1979; Toole,1973). Generally, there are three levels of specificity at which such long chain, highly charged molecules might act (Laurent, 1977): by nonspecific steric exclusion, by an electrostatic attraction between the negatively charged GAG and certain regions of the cell surface, or most specifically by binding between GAG and the cell involving a recognition site on the cell surface.

Steric exclusion has been proposed by Morris (1979) as a mechanism of GAG-induced cell aggregation. Comparison of the effects of a number of branched and linear, high molecular weight polymers with those of GAGs suggested that the GAGs act like the other polymers to enhance aggregation of embryonic neural retina cells by virtue of the comparatively large volume from which they effectively exclude the cells, forcing the latter into close proximity in the remaining space. Of note in this study, however, was that the influence of chondroitin

sulfate on cells suggested that this particular interaction might take some other form in addition to steric exclusion.

Chondroitin sulfate is one of the principal GAGs synthesized by chick neural retina cells, particularly after the seventh day of embryonic growth. Between days 7 and 14 of development the synthesis pattern of this GAG is changed, with increasing amounts of the 4- rather than 6- sulfated form produced (Morris et al.,1977). Localization studies (A. Millemann, unpub.) suggest that a higher percentage of total tissue GAG is found associated with the cell surface than in the cytoplasm at 14 days than at 7 days development. Most all of this GAG is probably associated with protein as proteoglycans and glycoproteins.

In cartilage, matrix chondroitin sulfate and keratan sulfate are covalently attached to protein cores, forming proteoglycan monomers. These two-dimensional "bottlebrushes" are often noncovalently associated via short link proteins with a hylauronic acid backbone (Hardingham & Muir,1973; Hascall & Heinegard,1974). Characterization of the proteoglycans found in neural retina is underway in our lab (Morris & Ting,1981): results so far indicate that the retina GAG is associated with a protein core but these units apparently do not associate with hyaluronic acid to form macromolecular aggregates as in cartilage (Morris & Birkholz-Lambrecht, in press).

The present study is an attempt to determine the level of specificity at which the interaction between cells and GAGs takes place. In order to localize the GAGs and thus more effectively control the interactions, the chondroitin sulfate and hyaluronic acid tested were covalently attached to Sepharose beads. These derivatized beads were incubated with neural retina cells from 10-day chick

embryos which *in vivo* were just ending their migratory and rearrangement stage and were forming intercellular contacts at the onset of final differentiation (Kahn, 1974).

Methods

Materials

The beads used in these experiments were ω-aminohexyl Sepharose 4B from Sigma. Also from Sigma were the 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide, chondroitin-6-sulfate (Ch6S) (from whale cartilage) and hyaluronic acid (HA) (from human umbilical cords). Saline solutions used in dissection were Hank's balanced saline solution (BSS) pH 7.3 from Gibco, and calcium/magnesium-free balanced saline solution (CMF) both buffered with morpholinopropane sulfonic acid (MOPS), 0.01M, as described by Morris & Moscona (1973). The cell culture medium was Eagle's Minimal Essential Medium with Earle's balanced salt solution (MEM) (M.A. Bioproducts) supplemented with 10% (v/v) fetal bovine serum (Sterile Systems, Inc.), 1% (v/v) of a penicillin-streptomycin solution (Difco) and 5 μg/ml DNase I (Sigma). Trypsin (beef pancreas) was from ICN Pharmaceuticals, Inc.

A gyratory shaker (Model G2, New Brunswick Scientific Co., Inc) was used for rotation of cell cultures and bead derivatizations; a Spencer Brightline Neubauer hemocytometer was used to determine cell culture density; and cells and beads were examined with an Olympus IMT inverted microscope.

Bead Preparation

Sepharose 4B beads were derivatized with glycosaminoglycan following the manufacturer's suggested protocol and using the GAG concentra-

tions suggested by either Tengblad (1979) for HA or Iverius (1971) for Ch6S. Typically, 0.75 gram bead powder was allowed to swell in 15 ml of 0.5M NaCl for at least 4 hours, after which the resulting gel was washed with 150 ml of 0.5M NaCl (200 ml/g powder). This washing removed the lactose and dextran packaged with the beads; the salt was then removed by washing with a similar amount of distilled water. Ligand solution was prepared by dissolving the glycosaminoglycan in distilled water to give 3 mg/ml HA or 2 mg/ml Ch6S; 10 ml of this were added to approximately 2 ml gel. After the pH of the GAG-bead solution was adjusted to between 4.5 and 6.0, 0.25g carbodiimide was added to make a 0.1M solution. The reaction mixtures thus obtained were put on a 50 rpm shaker at room temperature for 24 hours. The pH was checked at least three times during the first hour and three times during the remaining 23 hours and was adjusted to 4.5-6.0 if necessary by addition of 0.25N HCl. Samples of the reaction "supernatant" were taken at intervals during the 24 hour period and were later tested for uronic acid content using the Bitter Muir assay (1962) to determine the extent of bead derivatization. For all experiments, between 0.9 and 1.2 mg Ch6S was bound per ml of wet gel; HA bound at 1.0-1.2 mg/ml of wet gel. After 24 hours the beads were washed alternately with 0.1M $NaHCO_3$, pH 10, and 0.1M sodium citrate, pH 4, using 100 ml of each total. If stored, beads were left in the sodium citrate at $4^{o}C$; before use all were washed with at least 100 ml of distilled water and then suspended in culture medium.

Two types of non-derivatized beads were tested as controls for the GAG-derivatized ones. The first type was "washed beads", which had been carried sequentially through 0.5M NaCl and distilled water washes

to remove the manufacturer's lactose and dextran but which had not been exposed to the derivatizing agent. The second type was beads that were taken all the way through the derivatizing procedure, except that distilled water alone was used as the ligand solution. These were termed "pseudo-derivatized beads". Both types of non-derivatized beads had exposed uncharged amine groups as opposed to the negatively charged carboxyls and sulfates of the GAG-derivatized beads.

Preparation of Cells

Eggs from White Leghorn chickens were incubated 10 days at 37°C. Retinas were dissected out in BSS, washed three times in CMF with 0.01M MOPS, and incubated 15 minutes at 37°C on a shaker at 50 rpm. They were then suspended in 0.25% trypsin in CMF with 0.05M MOPS solution and incubated as before for another 15 minutes. Trypsinization was stopped by washing the retinas two or three times in culture medium.

Retina cells were suspended in 1-2 ml of medium and dissociated by trituration through pipettes of increasingly smaller bore. The density of the resulting cell suspension was determined and the suspension was diluted with culture medium as required. Some of these freshly dissociated cells were added to beads at this point while others were incubated prior to addition of beads.

For incubation, 1 ml of suspension (50×10^6 cells) was added to 5 ml of culture medium in a 50 ml Erlenmeyer flask. Such flasks were incubated at 37°C for 1.5 hours on a shaker at either 75 rpm (to permit aggregation of the cells) or at 125 rpm (to maintain a suspension of dissociated cells). Some cells were incubated with glycosaminoglycan which was dissolved in the 5 ml of culture medium prior to addition of

cells. Chondroitin-6-sulfate was added to give a final concentration of 10 mg/ml; hyaluronic acid was used at a final concentration of 2.5 mg/ml. Cells in medium containing GAG were incubated 1.5 hours on a shaker at 125 rpm before the addition of beads.

In some experiments, cells which had been preincubated in GAG were transferred directly to bead cultures as small aggregates suspended in the GAG-medium. Thus, exogenous GAG (6 mg/ml Ch6S or 1.5 mg/ml HA) was present in the medium in these bead cultures. In other experiments cells which had been preincubated in GAG were washed one or two times with 10 ml of GAG-free culture medium and then resuspended in 6 ml of the medium prior to their addition to beads.

Cell-Bead Cultures

Cells were mixed with beads in all cases to produce a suspension of $5x10^6$ cells/ml. For comparison with experiments in which cells preincubated in GAG were transferred directly without a wash, experiments were done wherein Ch6S (6 mg/ml) was added to half the culture flasks simultaneously with the cells and beads. Freshly dissociated cells were added directly to a series of concentrations of Ch6S (0.03-6.0 mg/ml) in medium containing the beads.

Beads were added to cultures in all cases to produce a 10% (v/v) suspension. The bead-cell suspensions were incubated at 37^oC on a shaker at 50 rpm. Samples were examined at intervals to check for aggregation between cells and between cells and beads.

Determination of Cell-Bead Interactions

Attachment of cells to the beads was demonstrated by a series of progressively disruptive tests. 1) The sample was swirled on the microscope slide while watching it under the microscope. "Unattached"

cells or cell clusters were those that moved away from beads upon swirling. 2) A glass needle was used to roll the bead-cell complexes around on the slide. Cells remaining with beads under this treatment but separating with the next treatment were said to be "loosely attached". 3) The bead-cell solution was filtered through a Nitex mesh of 62 μm pore size, which retained the beads and largest cell clumps but allowed single cells and small cell aggregates to pass through as the filtrate. The mesh was then washed with 1.5 ml of medium which was saved as "Wash". The beads and cells remaining on the mesh were then rinsed off with medium into another dish marked "Mesh". All three of these fractions--filtrate, wash and mesh--were examined under the microscope. Cells "firmly attached" to beads were those remaining attached in the mesh fraction.

Results

Overall, five types of differently treated cultures of retina cells were tested for the ability of the cells to attach to beads. Two types of cultures consisted of single cell suspensions (those freshly dissociated and those incubated in medium at 125 rpm), whereas the other three types consisted of small aggregates of cells (cells incubated with HA or Ch6S and cells incubated in medium at 75 rpm). Each of the five types of cultures was combined with each of the four kinds of beads (beads derivatized with HA or Ch6S or the two types of non-derivatized beads); each of these 20 possible combinations was tested on two to six different occasions. The possible effects of exogenous GAG at varying concentrations in the culture medium were also determined.

In the first experiment, Ch6S-derivatized beads were incubated with cells that had been previously incubated at 125 rpm in medium with or without Ch6S. Those cells which had been preincubated with Ch6S were not washed before culturing with the beads and, thus, were in the continued presence of GAG. The results were strikingly different with and without Ch6S: those cells exposed to Ch6S did not attach to the beads at all whereas "naive" cells attached so strongly to the beads that they were not removed by repeated washes on the Nitex mesh (Figure 1, d-g, l-o). However, this observed difference could be explained by the fact that cells incubated in medium without GAG were still dissociated when added to beads, whereas those incubated in Ch6S medium were grouped as small clusters. Attachment of aggregates to the beads could have been prevented simply by the greater shear forces acting on aggregates as opposed to single cells.

To determine the importance of prior cell-cell association to cell-bead interactions, cells were incubated in medium without GAG but at 75 rpm and then were added to Ch6S beads. These cells had formed aggregates similar in size to those preincubated with Ch6S at 125 rpm (see Figure 2). In three of four trials these aggregates did attach to the Ch6S beads; in the one case, larger clumps of cells seemed to be loosely attached to the beads but moved away from the latter when the sample was swirled. It is significant that this specific cell culture was inadvertently maintained at pH 7.8 instead of the usual 7.2 and some cell damage was evident.

The results of this bead-cell combination indicated that a cell's exposure to GAGs influences its behavior with respect to beads. Incubation with HA also blocked cell attachment to beads, although some

loose association between HA cell clusters and Ch6S beads was noted, as was found between Ch6S cells and HA beads (see Discussion and Figure 3).

To determine whether the interaction between single cells and beads depended on cell surface material that was removed during trypsinization but regenerated during preincubation, freshly dissociated cells were cultured with beads; as a control, cells were incubated 1.5 hours after dissociation at 125 rpm to maintain a single cell suspension as in the first experiment (Figure 1). No differences in attachment were seen between the freshly dissociated and preincubated cells.

No matter what type of bead was used (derivatized or nonderivatized), cells that were not incubated with either HA or Ch6S, whether in small aggregates or as single cells, attached equally firmly to all beads, while cells incubated with HA or Ch6S did not attach to any beads (Table 1).

Incubation with GAG thus dramatically altered the cells' ability to attach to any kind of bead. However, these experiments did not distinguish between the possible influence of GAG bound to the cell surface during preincubation and GAG present in solution during the adhesion test. To test whether bound GAG may have been responsible for the cells' failure to attach to beads the GAG-incubated cells were washed before addition to beads. The ability of GAGs to inhibit attachment was found to decrease with the number of washes. Moreover, preincubation in GAG was not necessary to block cell attachment to beads--addition of 6 mg/ml Ch6S along with beads to freshly dissociated cells also inhibited cell-bead attachment (Table 2).

Thus, the presence of GAG in the culture medium and/or preincubation with GAG blocked cell attachment to beads. When freshly dissociated cells were cultured with beads in the presence of Ch6S (added to the medium) it became apparent that cell attachment to beads was dependent on the concentration of Ch6S. The threshold level of Ch6S for inhibition of cell attachment was variable in different experiments, but in all cases was lower with Ch6S-derivatized beads than with pseudo-derivatized ones (Table 3).

Discussion

The results of this study as summarized in Tables 1-3 show that the attachment of neural retina cells to Sepharose beads is regulated by the levels of exogenous glycosaminoglycan to which the cells are exposed rather than being determined only by the surface of the bead. This suggests that GAG interacts with the cell surface to prevent cell attachment when present in high enough (threshold) concentrations by sterically excluding cell contact with other (bead) surfaces that do not also interact with the same GAGs in the same or a complementary manner.

In the cultures tested there were two sources of exogenous GAG to which the cells were exposed: that in the medium and that derivatized to certain beads. If GAGs in the medium were simply sterically excluding cells, crowding them together during their incubation period in GAG-medium to produce the small aggregates observed rather than interacting with the cell surfaces in some more specific way, it would be expected that these aggregates, after washing, would be identical to those formed during preincubation in plain medium at 75 rpm. How-

ever, those cells not exposed to GAG attached to all types of beads, whereas those preincubated with GAG did not attach to any beads if washed only once. When washed two times in GAG-free medium, these aggregates behaved like "naive" cells, attaching to all types of beads. Thus, there must have been some sort of interaction occurring between the GAG and cells during the incubation period that persisted through the latter's single washing in medium but which was disrupted by further washing.

Even though it is unlikely that GAGs, which are strongly polyanionic, interact directly with the negatively charged cell surface, the possibility remains that they may do so indirectly through the formation of calcium bridges. However, when beads with cells attached were placed in a small pipette that had Nitex mesh at the bottom and the resulting column was washed with several volumes of CMF, no cells were found in the eluate or unattached in the medium when the column was poured into a culture dish (data not shown). Several recent studies (Brackenbury et al.,1981; Grunwald et al.,1981; Takeichi et al., 1979; Magnani et al.,1981) have shown that two different mechanisms function in chick neural retina cells to promote aggregation--one, regulated developmentally between days 7 and 16 of embryonic life, is calcium-dependent while the other, unregulated through development, is not dependent on the presence of calcium. Trypsin-dissociation of cells destroys the calcium-dependent system, which is only partially restored after 1.5 hours. Thus, although we cannot exclude the possibility that GAGs bind to the cell surface by a calcium bridge, the cell-cell and cell-bead adhesions seen in this study are most likely independent of such binding and are occurring via the calcium-

independent mechanism.

Although simple charge interactions between GAGs and other molecules on the cell surface thus seem an unlikely explanation for the adhesion observed here, there is evidence that different GAGs may adhere to each other. Turley and Roth (1980) have shown that HA and ChS interact directly in solution, seemingly via their carbohydrate chains. This interaction is thus different from that found between HA and ChS in proteoglycans which occurs through a protein binding site (Hascall & Heinegard,1974). Turley and Roth showed that Dowex beads derivatized with HA agglutinated ChS-derivatized beads although homotypic associations of neither kind were seen. This sort of interaction obviously does not explain the attachment of freshly dissociated cells to underivatized beads. However, it is interesting that in the present study cells preincubated with Ch6S, grouped in large aggregates that otherwise did not attach to any sort of bead, did form loose associations with HA-derivatized beads. The shape of the aggregates in these cultures often was that of a crescent, into the curve of which fit a bead. No physical connection could be seen between the bead and cells by careful focusing of the microscope to optically section the interface, but the two entities would roll together when the medium was disturbed with a glass needle. In other cultures of large aggregates and beads, cells would move away from beads with similar disruptive treatment. This loose association was also seen, though to a lesser extent, between cells preincubated in HA and beads derivatized with Ch6S. Neither of these two loose associations was maintained when the culture was washed on Nitex mesh (see Figure 3).

The next level of interactive specificity is a direct binding of GAG to the cell surface. Underhill and Dorfman (1978) could inhibit the aggregation of mouse 3T3 cells with hyaluronidase, and other studies (Delpech & Halavent,1981; Underhill & Toole,1979,1981) have shown that exogenous HA binds directly to cells. Although HA reportedly represents only 2% of total GAG in the retina (Morris et al.,1977), if attached to the cell surface it could affect aggregation. Perhaps pertinent to this is the recent isolation from neural tissue of a large glycoprotein, hyalurononectin, which binds HA (Delpech ¢ Halavent,1981).

The experiments testing freshly dissociated cells in combination with either Ch6S beads or pseudo-derivatized beads where various concentrations of Ch6S were added to the culture medium further support the hypothesis that a direct interaction occurs between GAGs and the cells surface. The most interesting result from these experiments was that cells plus certain concentrations of Ch6S did not attach to Ch6S beads but did firmly attach to pseudo-derivatized beads. This is the only case where the bead surface seemed to affect cell attachment, all other culture conditions being identical.

This result and all others obtained in this study may be explained by a model suggesting that at certain threshold concentrations exogenous GAGs interact with (bind to?) the surface of neural retina cells and thereby block cell attachment to beads. Although the model does not necessarily imply GAG binding at specific receptor sites on the cell surface, evidence supports the occurrence of some sort of fairly stable interaction. Glycosaminoglycans thus associated with the cell surface may sterically hinder cell attachment to beads or may block

attachment by masking the appropriate cell adhesion site. Either of these two mechanisms explains the failure of freshly dissociated cells cultured with threshold levels of Ch6S to attach to Ch6S beads. This same concentration of GAG in the medium obviously did not mask all cell adhesion sites since similar cells attached to pseudo-derivatized beads. The Ch6S may have made it impossible for the unmasked adhesion sites to reach the derivatized bead surface, either because the bead GAG masked the available cell sites or because steric interference between cell- and bead-associated GAG was too great.

The apparent ability of exogenous GAG to promote cell aggregation during the preincubation periods of this study yet inhibit cell-bead interactions during subsequent incubation seems at first a paradox. This dual nature of GAG effects on cells seems less paradoxical however, when not the cells themselves, but rather what they are interacting with, is considered. The surface of another cell is infinitely more complex than the surface of even a derivatized Sepharose bead. Thus, although they might not interact directly with other homotypic GAG molecules, GAGs associated with one cell's surface may interact with some other molecules on another's surface to promote adhesion/aggregation.

As is evident in Table 3, the threshold level of Ch6S inhibition of cell attachment to beads differed significantly on the three occasions it was tested. However, in all cases the threshold was lower for cells in combination with GAG-derivatized beads than with pseudo-derivatized ones. Further work is in progress to more precisely determine these threshold levels. An additional study is also being

done to determine how the aggregates formed during preincubation in GAG are affected by the wash(es) with GAG-free medium since the ability of these aggregates to attach to beads seems to decrease with increased washing. These experiments should further define the nature of the GAG-cell interaction demonstrated in the work presented here.

	BEADS			
	"Washed"	"Pseudo-Derivatized"	Ch6S-	HA-
CELLS				
Freshly dissociated	++	++	++	++
Incubated in Medium, 75 rpm	++	++	++	++
Incubated in Medium, 125 rpm	++	++	++	++
Incubated in Ch6S, 125 rpm	0	0	0	+
Incubated in HA, 125 rpm	0	0	+	0

Table 1. Attachment of Neural Retina Cells to Derivatized Sepharose Beads. Cells preincubated in GAG were unwashed, thus those cultures contain either 6 mg/ml Ch6S or 1.5 mg/ml HA.

++ indicates cells firmly attached to beads, not dislodged by filtering

\+ indicates cells loosely attached to beads, dislodged by filtering

0 indicates no cells attached to beads.

CELLS	BEADS: "Pseudo-Derivatized"	BEADS: Ch6S-
Freshly Dissociated		
no GAG added	++	++
+ 6 mg/ml Ch6S	0	0
Incubated in Medium, 75 rpm		
no GAG added	++	++
+ 6 mg/ml Ch6S	0	0
Incubated in Medium, 125 rpm		
no GAG added	++	++
+ 6 mg/ml Ch6S	0	0
Incubated in Ch6S		
Unwashed	0	0
Washed once	0	0
Washed twice	++	++
Washed once + 6 mg/ml Ch6S	0	0
Incubated in HA		
Unwashed	0	0
Washed twice	++	++

Table 2. The Effects of GAG in the Medium on Cell Attachment in Cell-Bead Cultures. Ch6S was added where indicated as the cells were added to the beads. The washing of the GAG-incubated cells is described in the text.

++ indicates cells firmly attached to beads

0 indicates no cells attached to beads.

	BEADS					
	"Pseudo-Derivatized"			Ch6S-		
	A	B	C	A	B	C
Freshly Dissociated Cells						
no GAG added	++	++	++	++	++	++
+ 0.03 mg/ml Ch6S		++			++	
+ 0.06 mg/ml Ch6S	++	++	++	0	++	0
+ 0.10 mg/ml Ch6S		++	++		++	0
+ 0.16 mg/ml Ch6S		++			++	
+ 0.30 mg/ml Ch6S		++			++	
+ 0.60 mg/ml Ch6S	0	++	0	0	0	0
+ 1.00 mg/ml Ch6S		0	0		0	0
+ 3.00 mg/ml Ch6S		0			0	
+ 6.00 mg/ml Ch6S	0	0	0	0	0	0

Table 3. Concentration Dependence of Freshly Dissociated Cells' Attachment to Beads in the Presence of Ch6S in the Culture Medium. The results from three experiments (A,B,C) are shown. Although the threshold levels of Ch6S which inhibited cell attachment to beads were different in the three trials, in all cases this threshold of inhibition was lower for Ch6S-derivatized beads than for pseudo-derivatized ones.

++ indicates cells firmly attached

0 indicates no cells attached

All concentrations were not tested in every trial.

Figure 1. The five differently treated types of 10-day neural retina cells before (d,h,l,p) and after culture with Ch6S-derivatized beads. Freshly dissociated cells are shown after 1 hour culture (a,b) and after 24 hours (c). Cells incubated in medium at 125 rpm for 1.5 hours (d) are shown after 1 hour (e,f) and after 24 hours culture (g). Cells incubated in medium at 75 rpm to form small aggregates (h) are shown after 1 hour (i,j) and 24 hours (k) culture with beads. Small aggregates formed during incubation in Ch6S (l) were not washed before addition to beads; these are shown (m,n) after 1 hour and after 24 hours (o). Cells incubated in HA (p) also were not washed; these are shown after 1 hour culture with beads (q) and after 24 hours (r,s).

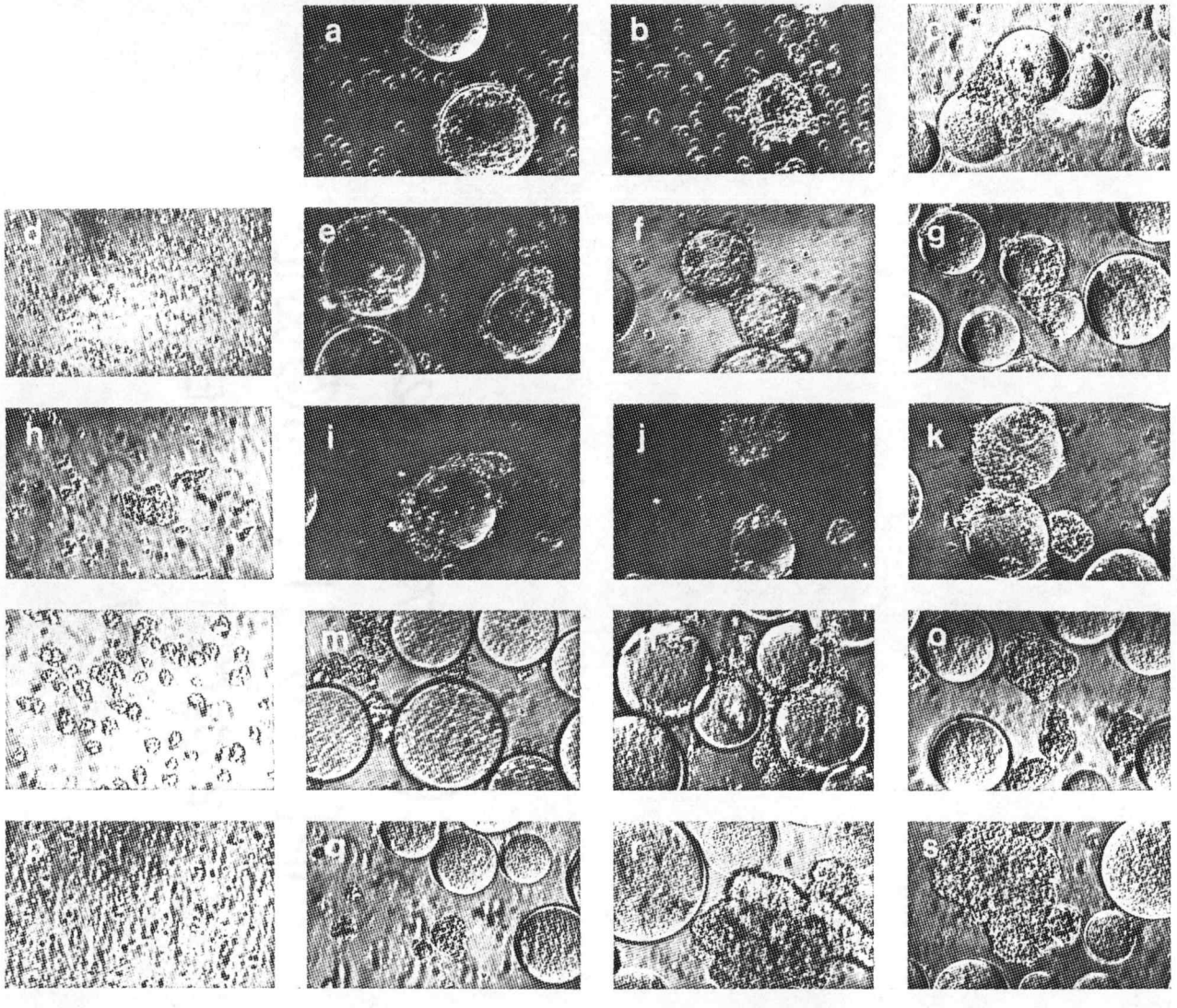

Figure 1

Figure 2. Cell attachment to beads did not depend on the type of derivatized bead used in culture. Cells are shown after 24 hours culture with "washed beads" (a,e,i), with "pseudo-derivatized beads" (b,f,j), with Ch6S-derivatized beads (c,g,k) and with HA-derivatized beads (d,h,l). The types of cell cultures shown here are cells incubated in medium at 125 rpm (a-d); cells incubated in medium at 75 rpm (e-h) and cells incubated in Ch6S (i-l). Cultures shown of Ch6S-incubated cells contain 6 mg/ml Ch6S in the medium since these cells were not washed prior to addition of beads.

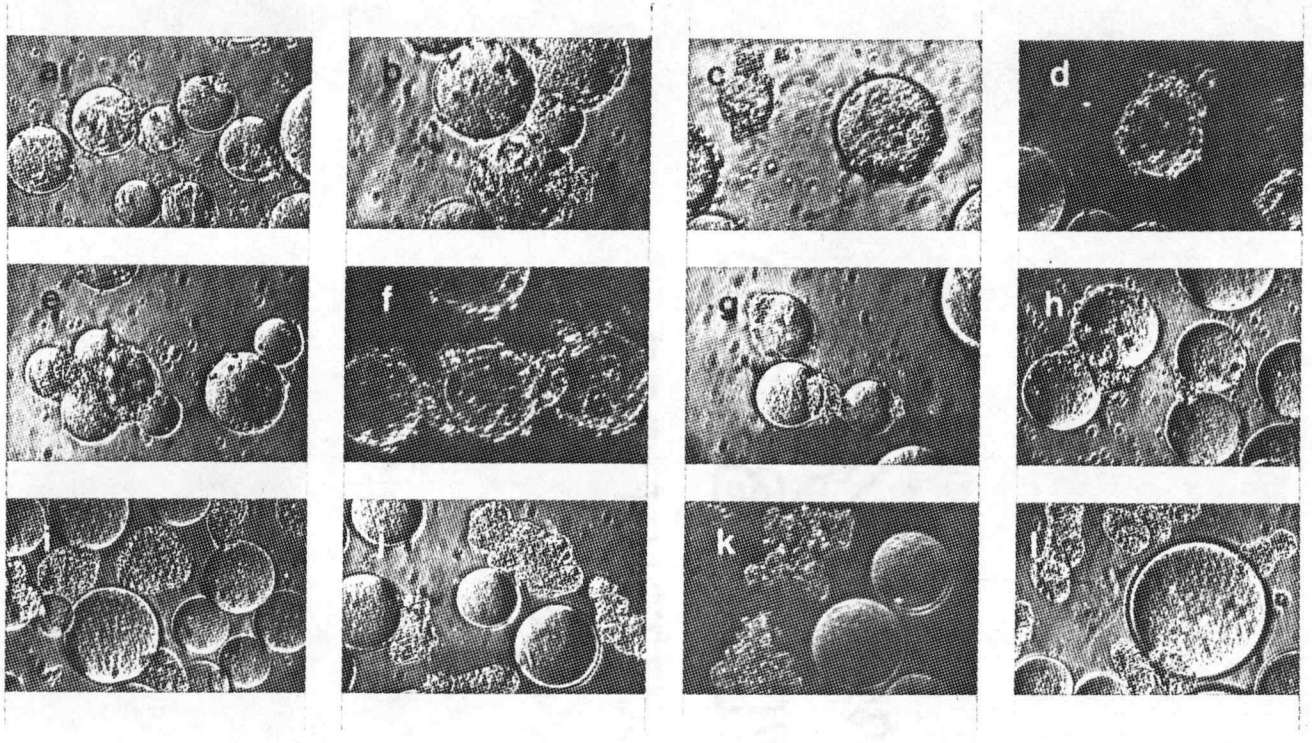

Figure 2

Figure 3. Examples of tests used to determine cell attachment to beads.

a-b: Swirling of Ch6S-incubated cell clusters and Ch6S-derivatized beads shows that though they are in close proximity (a) there is no attachment (b).

c-g: Greater disruption with a glass needle shows that HA-incubated cells and Ch6S-derivatized beads were not separated (c-d). However, filtering of similar cultures of HA-beads and Ch6S-incubated cells (e) shows that cell clumps and a small bead pass through the mesh as the filtrate (f) leaving naked beads and a few larger cell clusters on the mesh (g).

h-k: Similar filtering of Ch6S-incubated cells cultured with pseudo-derivatized beads (h). (i) shows the filtrate; large aggregates and naked beads are left on the mesh (j,k).

l-o: Filtering of 125 rpm-incubated cells cultured with pseudo-derivatized beads after 1 hour culture (l). The filtrate (m) contains a few single cells; beads with cells still firmly attached were left on the mesh (n,o).

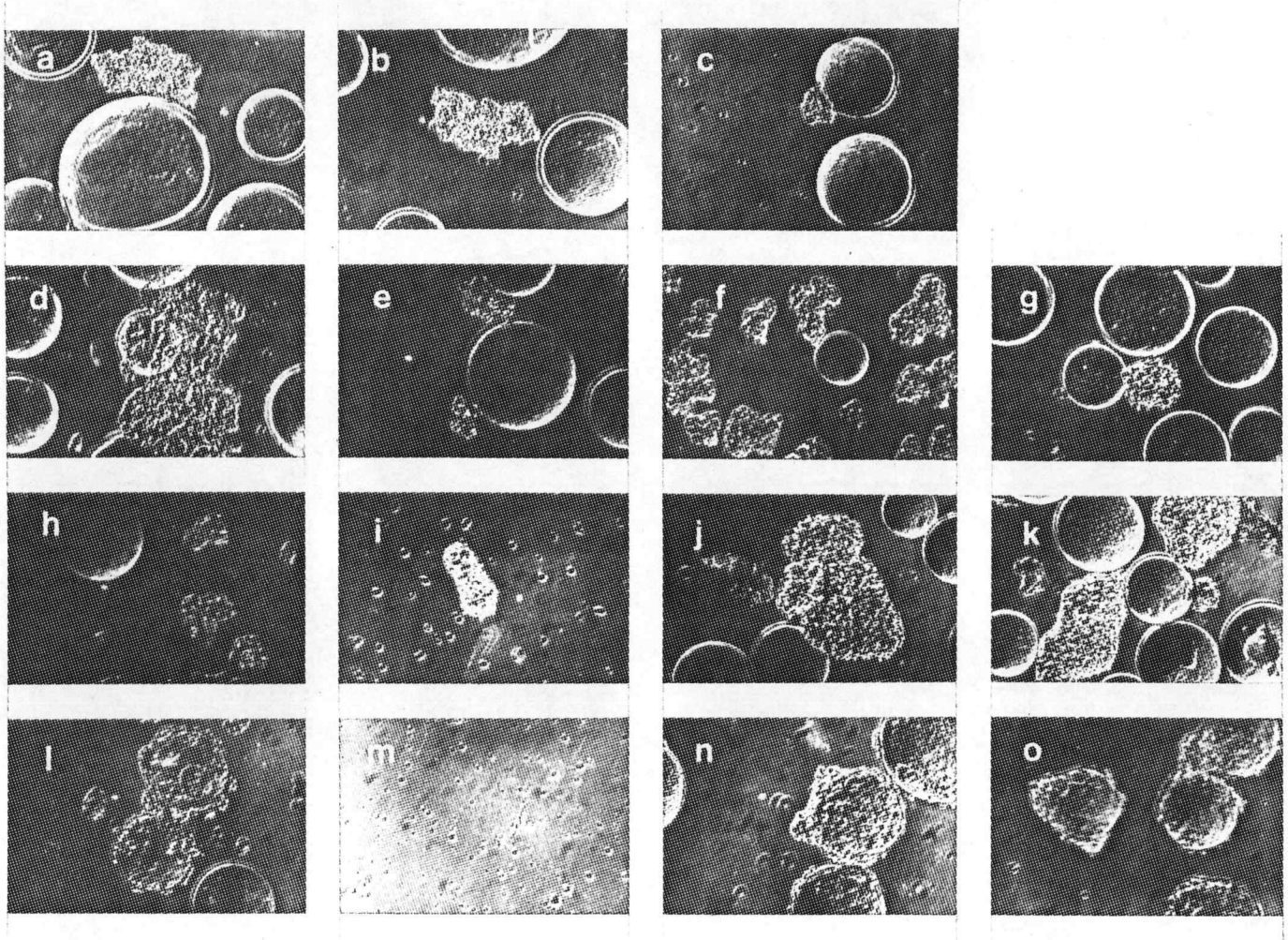

Figure 3

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APPENDICES

APPENDIX A. Isolation of Hyaluronic Acid and Other Glycosaminoglycans from Human Umbilical Cords

In an effort to find an inexpensive source of relatively pure glycosaminoglycan, the method of Jeanloz (1965) for the isolation of hyaluronic acid from human umbilical cord was attempted. Briefly, this technique involved lipid extraction from umbilical tissue with acetone, subsequent solubilization of the tissue with pepsin and trypsin and the removal of protein by chloroform-1-pentanol extraction, followed by ammonium sulfate precipitation to (theoretically) remove sulfate contaminants.

Umbilical cords (stored in acetone during the collection period) were obtained through the courtesy of Dr. T. Hart from the Obstetrics Department of Good Samaritan Hospital, Corvallis, Oregon. The major difficulties encountered in following Jeanloz's procedure involved controlling the smell of the protease-digested solution and the insolubility of the amount of ammonium sulfate suggested. Although all steps were completed as directed, the yield of "hyaluronic acid" was lower than predicted (1.23% rather than the expected 5.6% based on the weight of the dried cords) (see Figure A1). This could be explained in part by the fact that Jeanloz's procedure gives the sodium salt of HA whereas the product weighed here was in the protonated form. Furthermore, cellulose acetate strip electrophoresis (CASE) of the product (Morris et al.,1981) showed at least two bands (Figure A4), indicating considerable contamination of the HA with chondroitin sulfate-like material.

Two modifications of Jeanloz's procedure were also tested on the first batch of umbilical cords. For the first variation (Figure A2), a sample of the protease-digested solution was precipitated with trichloroacetic acid (TCA) and this redissolved precipitate was then subjected to precipitation with cetylpyridinium chloride (CPC). The CPC precipitate was dissolved in water and DNase was added to eliminate DNA. After enzymatic action was complete, further CPC precipitations were performed to remove the enzyme and purify the glycosaminoglycan.

The second variation on Jeanloz's procedure (Figure A3) involved CPC-precipitation of GAG material present in solution after the series of chloroform-1-pentanol extractions. As was done to finish the first modification, the CPC-precipitated material was finally dissolved in 2M NaCl and reprecipitated with 2.5 volumes 95% ethanol; this precipitate was dialyzed exhaustively against distilled water, lyophilized, and samples were run on CASE.

Unfortunately, neither modification produced pure hyaluronic acid--in both, as from the unmodified protocol, the "hyaluronic acid" produced at least two bands on cellulose acetate strips stained with Alcian blue, one running with the hyaluronic acid of the standard and the other near the standard unsulfated chondroitin (Figure A4). Indeed, there seemed to be more chondroitin-like material present than HA as judged by the intensity of the stained bands. The value of these variations is thus simply that they are shortcuts in Jeanloz's procedure to a product that, like his, is a mixture of glycosaminoglycans rather than pure hyaluronic acid.

In order to further purify the mixture obtained by Jeanloz's procedure, glycosaminoglycan fractionation based on the differential

solubility of their cetylpyridinium complexes was attempted following Schiller et al.'s modification (1961) of Scott's technique (1960). Glycosaminoglycan in solution was precipitated with CPC; this precipitate was repeatedly washed and then centrifuged down in the presence of (inert) Celite. Six such washes each were performed using 0.4, 1.2, and 2.1M NaCl concentrations with 1% CPC. The Bitter Muir assay (1962) was used to test each supernatant collected for uronic acid; glycosaminoglycan was generally found to be present in the first two washes of each salt concentration. Corresponding washes from several fractionations were pooled and ethanol precipitated; the precipitate was dialyzed exhaustively against water and lyophilized. Glycosaminoglycan recovered thus from the 0.4 and 1.2M NaCl washes represented between 34% and 92.5% of the original amount fractionated in different runs. Although the 1.2M NaCl fractions proved to be relatively pure chondroitin sulfate, migrating in one wide band along with the chondroitins of the standard, the 0.4M NaCl (HA) fractions again showed the same two bands of the prefractionation mix.

A second isolation was performed on fresh umbilical cords following Modification II of Jeanloz's procedure (Figure A3). Further modifications of the protocol were made: 1) umbilical cords were boiled in water after a wash in acetone to hydrate and thus facilitate their passage through the meat grinder, and 2)DNase was never added, thus only one CPC precipitation was performed. Difficulties were encountered getting CPC-GAG complexes to form. After numerous incubations and centrifugations of material, the supernate and precipitate were extensively dialyzed against 2M NaCl to displace CPC and the solution was filtered in the cold. According to Scott (1960) the precipitate thus

obtained should be only CPC and not CPC-GAG complexes. The supernate was then exhaustively dialyzed against distilled water, lyophilized, and weighed. From 1.74 kg wet weight of umbilical cord, (roughly 45 cords), 1.1777g "HA" was obtained, representing 0.07% rather than the 0.77% yield expected, based on wet weight of initial tissue.

To further purify the glycosaminoglycan mixture obtained from the second batch of umbilical cords as well as some of that remaining from the first attempt, ion exchange chromatography was used instead of the differential CPC fractionation technique. Column chromatography using DEAE Sephacel proved to be a very simple technique which gave good separation of chondroitin sulfates and hyaluronic acid. After trying several increasing amounts of sample and column material, it was found that a one liter column bed would effectively separate 400 mg GAG in one run. The column was equilibrated with the starting buffer (0.02M sodium acetate, pH 5.9) and the sample, dissolved in the same buffer to give a concentration of 5 mg/ml, was applied to the top of the column. When it was adsorbed, a 3600 ml gradient of 0.02M sodium acetate buffer with 0M to 1.5M NaCl was run. Ten ml fractions were collected and assayed for uronic acid content using the Bitter Muir technique (1962). The results of one such run are shown in Figure A5.

Peak I fractions were pooled for all columns, as were those of Peak II. As this represented two samples of considerable volume, both were concentrated on a rotoevaporator before exhaustive dialysis against distilled water and lyophilization. Final weights obtained for each peak indicated that 80% recovery of material run on the columns was achieved, and that indeed, roughly 60% of that material was chondroitin sulfate-like, and not HA. CASE showed each peak moving as

one band, Peak I corresponding to the standard HA and Peak II to standard chondroitin sulfates (Figure A4).

Based on the results obtained by these various protocols, it is recommended that future isolations of glycosaminoglycans from human umbilical cords be attempted following Modification II of Jeanloz's procedure with subsequent column chromatography to purify the GAG mixture.

Figure A1. Isolation of Hyaluronic Acid from Human Umbilical Cords by Jeanloz's Procedure.

Tissue Preparation

45± 5 human umbilical cords were collected over a 6 week period, stored in acetone.

Cords were acetone-rinsed, scrubbed to remove blood, air dried, and weighed. Dry weight: 161.8 grams.

Tissue was ground using medium bit on hand-operated meat grinder; 1.6 liters distilled water added to produce oatmeal-like slurry.

Protease Digestion

pH adjusted to 2.0 with 2N HCl; Sodium azide added to make 2%; 4 grams pepsin added 2x at 8 hour intervals during 37°C incubation on shaker.

Ph adjusted to 7.4 with 8N NaOH; 4 grams trypsin added at 18 hour intervals 3x during next 2.5 days' 37°C incubation on shaker.

Note: the solution really smells terrible.

Ethanol Precipitation I

pH adjusted to 2.0, 2 volumes 85% EtOH added to precipitate GAGs overnight at 4°C.

Resulted in a floating gel that spun down when centrifuged 6000 rpm, 20 minutes on Sorvall RC-5B Refrigerated Superspeed centrifuge.

Dissolved gel in water, dialyzed overnight vs. running tap water to give 1050 ml solution.

*Removed 150 ml solution to use in Modification I (Figure A2).

Concentrated remaining 900 ml to 400 ml by rotoevaporation.

Protein Extraction using Chloroform-1-Pentanol

To 400 ml sample added 400 ml of this solution: 60 ml (64g) acetic acid, 120 g sodium acetate, 220 ml water.

To this added 1320 ml chloroform and 680 ml 1-pentanol (amy alcohol).

Shook mixture 10 minutes, centrifuged 4080xg, 30 minutes.

Saved yellow supernatant phase (600-650 ml) for further extraction; repeated extraction 4 times, resulting in 400 ml solution.

*Took 10 ml of this for use in Modification II (Figure A3).

Ethanol Precipitation II

To 360 ml sample added 4 volumes 95% EtOH at 4°C, left overnight

Centrifuged precipitate down, 4080xg, 25 minutes.

Dissolved precipitate in 450 ml water, dialyzed this vs. running tap water overnight.

Figure A1. (Continued)

Pyridine-Ammonium Sulfate Precipitation

Diluted sample to 800 ml with water; added 800 grams $(NH_4)_2SO_4$ to saturate solution; it wouldn't all dissolve.

Added 80 ml pyridine, stirred vigorously 15 minutes; left at 4^oC for 24 hours; ammonium sulfate never did all dissolve.

Centrifuged gel-like layer several times 5000xg, 1 hour but got no precipitate; let this stand overnight, recentrifuged and filtered supernate through Whatman filter paper.

Washed precipitate with EtOH, dissolved it in 200 ml water, dialyzed vs. water.

Repeated pyridine-ammonium sulfate precipitation on dialyzed solution; washed precipitate with EtOH, dissolved it in water, dialyzed against vs. tap water overnight, then exhaustively against distilled water.

Lyophilized sample, weighed: 1.7 grams.

Final "HA" weighed 1.991 grams, 1.23% of original dry weight. (This included that islated by Modifications I and II).

Figure A2. Isolation of HA by Modification I of Jeanloz's Procedure.

Tissue Preparation, Protease Digestion and Ethanol Precipitation I were performed as part of procedure outlined in Figure A1.

TCA Precipitation

Added 50 ml 20% TCA to 150 ml sample to precipitate.

Centrifuged 4080xg, 30 minutes.

Dialyzed yellow supernate overnight vs. running tap water, then vs. distilled water exhaustively.

CPC Precipitation, Ethanol Precipitation

Made 475 ml sample 0.02M NaCl by addition of salt.

Incubated sample at 37°C, added CPC to make solution 0.2% CPC; incubated 15 minutes more to get precipitate.

Centrifuged 4080xg, 15 minutes; dissolved precipitate in 90 ml 2M NaCl, heated to dissolve.

Added 4 volumes 95% EtOH to precipitate at 4°C overnight.

Centrifuged 4080xg, 20 minutes; dissolved precipitate in 120 ml 0.02M NaCl.

DNase, CPC Precipitation, Ethanol Precipitation, Lyophilization

Added DNase to give 0.5 μg/ml solution; incubated 1 hour at 37°C.

Added CPC to make it 0.2%, incubated at 37°C 15 minutes; centrifuged 4080xg, 20 minutes.

Dissolved precipitate in 2M NaCl, added 4 volumes EtOH at 4°C, 6 hours.

Centrifuged 4080xg, 20 minutes; dissolved precipitate in 0.02M NaCl, added 4 volumes EtOH to precipitate at 4°C, 6 hours.

Centrifuged 4080xg, 20 minutes; dissolved precipitate in distilled water, dialyzed exhaustively.

Lyophilized sample, weighed: 0.2440 grams.

Note: Based on this yield, Jeanloz's procedure (as in Figure A1) should have yielded $.2440 \frac{(1050)}{150} = 1.708$ grams.

Figure A3. Isolation of HA by Modification II of Jeanloz's Procedure

Tissue Preparation, Protease Digestion, Ethanol Precipitation I, and Protein Extraction with Chloroform-1-Pentanol were performed as part of the procedure outlined in Figure A1.

CPC Precipitation

Diluted 10 of sample to 40 ml, added salt to make 0.02M NaCl.

Added CPC to make 0.2% solution, incubated 15 minutes, 37°C.

DNase, Ethanol Precipitation

Added DNase to give 0.5 μg/ml solution; this was a mistake.

Centrifuged 809xg, 15 minutes in Sorvall GLC-2 centrifuge.

Dissolved precipitate in 10 ml 2M NaCl by heating; added 4 volumes 95% EtOH at 4°C overnight.

Centrifuged 809xg, 15 minutes; dissolved precipitate in 10 ml 0.02M NaCl.

CPC and Ethanol Precipitations

Repeated CPC precipitation as before; centrifuged 10 minutes 300xg; dissolved precipitate in 15 ml 2M NaCl.

Added 35 ml 95% EtOH to precipitate at 4°C.

Centrifuged 15 minutes, 809xg; dissolved precipitate in 12 ml distilled water; dialyzed this exhaustively vs. distilled water.

Lyophilized sample, weighed: 0.0470 grams.

Note: Based on this yield, Jeanloz's procedure (as in Figure A1) should have yielded 2.1963 grams.

Figure A4. Electrophoresis of Glycosaminoglycan Samples on Cellulose Acetate Strips (CASE). The strips were stained in Alcian blue dye after electrophoresis. (1) shows the GAG mixture obtained from Jeanloz's procedure (unmodified); (2) is that obtained from Modification I and (3) is that from Modification II of Jeanloz's procedure. (4) is a sample of Peak I from DEAE Sephacel chromatography; (5) is a sample of Peak II. "St" bands are those of the standard mix of GAGs used as a reference in all electrophoretic runs.

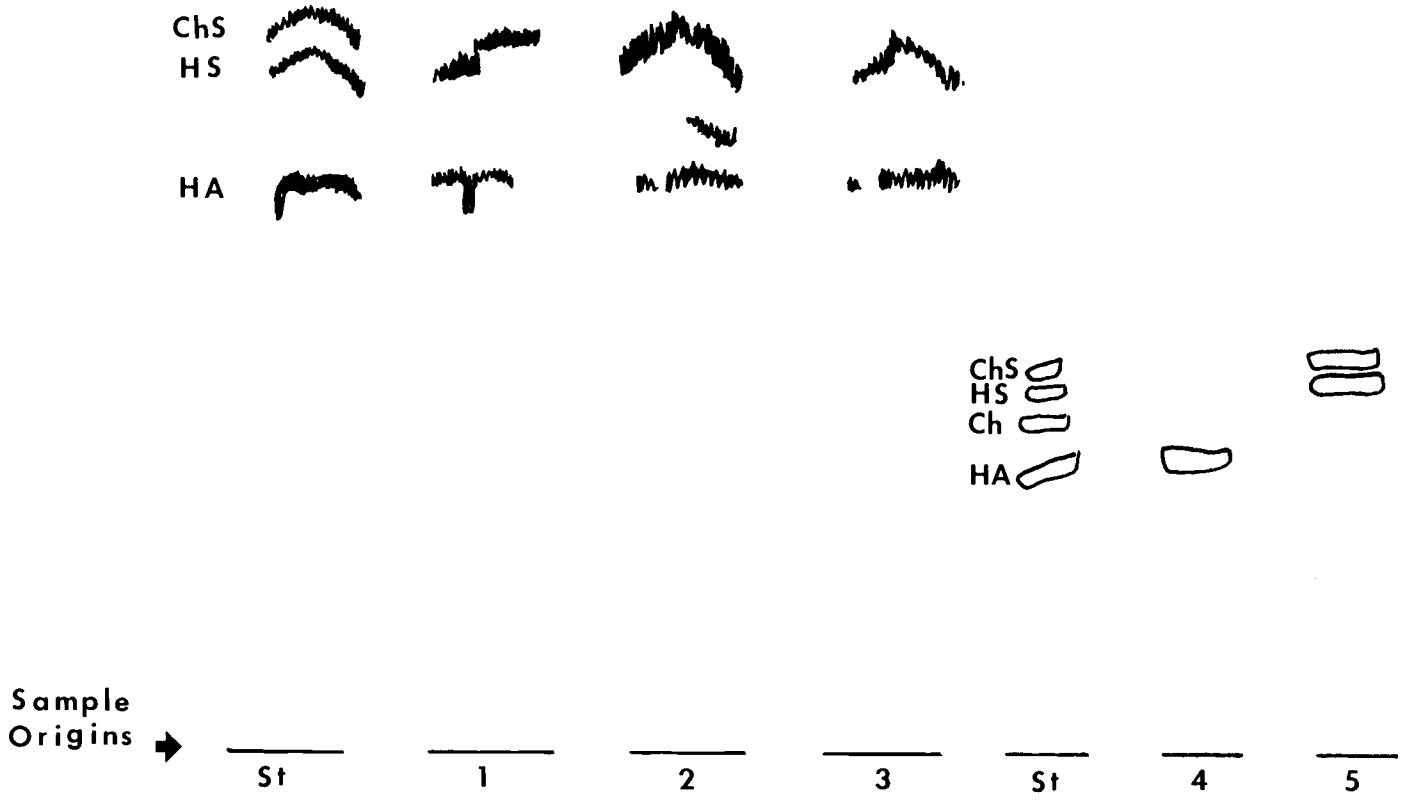

Figure A4

Figure A5. Separation of HA and ChS using DEAE Sephacel. 10 ml fractions were collected; fraction numbers are shown on the abscissa. GAG concentration is plotted on the ordinate as μg/ml as determined by the Bitter Muir assay for uronic acid. Peak I is hyaluronic acid; Peak II is chondroitin sulfates, as determined by CASE (see Figure A4).

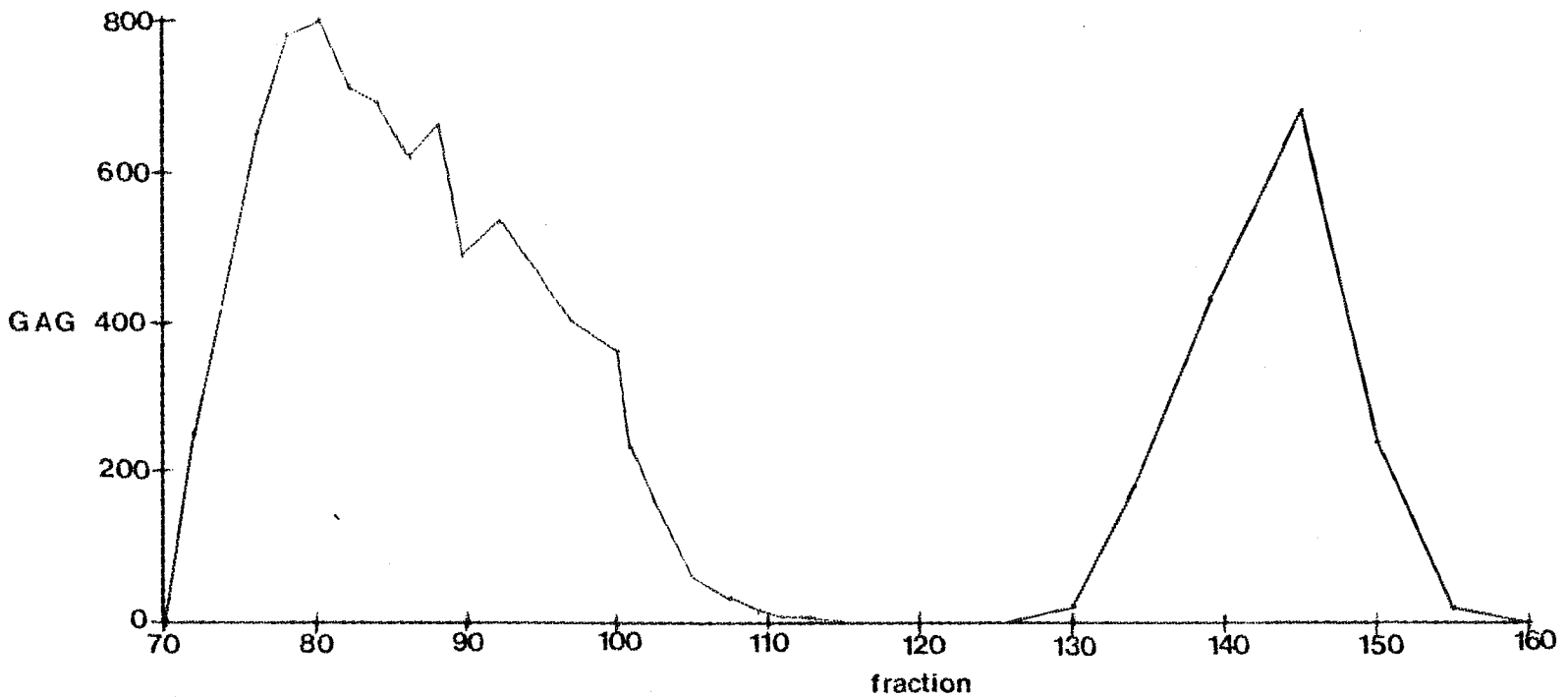
800
600
GAG 400
200
0
70
80
90
100
110
120
130
140
150
160
fraction

Figure A5.

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APPENDIX B. Cells, Gels, and Frustration

Glycosaminoglycans, when added to cultures of neural retina cells, exert a marked effect on cell behavior with respect to cell adhesion and aggregation. Morris has reported (1976) that exogenous ChS transiently promotes nonspecific adhesion of retina cells to the substrate while HA promotes cell-cell aggregation. These differences in effect take on greater potential significance when the differential GAG synthesis pattern of retina cells during development is also considered (see Introduction). Since the concentrations of the different GAGs in the extracellular matrix are most likely also changing during development, one line of research pursued in this thesis involved efforts to determine if gradients of glycosaminoglycans affected the behavior of cultured neural retina cells.

To begin this research, several practice cultures were performed wherein retina cells of varying ages were trypsin-dissociated using standard techniques (Morris, 1971) and cultured in plastic petri dishes. Fetal bovine serum was included as 10% of the culture medium, and in some cases the culture medium was supplemented with GAG. A series of photographs was taken of these cultures to document typical differentiation of cells under such conditions; Figure B1 shows differentiation of retina cells from 8-day embryos cultured in GAG-free medium; Figure B2 shows 8-day retina cells cultured in medium containing glycosaminoglycan.

Retina cells like plastic--that is, they readily adhere to it and spread, sending out cellular processes (neurites) and forming aggregates

whose morphology is characteristic of the cells' age at dissociation (Sheffield & Moscona,1970). However, to better approximate *in vivo* conditions *in vitro* it was decided to try using collagen (covering the plastic dishes as a gel) as a substratum for cells in culture. Collagen also presented an advantage in that gradients of GAG could (theoretically) be stabilized in its matrix, thus furthering the simulation of more natural cell environments.

Isolation of Collagen

Type I collagen was isolated from rat tail tendons following Elsdale and Bard (1972) or Schor (1978,1980). Elsdale and Bard's technique involved extraction of collagen from tendons in 0.5M acetic acid at 4°C over several days, followed by dialysis vs. one-tenth strength Eagle's medium at pH 4. Schor modified this only in that the extracted collagen was dialyzed against distilled water (1978). One batch of collagen was prepared following another of Schor's techniques (1980)--this included further purification of the collagen by NaCl precipitation to remove basement collagens. As no differences were observed between cells cultured on these different collagen preparations, the salt precipitation step was omitted in subsequent isolations. The absorbances of several (diluted) samples of the collagen solutions were measured on a Varian spectrophotometer at 230 nm. Comparison of these values with those obtained using known concentrations of collagen in solution showed that the preparations routinely contained 1-2 mg/ml of collagen.

Preparation of Collagen Gels

By raising the ionic strength and/or pH of these collagen solutions a gel was easily obtained. Initial gels were made from collagen

prepared in water; 35 mm Petri dishes (Falcon) containing 2 ml of solution were placed in chambers containing concentrated ammonium--dissolution of the ammonium vapor in the collagen solution caused gelation (Bornstein, 1958) However, although such gels were extensively washed in culture medium before experimental use, the retina cells did not usually survive more than 24 hours on or in the gels. Several other types of chick embryo cells were cultured on these gels to determine whether culture success depended on the cell type or substratum; as even fibroblasts and heart cells did not appear healthy after several days' culture on collagen the ammonium technique was discontinued.

Subsequent gels using collagen prepared either in water or in one-tenth strength medium were made by Elsdale and Bard's method of simultaneously adding the appropriate amounts of ten-strength medium (to adjust the ionic strength to physiological conditions) and 0.5M sodium bicarbonate (to adjust the pH from 4.0 to 7.2). No toxicity was encountered with this technique. However, when glycosaminoglycans were added (dissolved in the ten-strength medium) the collagen sometimes failed to gel and instead remained as a viscous slurry containing precipitated clumps of denatured collagen. The variability of success of gelation was confusing as it didn't seem to follow any consistent pattern. It could have been, however, that unnoted differences in the speed or order of addition of 10x medium plus GAG and sodium bicarbonate in different gel preparations accounted for the variability in their success. Snowden and Swann (1980) found that chondroitin-6-sulfate, hyaluronic acid, and dermatan sulfate all affect the kinetics of collagen precipitation. They also showed that the presence of Ch6S at physiological conditions does not affect the formation of the

characteristic banding patterns seen in collagen fibrils with the electron microscope, but that the banding is not seen in the precipitate formed with Ch6S at acidic conditions. Thus if the 10x medium with GAG were added to the collagen long enough before the pH was adjusted to allow some precipitate to form, abnormal collagen gels would result.

Cells and Gels

Numerous experiments were attempted using the above method for making collagen-GAG gels. GAGs--either Ch6S, Ch4S, or HA--were added as part of the 10x medium to give final concentrations in culture varying from 5 to 30 mg GAG/ml of gel. Either 1 or 2 ml of collagen gel was present in each 35 mm dish. Trypsin-dissociated neural retina cells from chick embryos of various ages were cultured on top of successful gels or in collagen slurries. In other trials, cells were added to collagen solutions immediately after the pH and ionic strength were adjusted and before gelation--in this way they were incorporated into the gel. Theoretically this incorporation is an interesting approach, as the cells are thus surrounded by matrix as in vivo. In practice, however, it proved to be of little value as the cells' refractive index was very nearly that of the collagen, and even using phase optics it was thus extremely difficult to locate the cells. Using thinner gels (1 ml of gel per 35 mm dish) helped somewhat, but clear resolution of morphological detail was still lacking.

Figure B3 shows such cultures after one day incubation. Although it was evident that cells cultured in collagen with GAG formed aggregates that were both larger in size and more numerous than those formed in plain collagen, the differences in cell morphology between these

cells and cells of the same original preparation cultured on plastic were also striking. While cells cultured in/on collagen were rounded up and appeared generally less healthy, those cultured on plastic for the same time period had formed numerous processes linking aggregates and were otherwise more differentiated. This contrast obviously presented serious implications regarding the affinity of retina cells for collagenous substrata. Nevertheless, some method for stabilizing GAG gradients was needed, so several experiments were attempted using collagen for this purpose and taking into consideration the possibility that collagen retarded differentiation of retina cells in culture.

Glycosaminoglycan Gradients?

A truly satisfactory way to establish GAG gradients in culture was never found. Several attempts were made to construct micrograd ient makers out of plexiglas and/or plastic syringes with the intention of mixing a gradient of GAG (in 10x medium) with a stream of collagen as they both were being poured or layered into a culture dish. One major difficulty encountered was that the volume of the connecting tube between the two chambers of the gradient maker (dead space) could not be reduced enough so that it wouldn't account for a significant part of the total gradient volume. Problems were also encountered with the collagen itself--on many occasions it wouldn't gel fast enough as the gradient was poured to ensure that the GAGs did not diffuse and thus eliminate the gradient. On other occasions, by the time that the end of the gradient was being poured the collagen had gelled and thus mixing with the 10x medium solution was unsatisfactory. In any case, even if the gradient gel was successfully formed, there was no way to determine

if a gradient really existed. The concentrations of GAGs were so low that they were undetectable by the Bitter Muir assay (1962), and spectrophotometric analysis of GAG concentrations in Alcian blue-stained gels (Gold,1979) was unreliable.

Another method of establishing gradients was also tested, although in this case also there was no satisfactory way to assure the existence of a true gradient. This second method involved drying solutions of varying concentrations of GAG as drops along a line of increasing concentration or as lines in a square wherein GAG concentration increased from one side to the opposite side (Figure B4). Drops and lines were repeatedly applied after the previous ones had dried until the volume of the concentration of GAG in each particular area was equal to the volume of collagen that was subsequently layered over that area of the dried gradient. It was assumed that the dried GAG could dissolve into the collagen solution during gelation and would thus be fixed in the collagenous matrix. Retina cells were mixed in with the collagen at the time it was layered on the dish.

The same difficulties with visualization of cells were encountered with these cultures as with the others. Photographs were taken all along the gradients; several series are shown in Figures B4 and B5. That the GAGs are influencing the cells is clear from the readily observable differences between line cultures containing GAG wherein aggregates are formed and control lines in which single cells are primarily seen. In all of four replicates an optimum GAG concentration for cell aggregation was apparent--aggregates were larger and more frequently encountered at around 20 mg/ml Ch4S, at 10 mg/ml Ch6S, and at 2.5 mg/ml HA. The highest concentrations tested of all of these

three GAGs seemed to adversely affect cell health; in cultures including HA cells in general again appeared much less healthy than those cultured with either form of chondroitin sulfate.

This sort of experiment does not allow one to draw any conclusions about the nature of the GAG-cell interactions that may be producing such visible differences in cultures. However, the possible existence of differing optimal GAG concentrations for cell aggregate formation, coupled with the fact that the molecular weight of the Ch6S used was twice that of the Ch4S, suggests that steric exclusion or viscosity effects of the GAGs in solution may be in part responsible for the observed effects. Further work on the viscosity of GAGs in solution is presented in Appendix C.

No further work on the culture of retina cells on collagen-stabilized GAG gradients was performed. Three major difficulties were encountered in this research: 1) it was very difficult to successfully repeat each experiment due to problems with the gels; 2) it was extremely frustrating to try to quantitatively analyze the cultures due to poor visualization of the cells in the gels, and 3) it was impossible in any case to determine if GAG gradients really existed in the cultures. Added to the above was the fact that collagen did not prove to be a very good substrate on which to culture neural retina cells. It is strongly recommended that future studies of retina cell behavior over glycosaminoglycan gradients do not attempt to stabilize the GAGs in collagen. Perhaps some other method such as cross-linking the GAGs themselves to form gels of differing density would be a more suitable technique.

Figure B1. Trypsin-dissociated neural retina cells from 8-day chick embryos cultured up to 25 days on plastic. (a) 5 hours culture, (b) 1 day, (c) 2 days, (d) 5 days, (e) 6 days, (f) 13 days, (g) 14 days, (h) 16 days, (i) 25 days.

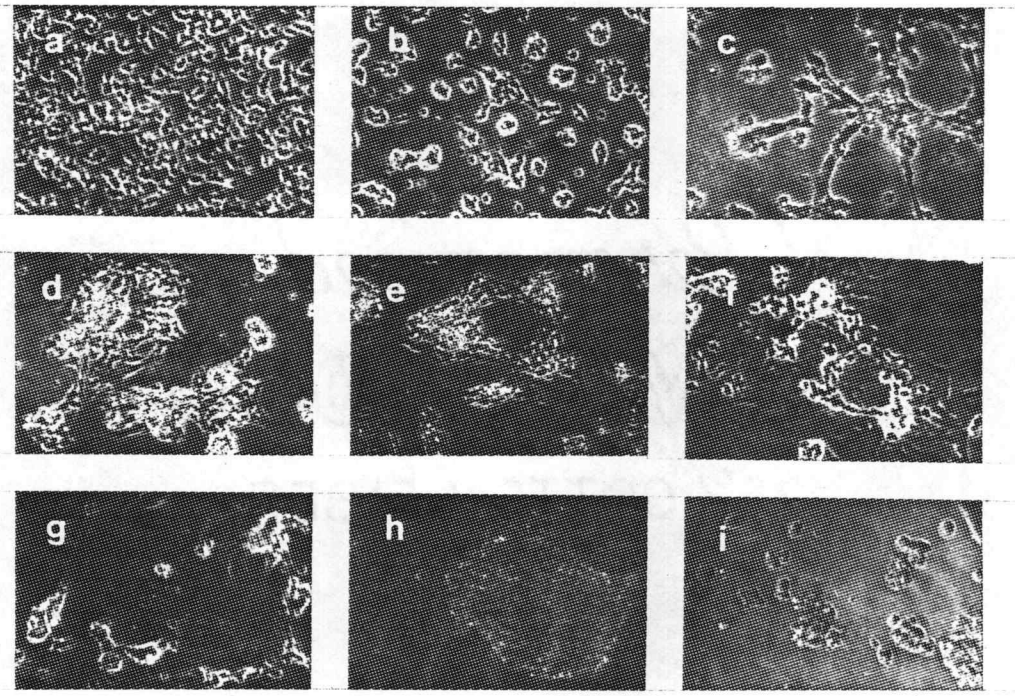

Figure B 1

Figure B2. Neural retina cells from 8-day chick embryos cultured on plastic with and without glycosaminoglycan. a, b, c show cells cultured for 1, 3 and 4 days respectively in plain medium; d, e, f show cells cultured 1, 3 and 4 days in medium containing 5 mg/ml hyaluronic acid; g, h, i show cells cultured in 5 mg/ml chondroitin-4-sulfate; and j, k, l show cells cultured 1, 3 and 4 days in 5 mg/ml chondroitin-6-sulfate.

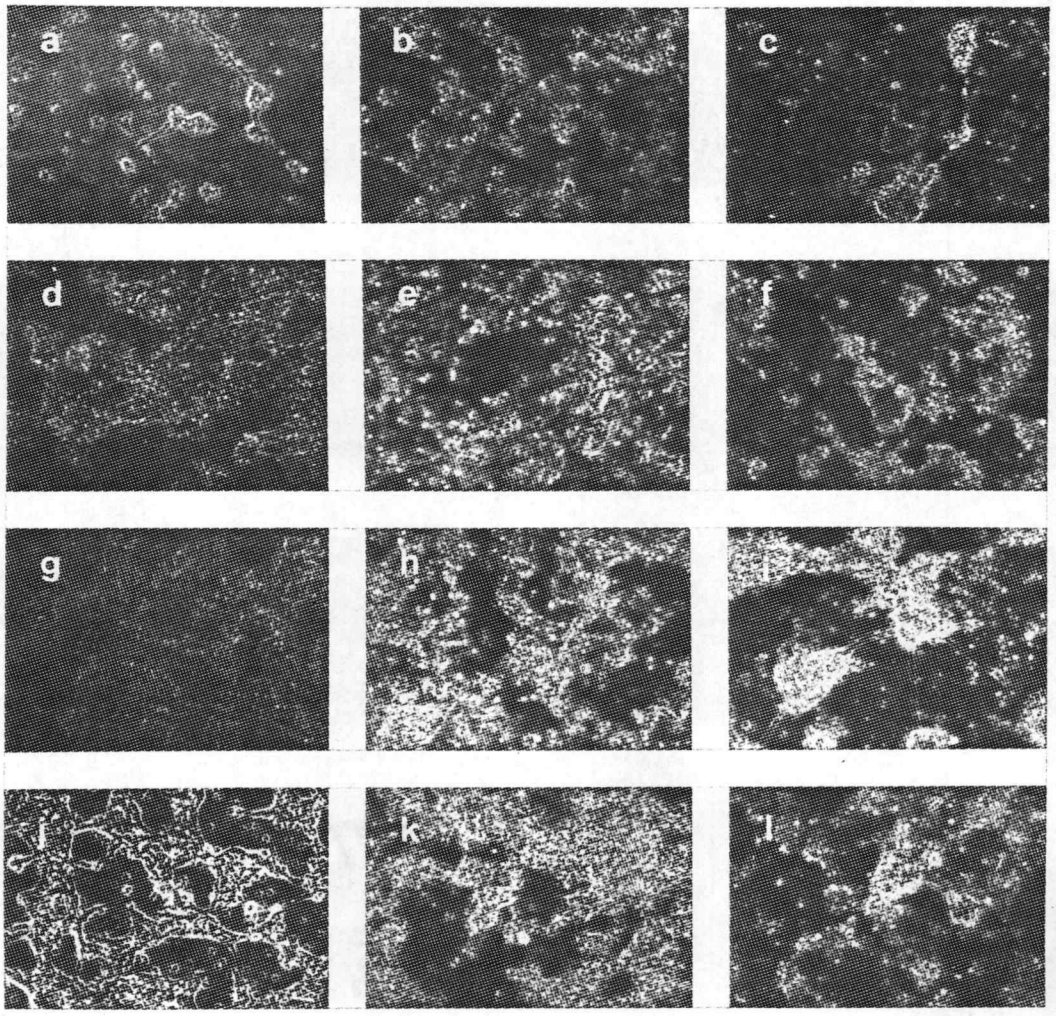

Figure B2

Figure B3. Neural retina cells from 10-day chick embryos after culture for 1 day on collagen or collagen with 5 mg/ml GAG. Freshly dissociated cells (a) are healthy; some have retained their processes during trypsinization. Some of these cells were cultured on plastic (b) to provide a reference culture. Their morphology is strikingly different from that of other cells cultured on collagen (c) or collagen with HA (d), with Ch4S (e) or with Ch6S (f).

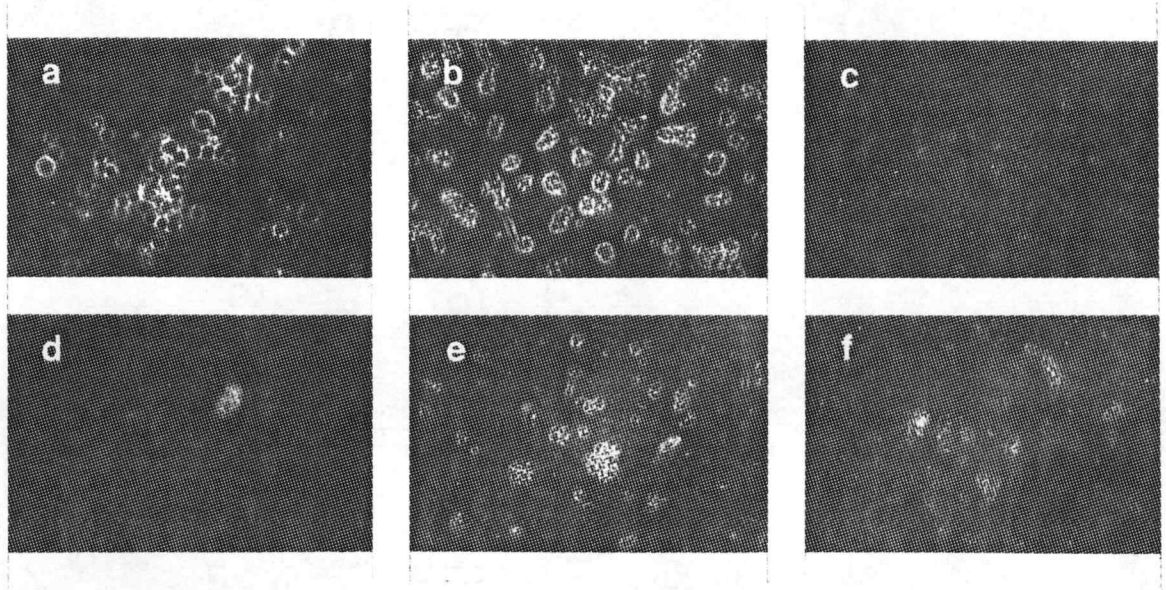

Figure B3

Figure B4. Culture of retina cells on collagen containing glycosaminoglycan gradients. A GAG gradient was dried on 35 mm plastic dishes as (a) drops on a line of increasing concentration or (b) lines on a square. Lines of cells in collagen without an underlying GAG gradient were used as controls (c-f). Cells appear uniformly distributed along the line with few or no aggregates formed after 1 day culture. In contrast, cells cultured 1 day on HA line gradients show aggregation at around 2.5 mg/ml HA. One such HA line gradient is shown (g-m). For (a), (b), and (g-m) numbers indicate GAG concentrations in mg GAG/ml collagen gel; arrows indicate the direction of increasing GAG concentration in the gradient.

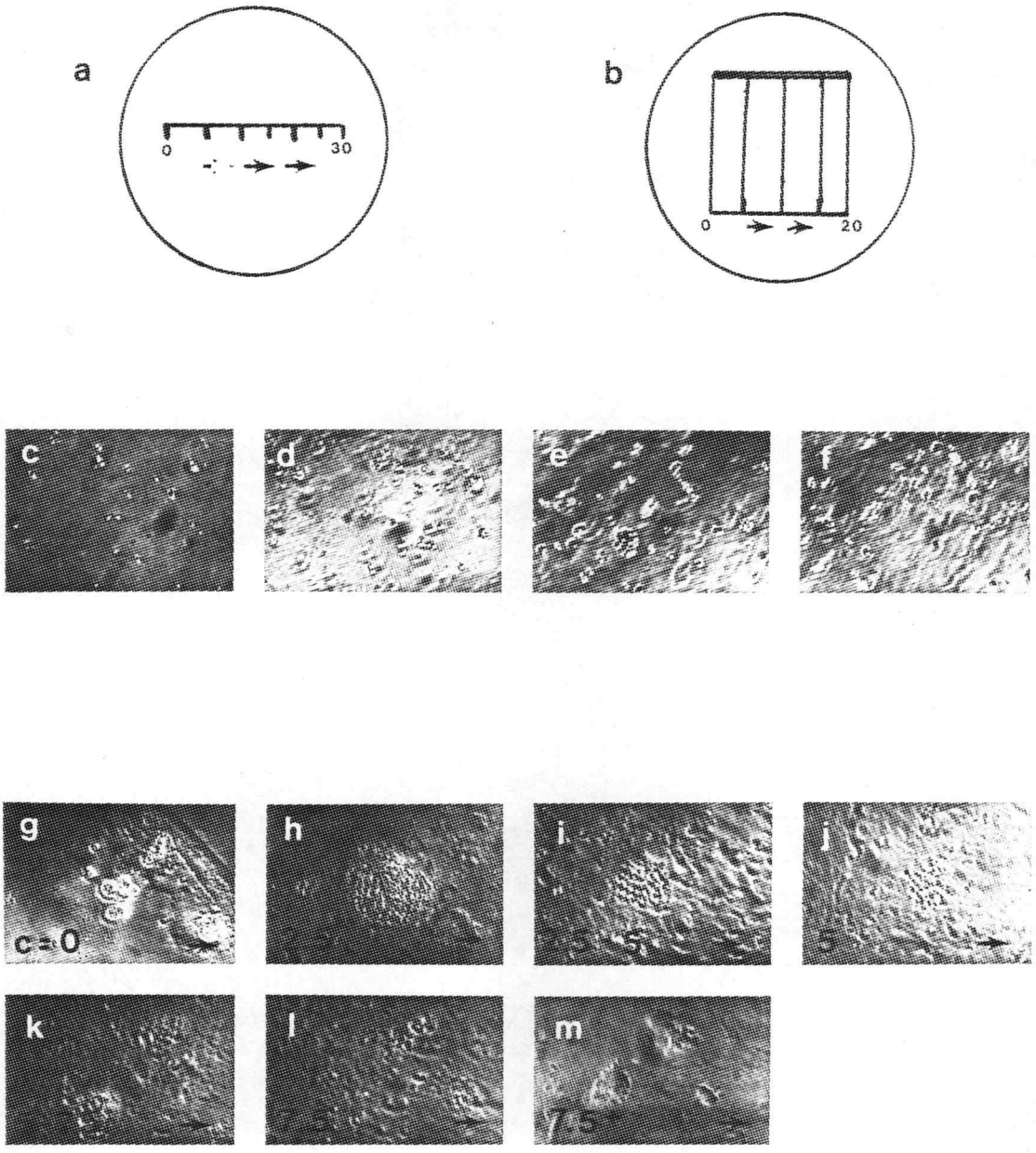

Figure B4

Figure B5. Retina cells cultured in collagen lines containing GAG gradients. (a-h) show cells cultured on a gradient of Ch6S; (i-p) show cells on Ch4S. Numbers indicate GAG concentration in mg/ml collagen gel; arrows indicate the direction of increasing GAG concentration in the gradient. Note the aggregate formation at around 10 mg/ml Ch6S and at around 20 mg/ml Ch4S in these two gradients.

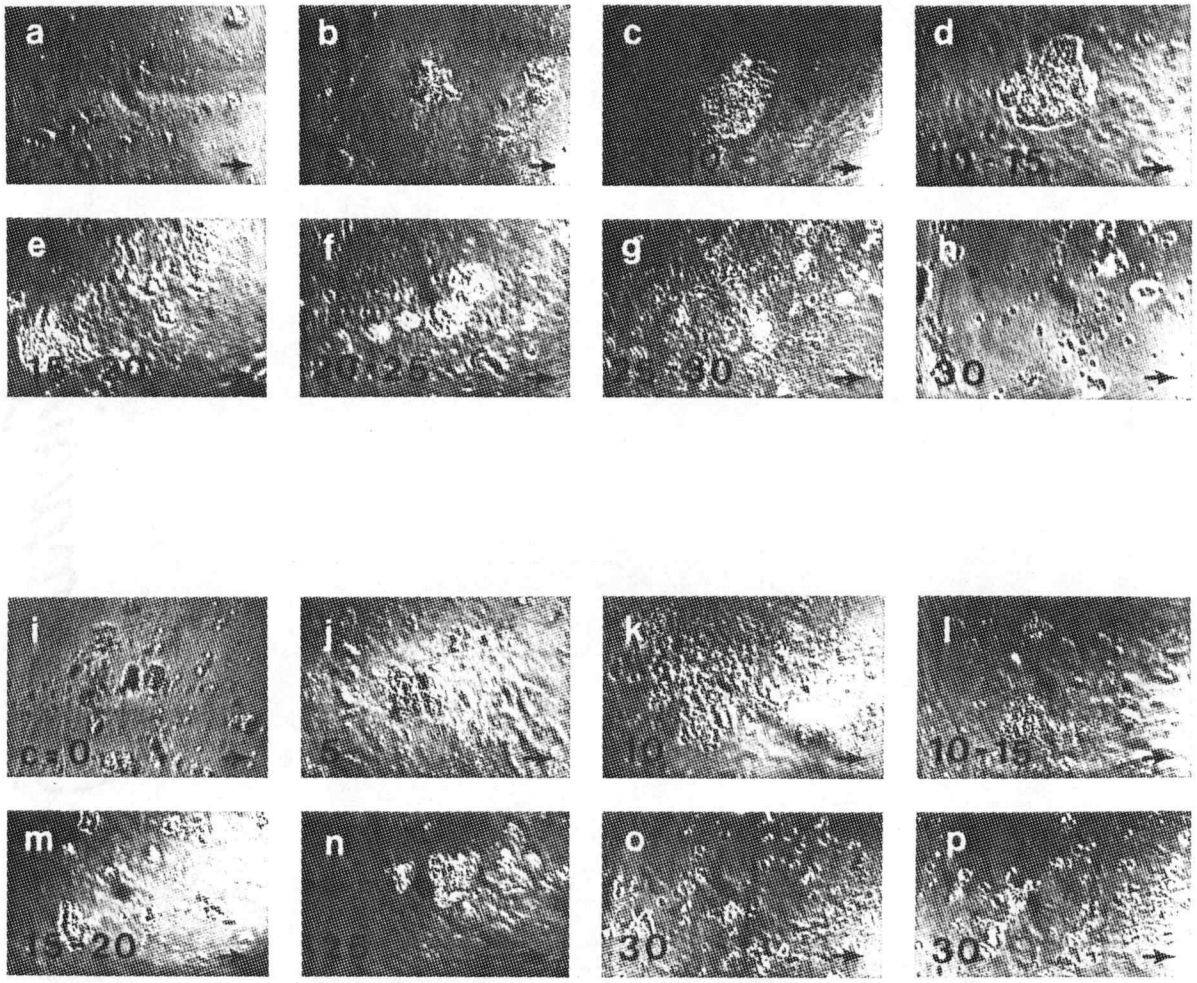

Figure B 5

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APPENDIX C. Determination of the Relative Viscosity of Glycosaminoglycans in Solution

Numerous studies have suggested that glycosaminoglycans, particularly hyaluronic acid, are involved in cell migratory behavior (see Introduction). These large, polyanionic molecules are highly hydrated in solution and may therefore create a favorable environment through which cells can move. On the other hand, adding GAGs to a solution greatly increases its viscosity. Although a more viscous environment might aid cell motility by providing extra traction, it would seem likely that after a certain point viscosity increases would retard cell movement, the glycosaminoglycans acting then as an intercellular glue. With these role changes in mind, it seemed of interest to measure the viscosity of different GAGs in solution to see 1) what sort of relative changes in viscosity are obtained by increasing GAG concentration and also 2) how a solution's viscosity is affected by addition of mixtures of different GAGs since mixtures rather than individual GAGs are typically found in extracellular spaces *in vivo*.

Glycosaminoglycan solutions were made by dissolving the appropriate amount of a particular GAG in 4 ml of Balanced salt solution (BSS), pH 7.2 (Gibco). Chondroitin-4-sulfate from shark cartilage (Sigma), chondroitin-6-sulfate from whale cartilage (Sigma) and hyaluronic acid from human umbilical cord (Sigma) were tested. GAG was weighed on a Mettler H10 balance (Scientific Products); weights were correct as measured to 10^{-4} grams. When mixtures of GAGs were tested, a stock solution of HA was prepared in BSS and the ChS was added to 4 ml of

this for each sample. For samples containing 10% fetal bovine serum (FBS) (Sterile Systems), the amount of GAG to give the proper concentration in 4 ml was dissolved in 3.6 ml of BSS; 0.4 ml of FBS was then added to this as incubation was begun.

Samples thus prepared were incubated in a 37°C water bath for at least 30 minutes before testing; the Ostwald glass capillary viscometer itself was immersed in the same water bath. Measurements were taken with a digital electronic stopwatch (VWR) to give the number of seconds it took the solution meniscus to pass between the two lines etched on the glass tube (efflux time). This was thus a measurement of the solution's kinematic viscosity, ν, which gives the viscosity η when multiplied by the density ρ:

$$\eta = \nu\rho \qquad \text{(Van Wazer, 1963).}$$

Each sample was tested three times (consecutively) while in the viscometer; these numbers were then averaged to give the data presented in Figure C1.

Several assumptions were made for the purpose of this study. First, it was assumed that the GAG solutions behaved in a Newtonian manner. In fact, since these are such large molecules and cannot be considered as compact, noninteracting spheres, their behavior was probably not Newtonian but rather simulated that of a pseudoplastic fluid. However, since the mathematics involved in determining the viscosity of pseudoplastic fluids is quite complex and because the interest here lay primarily in relative rather than exact viscosities, the equations for Newtonian fluids were used.

A second assumption was made regarding the density of the GAG solutions. Since the solutions tested were of relatively low

concentration (the highest was 20 mg/ml), it was assumed that their density was equal to that of water, or ρ = 1 g/ml. The error thus possibly introduced was no more than 2%. Thus the seconds measured could be used to calculate the viscosity η using the equation:

$$\eta = \theta(k\rho + \mathcal{L}H) - K/\theta^m \qquad \text{(Van Wazer, 1963)}$$

where H is the applied pressure head, k, $\mathcal{L}$, and K are constants, m=2 for the viscometer used, and θ =efflux time in seconds. Since no pressure was applied in these runs, H=0 and thus:

$$\eta = \theta k - K/\theta^2 \quad .$$

For efflux times greater than 200 seconds, K/θ^2, the kinetic energy correction term, is negligible (Van Wazer, 1963). Because most times measured were on the order of 100 seconds, this second term was omitted in all cases, leaving

$$\eta = \theta k \quad .$$

k is a constant whose value depends on the dimensions of the viscometer; since the same viscometer was used for all samples, this was disregarded also and the equation simplified finally to $\eta \propto \theta$. Thus the data ($\eta\theta$) are presented in Figure C1 as seconds efflux time; these numbers can be considered to be directly proportional to the viscosity.

There are many sources of error inherent in measuring viscosity using a glass capillary viscometer, most of which arise from solution-viscometer interactions (i.e. wall, shear, and surface tension effects). This fact, plus the probable non-Newtonian behavior of GAG solutions, mean that the data presented cannot be taken as the actual viscosity η . However, it <u>can</u> be used to calculate the relative viscosities of the different GAGs in solution, which were the interest of this study

anyway. The relative viscosity η_r is simply the viscosity of the solution η_s divided by the viscosity of the solvent alone η_o :

$$\eta_r = \eta_s / \eta_o$$ (Van Holde, 1971).

The solvent in all cases in this study was BSS or BSS + 10% FBS. The relative viscosities of the GAG solutions are presented in Figure C2 as calculated from the data in Figure C1.

As can be seen in Figure C1, solution viscosities did increase with increasing concentration of glycosaminoglycan. The rate of increase of viscosity was characteristic of the type of GAG measured--linear regression analysis of the lines obtained by plotting viscosity (η_θ) as a function of GAG concentration (Figures C3 and C4) showed that the data fit straight lines whose slopes were distinctly different, being about 4 for Ch4S and 10.6 for Ch6S. This was true regardless of the presence or absence of 10% FBS in the samples. The roughly 2.5-fold difference in slope between the chondroitin sulfates may have been due to differences in the molecular weights of the two preparations tested. The molecular weight of the Ch4S used was approximately 25-50 x 10^3 while that of the Ch6S was 40-80 x 10^3 (Sigma Technical Services, personal communication).

The data from samples containing 10% serum are somewhat confusing. It might be expected that adding another component (FBS) to the solvent (BSS) with a GAG would increase the latter's viscosity; this was found to be the case when serum was added to ChS samples (except for the 10 mg/ml samples when the η_θ obtained was lower than that without serum). That the relative viscosities for ChS+serum+BSS samples were very close (+1.3-6% differences) to those measured for the ChS+BSS samples suggests that the serum did not affect the GAG under

consideration. By contrast, for the ChS-HA mixed solutions containing serum the measured η_s were 1.6-3.7% less than those of corresponding GAG concentrations in BSS. One explanation for this is that, if the serum contained hyaluronidase (whose activity was evidently different in different samples), degradation of the GAGs may have been occurring during the incubation period which would, of course, have reduced the solution's viscosity. Indeed, as the HA (single GAG) determinations were being made the viscosity declined with each consecutive trial, and measurement of the same samples on consecutive days gave slightly decreased viscosities the second day (Figure C3). However, this interpretation must be accepted cautiously. Not only were the decreases observed of questionable significance since the original error due to sample weight could have approached 2%, but a slight dilution of the sample may have occurred simply through its accumulative absorption of water from the sides of the capillary tube during each testing, which would also explain the viscosity decreases. A true test of serum's ability to degrade the GAGs would be to measure the viscosity changes using 10% FBS which had been heat-inactivated.

Although an increase in viscosity would be expected with increasing concentration and was, in fact, roughly additive for each individual GAG, mixing of two different GAGs produced an increase in viscosity that was greater than a simple additive effect. For a comparison of the theoretical viscosity, determined by adding the viscosities of appropriate single GAG solutions, to the observed viscosity of the mixed GAG solution, the specific viscosity η_{sp} of each GAG solution was calculated as

$$\eta_{sp} = (\eta_s - \eta_o)/\eta_o$$ (Van Holde, 1971).

By using the specific viscosity, the contribution of the solvent to the solution's viscosity was thus eliminated. These values are shown in Figure C5. Figure C6 shows that for samples containing two GAGs in BSS or in BSS + FBS, the observed specific viscosity exceeds the theoretical for all samples except those of low Ch4S concentrations. In fact, the observed specific viscosities exceeded the theoreticals by as much as 44% (though for most samples it was less than that). The difference between the theoretical and observed viscosities increased with increasing ChS concentration.

It is impossible, using the data presented here, to explain this difference in a specific mechanistic way and although it is tempting to do so, it is not safe to conclude that the difference reflects some sort of interaction between the two GAGs in solution. Specific viscosities are additive (as assumed when calculating the theoretical values) only at low solute concentrations. Another way to express the specific viscosity is in terms of the concentration c (Van Holde, personal communication):

$$\eta_{sp} = [\eta]c + Kc^2 \quad .$$

At high concentrations the kc^2 term becomes large enough so that the plot of η_{sp} vs. concentration becomes non-linear. This is also easily seen in Figure C3 where the data for all single GAG solutions plotted give lines that deviate from linearity at high concentrations.

By dividing the specific viscosity by the concentration (η_{sp}/c) and plotting this value vs. the concentration (Figures C7 and C8), the upward swing in the η_{sp} curve is somewhat diminished. The y-intercept of this line represents the intrinsic viscosity.

The importance of these data to culture systems lies primarily in the differences in viscosity observed between solutions of commonly used commercial preparations of the chondroitin sulfates and hyaluronic acid. Although the latter GAG is quite obviously more viscous in solution than ChS, it is important to note also that its viscosity is a more sensitive function of concentration than the others' as shown by the dramatic slope of that line. The difference between the 4- and 6-sulfated forms of chondroitin is remarkable too, despite the fact that it is probably due to differences in the molecular weight of the samples tested. It is now obvious that a comparison of two cell cultures including identical concentrations of Ch4S or Ch6S must take into account the possibility of differential viscosity effects on the cells if the molecular weights of the two preparations are not the same.

Figure C1. η_θ, seconds efflux time. GAG concentrations are given as mg/ml.

	A GAG in BSS	B GAG in BSS + 10% FBS	C Samples from B 1 day later	D GAG + 1 mg/ml HA in BSS	E GAG + 1 mg/ml HA in BSS + 10% FBS
BSS	60.73	61.7-FBS 62.1+FBS	62.1+FBS	61.7	61.1-FBS 62.4+FBS
Ch4S					
1.25	64.8			180.2	178.7
2.5	68.6	71.4	71.2	183.9	183.1
5.0	77.1	78.7	77.4	197.6	195.5
7.5	85.1			213.7	210.8
10.0	95.0	92.6	90.6	231.0	226.3
15.0	115.0	117.3	113.7	267.2	260.7
20.0	139.6	140.8	137.9	308.3	301.7
Ch6S					
1.25	68.6			189.3	192.4
2.5	77.8	79.1		206.5	206.6
5.0	95.0	99.7		237.3	238.7
7.5	116.2			276.4	279.8
10.0	141.2	136.1		317.2	314.3
15.0	190.0	192.4		410.6	414.5
20.0	267.7	278.4		532.6	518.8
HA					
(BSS)	60.1				
1.0	150.6			177.5	184.2
1.25	233.9				
2.5	546.5				
4.0	1577.6				
5.0	3278.2				

Figure C1.

Figure C2. Relative viscosities, η_r . GAG concentrations are given as mg/ml; η_o is BSS ± FBS.

	A GAG in BSS	B GAG in BSS + 10% FBS	C Samples from B 1 day later	D GAG + 1 mg/ml HA in BSS	E GAG + 1 mg/ml HA in BSS + 10% FBS
Ch4S					
1.25	1.07			2.92	2.86
2.5	1.13	1.15	1.15	2.98	2.93
5.0	1.27	1.27	1.25	3.20	3.13
7.5	1.40			3.46	3.38
10.0	1.57	1.49	1.46	3.74	3.63
15.0	1.89	1.89	1.83	4.33	4.18
20.0	2.30	2.27	2.22	5.00	4.83
Ch6S					
1.25	1.13			3.07	3.08
2.5	1.28	1.27		3.35	3.31
5.0	1.57	1.61		3.85	3.31
7.5	1.91			4.48	4.48
10.0	2.33	2.19		5.14	5.04
15.0	3.14	3.10		6.65	6.64
20.0	4.41	4.48		8.63	8.31
HA					
1.0	2.51			2.88	2.95
1.25	3.89				
2.5	9.09				
4.0	26.05				
5.0	54.50				

Figure C2.

Figure C3. η_θ as a function of GAG concentration. η_θ is seconds efflux time; GAG concentrations are given as mg/ml. All solutions plotted here are in BSS; BSS + FBS data give nearly identical plots.

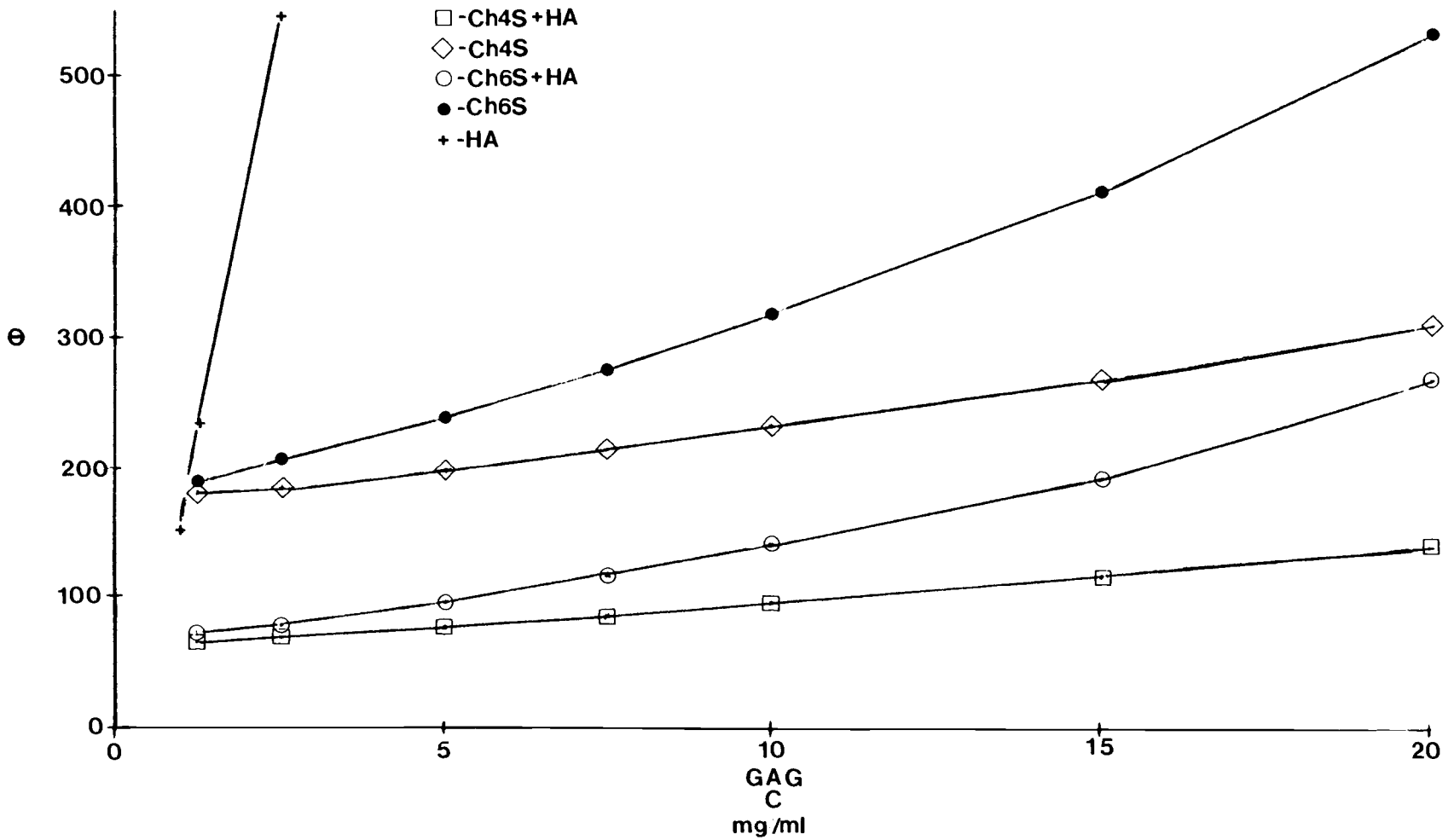
□ -Ch4S+HA
◇ -Ch4S
○ -Ch6S+HA
● -Ch6S
+ -HA
500
400
300
200
100
0
Θ
0
5
10
15
20
GAG
C
mg/ml

Figure C 3

Figure C4. Linear regression analysis of lines obtained by plotting η_{sp} (Figure C1) as a function of GAG concentration as shown in Figure C3.

	η_θ Ch4S in BSS	η_θ Ch6S in BSS	η_θ HA in BSS	η_θ Ch4S in BSS + 10% FBS	η_θ Ch6S in BSS + 10% FBS	η_θ Ch4S + HA in BSS	η_θ Ch6S + HA in BSS	η_θ Ch4S + HA in BSS + 10% FBS	η_θ Ch6S + HA in BSS + 10% FBS
SLOPE	3.95	10.35	716.4	3.98	11.03	6.90	18.01	6.56	17.41
Y-INTERCEPT	57.64	46.20	-812.73	58.40	41.31	165.6	152.4	165.0	157.0
CORRELATION COEFFICIENT	.9968	.9904	.9438	.9925	.9857	.9964	.9933	.9956	.9953
NO. OF DATA POINTS	7	7	5	5	5	7	7	7	7
ρ	.001	.001	.05	.001	.01	.001	.001	.001	.001

Figure C4.

Figure C5. Specific Viscosity, η_{sp}, calculated as $\eta_{sp}=(\eta_s-\eta_0)/\eta_0$ using values from Figure C1.

	A GAG in BSS	B GAG in BSS + 10% FBS	D GAG + 1 mg/ml HA in BSS	E GAG + 1 mg/ml HA in BSS + 10% FBS
Ch4S				
1.25	.07		1.92	1.86
2.5	.13	.15	1.98	1.93
5.0	.27	.27	2.20	2.13
7.5	.40		2.46	2.38
10.0	.57	.49	2.74	2.63
15.0	.89	.89	3.33	3.18
20.0	1.30	1.27	4.00	3.83
Ch6S				
1.25	.13		2.07	2.08
2.5	.28	.27	2.35	2.31
5.0	.57	.61	2.85	2.83
7.5	.91		3.48	3.48
10.0	1.33	1.19	4.14	4.04
15.0	2.14	2.10	5.65	5.64
20.0	3.41	3.48	7.63	7.31
HA				
1.0	1.51		1.88	1.95
1.25	2.89			
2.5	8.09			
4.0	25.25			
5.0	53.50			

Figure C5.

Figure C6. Comparison of observed η_{sp} (columns D & E, Figure C5) with the theoretical values based on single GAG solutions. Columns F & G (when multiplied by 100) represent the % that the observed values account for the theoretical η_{sp}'s.

	D	D_T	F	E	E_T	G
	GAG + 1 mg/ml HA in BSS	Theoretical D ($A+n_{sp}HA$)	D/D_T	GAG + 1 mg/ml HA in BSS + 10% FBS	Theoretical E ($B+n_{sp}HA$)	E/E_T
Ch4S						
1.25	1.92	1.95	.985	1.86		
2.5	1.98	2.01	.985	1.93	2.10	.919
5.0	2.20	2.15	1.02	2.13	2.22	.959
7.5	2.46	2.28	1.08	2.38		
10.0	2.74	2.45	1.12	2.63	2.44	1.08
15.0	3.33	2.77	1.20	3.18	2.84	1.12
20.0	4.00	3.15	1.27	3.83	3.22	1.19
Ch6S						
1.25	2.07	2.01	1.03	2.08		
2.5	2.35	2.16	1.09	2.31	2.22	1.04
5.0	2.85	2.45	1.16	2.83	2.56	1.11
7.5	3.48	2.79	1.25	3.48		
10.0	4.14	3.21	1.29	4.04	3.14	1.29
15.0	5.65	4.02	1.41	5.64	4.05	1.39
20.0	7.63	5.29	1.44	7.31	5.43	1.35
HA						
1.0	1.88			1.95		

Figure C6.

Figure C7. η_{sp}/c values calculated from η_{sp} data in Figure C5. These values are plotted vs. GAG concentration in Figure C8.

	A	B	D	E
	GAG in BSS	GAG in BSS + 10% FBS	GAG + 1 mg/ml HA in BSS	GAG + 1 mg/ml HA in BSS + 10% FBS
Ch4S				
1.25	.056		1.53	1.48
2.5	.052	.060	.792	.772
5.0	.054	.054	.440	.426
7.5	.053		.328	.317
10.0	.057	.049	.274	.263
15.0	.059	.059	.222	.212
20.0	.065	.064	.200	.192
Ch6S				
1.25	.104		1.65	1.66
2.5	.112	.108	.940	.924
5.0	.114	.122	.570	.566
7.5	.121		.464	.464
10.0	.133	.119	.414	.404
15.0	.143	.140	.377	.376
20.0	.171	.174	.382	.365
HA				
1.0	1.51		1.88	1.95
1.25	2.31			
2.5	3.24			
4.0	6.31			
5.0	10.7			

Figure C7.

Figure C8. η_{sp}/c (from Figure C7) plotted as a function of GAG concentration (mg/ml). These lines do not swing upward as those do in Figure C3.

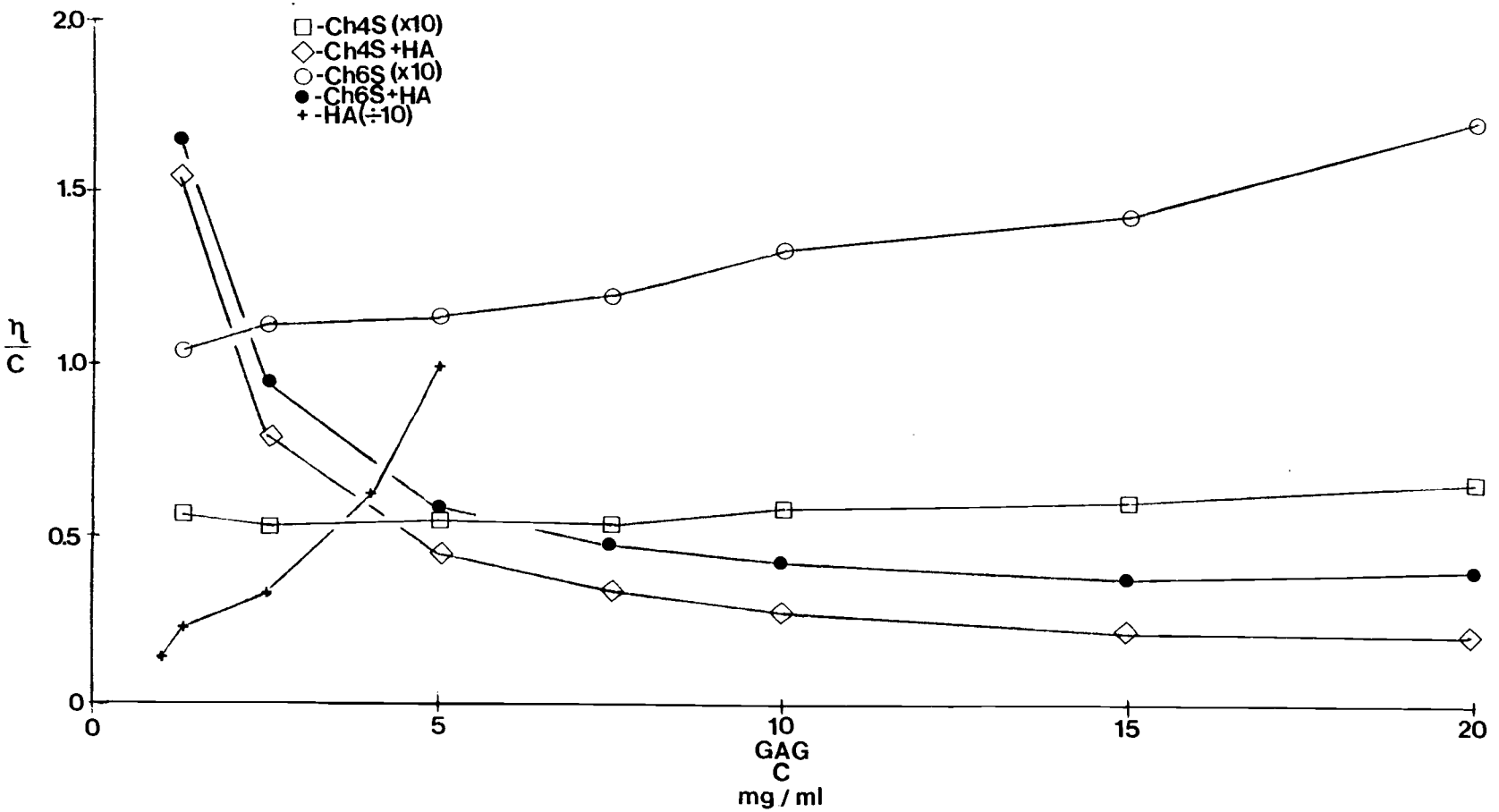
□-Ch4S (x10)
◇-Ch4S +HA
○-Ch6S (x10)
●-Ch6S+HA
+ -HA(÷10)
η/C
2.0
1.5
1.0
0.5
0
0
5
10
15
20
GAG
C
mg / ml

Figure C 8

References

1) Van Holde,K. (1971) Physical Biochemistry. Prentice-Hall, Inc. pp.141.

2) Van Wazer,J.R., J.W.Lyons, K.Y.Kim, R.E.Colwell (1963) Viscosity and Flow Measurement: A Laboratory Handbook of Rheology. Interscience Publishers. pp. 187-262.