Almost all mammalian cells contain glycosaminoglycans as a part of their coat and extracellular matrix (25). Glycosaminoglycans have been studied for many years as components of the extracellular matrix. It is now well recognized that they are not merely a support medium for cells, but play an important role in the development of growth of normal (26) and neoplastic tissue (27,28). Glycosaminoglycans are heteropolysaccharide in nature which are long linear, polyanionic polysaccharide molecules in covalent linkage to a protein core. This protein core and branches form a macroproteoglycan. Glycosaminoglycans are highly charged polyanions composed of repeating disaccharides units which bear either a carboxyl or sulphate group or both and which, except for keratan sulphate, consists of uronic acid and hexosamine moieties. Eight GAG's have been studied in detail: hyaluronate, chondroitin, chondroitin 4 and chondroitin 6 sulphate dermatan sulphate, keratan sulphate, heparan sulphate and heparin. They can be classified into sulphated and non-sulphated GAG's. The molecular weight of sulphated GAG's are usually in the range 1-2 x 10^4, but hyaluronate can be as large as 1-2 x 10^7.

Hyaluronic acid, an unbranched helical polymer composed of repeat disaccharide units of β-N-acetyl-D-glucosamine and β-D-glucuronic acid, as shown below:
In solution, it has the property of relatively stiff random coil (29) with a very large hydrodynamic volume, apparently thousand times greater than its volume in the dry state (30, 31). Hyaluronate is found in high concentrations in developing embryonic tissues such as cornea (32) vertebral column (1, 33) neural crest (34, 35, 39), heart (36), primary mesenchyme (1, 2, 37) and mammalian remodelling tissues (38-40). The core of hyaluronic acid is known to be attached covalently to hundreds of individual proteoglycan thus forming an aggregate of 100-150 x 10^6 MW (41, 42).

With the exception of hyaluronate, GAG do not usually exist in vivo as isolated polysaccharide chains, but as proteoglycans where the GAG chains are linked covalently to protein (43-48). In contrast to sulphated GAG, hyaluronate may not exist as a proteoglycan, but as individual GAG chains in the extracellular matrix (49).

**HYALURONATE BIOSYNTHESIS**

a. **Precursor activation**

Glycosaminoglycan are synthesized through uridine sugar nucleotide precursors, namely, uridine diphosphate glucuronic acid as shown below:

\[
\begin{align*}
\text{UTP} + \text{N}-\text{Acetylglucosamine 1-P} & \rightarrow \\
\text{UDP} - \text{N}-\text{Acetylagucosamine} + \text{P-P}
\end{align*}
\]

The key intermediate compounds related to GAG's are UDP-glucose and UDP-N-Acetylglucosamine (50). Both of these can be formed by reaction of UTP with sugar 1-P (either glucose-1-P or N-acetylglucosamine-1-P) in
the presence of homogenates from proteoglycan producing tissues. UDP-Glucose can be oxidized to form UDP-glucuronic acid which can in turn be decarboxylated to form UDP-xylose. (51). Both of UDP-glucose and UDP N-acetylgalactosamine can undergo epimerization at the C-4 position to form UDP-galactose and UDP-N-acetylgalactosamine (52). Thus, the synthetic steps in the formation of all the sugar nucleotides necessary for synthesis are known to occur in proteoglycan producing tissues. The demonstration of the cell free synthesis of HA in a preparation obtained from Group A streptococci represented the first unequivocal example of a cell free system of a complex polysaccharide synthesis (53). Subsequently, systems for the study of HA in preparations from eukaryotic cells have been developed by Hopwood et al. (54) and Appel et al. (55).

Two of the sugar nucleotides have been shown to act as feedback inhibitor of key enzymes in the pathway of sugar nucleotide formation in liver. It is likely that they function similarly in GAG producing tissues. UDP-N acetylgalactosamine has been shown to inhibit the formation of glucosamine (56). Thus, an excess of UDP-N-acetylgalactosamine would turn off its own synthesis and control the amount of hexosamine available for GAG formation.

It is assumed that all cells capable of synthesizing proteoglycans contain the machinery for synthesizing all the necessary precursors since phosphorylated sugars and sugar nucleotides do not readily enter into the cell.
b. **Protein core:**

It has been established that synthesis of protein is necessary before formation of glycosaminoglycan can take place. This has been demonstrated by pulse chase experiments (57), and by experiments showing that puromycin inhibited synthesis of proteoglycans, but did not inhibit the transfer of sugars to an endogeneous proteoglycan acceptor (58).

The mechanism of HA synthesis remains poorly understood particularly with respect to chain initiation HA formation is insensitive to inhibition of protein synthesis in streptococci (59). Dolichol pyrophosphate derivatives isolated by Turco and Heath (60) and Hopwood and Dorfmann (54) do not seem to be involved in HA synthesis which is not inhibited by tunicamycin. Sugahara et al. (61) attempt at transferring individual monosaccharides to oligosaccharides have so far been unsuccessful.

As HA is requisite for formation of the aggregates of proteoglycan characteristic of cartilage, it is to be expected that some coordination of HA and proteoglycan biosynthesis must exist.

c. **Oligosaccharide linkage:**

The initial step for the synthesis of hyaluronic acid is the transfer of xylose from UDP-xylose to a serine moiety in the core protein acceptor(62,63) followed by serial alternate additions of glucuronic acid and N-acetyl glucosamine from sugar nucleotide to the non-reducing end of nascent oligosaccharide(64,65). The sugar moiety of GAG arise from glucose(66). In addition,
alternate pathways have been found in liver involving the phosphorylation and acetylation of glucosamine with subsequent conversion to the appropriate sugar nucleotides (67, 68). These pathways might also be involved in other tissue but have not as yet been demonstrated.

d. Glycosaminoglycan polymerization:

As with the formation of oligosaccharide link the glycosaminoglycan chains are synthesized from sugar nucleotide precursors. Thus UDP-glucuronic acid and UDP-N-acetylglucosamine are precursors of hyaluronic acid (69), heparin (70) and presumably heparan sulphate and UDP-glucuronic acid and UDP-N-acetylglalactosamine are precursors for chondroitin sulphate (71, 72).

Initially the experimental data had been shown where biosynthesis mechanism of hyaluronate are independent of intact protein biosynthesis (73, 74). However, the existence of a hyaluronate protein complex was established afterwards. In rat skin inhibition of hyaluronate biosynthesis has only been observed after a long preincubation with puromycin (75) and in cultured human fibroblasts, cycloheximide causes only a minimal inhibition of hyaluronate labelling (76). These experimental findings rule out an obligatory protein biosynthesis during the formation of hyaluronate. \( \beta \)-Diazo-5-oxo-L-norleucine (DON) affects the biosynthesis of purines and pyrimidine nucleotides of RNA (77). Thus, maintenance of hyaluronate synthetase activity appears to require both unaffected biosynthesis of nucleotides and of nucleic acids (RNA). Biochemical loci through which hyaluronic acid synthesis is regulated have not been
firmly established but would include the steps after phosphorylation of glucose through the assembly of hyaluronic acid from monosaccharides.

Investigators have shown that control of synthesis is exerted through hyaluronic acid synthetase(59,78-83) considered as multiglycosyltransferase. Identification of HA synthetase rests on activity exhibited by cell lysates wherein monosaccharides units are transferred to nascent hyaluronic acid chains(59). It is bound to particulate fraction of cells and attempts to purify hyaluronate synthetase have failed(86). Cyclic AMP and prostaglandin E2 and estrogen stimulated hyaluronic acid synthetase activity in rat fibroblasts but the response to cyclic nucleotide was blocked by cycloheximide(84). It has been established that synthesis of proteins is necessary before formation of the glycosaminoglycan can take place. This has been demonstrated by pulse-chase experiments(57) and by experiments showing that puromycin inhibited synthesis of proteoglycans, but did not inhibit the transfer of sugar to an endogenous proteoglycan acceptor(58).

Certain aspects of biosynthesis such as the formation of the protein portion, the organization of the synthesis within the cell and the control of the synthetic precursors are largely unsolved. These may prove to be of great importance, since it has become increasingly apparent that pathological processes are generally related to alterations of aspects of control and metabolic organization rather than simple direct effects on individual steps in the metabolic pathways. This would be expressed on a cellular basis resulting in multiple changes
rather than a single, easily described enzyme effects, it is probable that most effects on protoglycan or GAG formation are secondary to more widespread changes in the cell. Changes in GAG production have been described in cultures of skin fibroblasts from patients with Marfan's syndrome(83) and diabetes(84). In each of these cases, there were changes in the amount of one or another of the GAG.

Many details of the biosynthesis of hyaluronic acid remain to be resolved. For example, the role of lipid intermediates in the synthesis of this polysaccharide has been suggested(85). Failure of tunicamycin to inhibit synthesis of hyaluronic acid in fibrosarcoma tissues and in cell free extracts of fibrosarcoma(55) or in the streptococcal system(61) suggests that a dolichol pyrophosphoryl intermediate is not involved. It has also been suggested that there is a cooperative effect of the two membrane bound glycosyltransferases with a growing hyaluronate chain which promotes polysaccharide synthesis(53,59).

DEGRADATION OF GLYCOSAMINOGLYCANS

The best defined glycosaminoglycan lyase is the hyaluronidase that is found in testicular tissue and also in other tissues, e.g., liver(86), bones lysosomes(87), and brain(88). This enzyme degrades hyaluronic acid to a family of oligosaccharides ranging in size from tetrasaccharide to larger even-numbered oligosaccharides(89). When hyaluronic acid is incubated with the enzyme, the primary product is the tetrasaccharide with a small amount of larger oligosaccharide and a small number of disaccharide. The disaccharide has been identified as N-
N-acetyl hyaluronic acid and the tetrasaccharide consists of 2 N-acetylhyaluronic acid units linked in the same fashion as they are linked in the intact hyaluronic acid molecule. The enzyme has been found to be an endo-β-N-acetylhexosaminidase, attacking β-linked N-acetylglucosaminy1 groups at random within the chain with a preferential reaction on the larger molecules rather than on smaller oligosaccharides(90). Thus, the hyaluronic acid molecule initially can be depolymerized rapidly, with consequent loss of viscosity. Further, degradation results in release of measurable amounts of reducing sugars but completion of degradation requires a longer time. The enzyme has been shown to be a transglycosylase capable of transferring disaccharide units from one molecule to another(90,91). Thus, when hexasaccharide is incubated with testicular hyaluronidase, some octasaccharide can be found as well as tetrasaccharide.

ROLE OF HYALURONIC ACID IN MORPHOGENESIS:

Formation of a precisely organized functional tissue or organ is the culmination of a complex series of specific cellular events, usually termed morphogenesis. These events involve several common types of cell behavior, notably movement, proliferation, shape change, recognition and adhesion. At each stage of morphogenesis of an organ or tissue, the macromolecules present in the extracellular matrix or associated with the external cell surface are important in providing structural support for and environmental signals to the cells involved. These contributions in turn exert a considerable influence on the course of morphogenesis.
Previous studies of hyaluronate metabolism at discrete stages of early development have shown the onset of differentiation are associated with a dramatic decrease in extracellular hyaluronate concentration. However, these studies have usually been addressed to general maturation or aging trends in tissue structure, e.g., in water content, rather than to morphogenic sequences. By studying hyaluronate metabolism at discrete stages during early development, at times and in tissues where striking morphogenic cell movement and differentiative changes are taking place, close correlations between hyaluronate synthesis and cell migration and between hyaluronidase activity and cell differentiation have now been revealed (92).

Hay and Revel (93) have described the course of events during early corneal morphogenesis in chick embryo in detail. It is interesting to note that the leading migratory mesenchymatous cells always move adjacent to the corneal epithelium (94) which is the source of hyaluronate. The appearance of hyaluronidase activity in corneal tissue coincides with the cessation of migration and deswelling (32) which follows the further development along with the change in major GAG from hyaluronate to sulphated GAG subsequently (95, 96). In support of this view it has been shown further (97, 98) that the enzyme hyaluronidase appears precisely at the time when cell begins to produce cartilage matrix after concluding their migration around the notochord. Pratt et al. (35) have shown that a major component of the extracellular matrix into which cranial neural crest cells migrate is hyaluronate, and these investigations correlate the production of hyaluronate closely with a large increase in volume of extracellular matrix and the initiation of crest cell
migration cartilage redifferentiation is maximal when hyaluronidase activity is maximal.

A second example is the dissociation of sclerotomal cells from the chick embryo somite and the translocation of these cells to the notochord. Kvist and Finnegan (97, 98) found that hyaluronate was the major GAG in the matrix surrounding the migrating sclerotomal cells at the site of cartilage formation, around the notochord chondroitin sulphate, was more than hyaluronate. In addition, Toole (33) found decreased synthesis of hyaluronate and increased hyaluronidase activity to occur precisely at the time that the cells began to produce cartilage matrix after concluding their migration around the notochord.

Similar correlations between the presence of hyaluronate and morphogenetic movements have been made during the development of several other embryonic tissues. Ectodermal cells at gastrulation (1), neural crest cells (34, 35, 99) and kidney mesenchyme (100) all move and proliferate in a hyaluronate-rich matrix. In most of these cases, subsequent differentiation has been shown to be associated with decreased hyaluronate levels. In addition to embryonic development, cell movement and proliferation taking place during salamander limb regeneration (101, 102) tendon remodelling (5), bone and skin wound healing (40, 103) are also associated with high levels of hyaluronate and subsequent differentiation with decrease in hyaluronate. Thus, according to Toole et al. (10) and others (1, 2), the beginning of the morphogenetic stage is characterized by accumulation of hyaluronic acid and migration of mesenchyme cells. These cells then differentiate into fibroblasts and cells
characteristic of the tissue being formed. At this stage a marked decrease of hyaluronic acid occurs. Furthermore, Sampaio and Dietrich (26) reported recently the marked changes of chondroitin-6-sulphate concentration and hyaluronidase activity after fetal development. Also, the peak of maximum hyaluronidase and chondroitin sulphate concentration coincides with the stage of cytodifferentiation in bovine. It may be correlated also that the change in major GAG was from hyaluronate early in development to sulphated GAG.

During morphogenesis hyaluronate accumulates in the tissues at a stage characterised by extensive cell migration (32, 33, 101). Subsequent differentiation is preceded by enzymatic removal of hyaluronate and this removal appears to be essential to normal development of the tissues. Specific inhibition of differentiation expressed as lack of morphological signs of chondrogenesis, was indeed noted when hyaluronate was added to cultures of embryonic chick somite cells. This effect could not be reproduced with other biological polyanions, such as chondroitin sulphates, heparin or nucleic acids (90, 104). Hyaluronate also inhibited the incorporation ($^{35}$S)-sulphate into polysaccharides and this effect was studied further on fully differentiated chondrocytes grown in culture (105-107). Hyaluronate was found to be a very potent inhibitor of proteoglycan synthesis with as little as 0.005 μg/ml significantly decreasing ($^{35}$S)-sulphate incorporation into glycosaminoglycans. Again, other polyanions were incapable of eliciting this effect. Partially depolymerized hyaluronate, but not small oligomers such as tetra or hexasaccharides retained inhibitory activity (107). The effect was restricted to chondrocytes.
No effect was noted on the production of sulphated polysaccharides by fibroblasts, even at relatively high concentrations of hyaluronate (10 μg/ml). During incubation of chondrocytes with (C\textsuperscript{14}) hyaluronate level was taken up by the cells, and reached a plateau within about an hour; Moreover, addition of unlabelled hyaluronate reduced this uptake. Most of the label appeared to be associated with the cell surface as it could be largely removed by mild trypsin treatment of the cells. Such treatment abolished the inhibitory effect of hyaluronate on proteoglycan synthesis\textsuperscript{(107)}. Taken together, these findings suggests that hyaluronate binds to specific sites on the surface of chondrocytes and thereby inhibits the synthesis of proteoglycan, but did not affect chondroitin (S\textsuperscript{35}) sulphate synthesis when supplied to cells along with benzyl-B-D-xyloside, an exogenous initiator and indicator of chain polymerization\textsuperscript{(108)}. It was therefore, concluded hyaluronate acts as interfering with glycosaminoglycan production is mainly regulated by the level of hyaluronic acid chain initiation and hence proteoglycan biosynthesis, by specifically inhibiting either the formation of normal core protein or the xylosyl-transferase, which initiates synthesis of chondroitin sulphate chains on the protein. The findings of Uzuka\textsuperscript{(109)} suggests that hyaluronic acid production is mainly regulated by the level of hyaluronic acid synthetase. Furthermore, Sisson et al.\textsuperscript{(110)} infer that in some circumstances, the availability of glucosamine may be rate limiting factor in the synthetic pathway of HA, but in their experiments, this amino sugar precursor appears to express itself by inducing the synthetase enzyme, the activity of which may be the ultimate regulator of HA synthesis.

In contrast to hyaluronate, sulphated GAS's were found to exert a stimulatory effect of GAG synthesis in both chondrocytes\textsuperscript{(111,112)} and corneal\textsuperscript{(113)} comparatively,
high polysaccharide concentrations were required to obtain significant effects and no evidence for a receptor mediated mechanism was obtained.

**HYALURONATE BINDING TO CORE PROTEIN OF PROTEOGLYCAN**

Proteoglycans are complex macromolecules that contain a core protein to which at least one GAG is covalently bound. This simple definition encompasses a wide range of structures involving different core proteins, different GAG and different numbers and lengths of GAG chains. At present proteoglycan isolated from cartilage have been widely studied. The pioneers to work on the organization of these aggregates were Sajdera and Hascall (12). They separated the proteoglycan monomer from nonproteoglycan component by using density gradient centrifugation and demonstrated that hyaluronate, one of the components, had a unique role in aggregation, individual strands bind as many as hundred proteoglycan monomer molecules (13,25,114-117). This interaction may be demonstrated in the absence of additional components (13,114) but is apparently stabilized by two link proteins that form part of the native aggregates (12-15,25). The link proteins are structurally closely related to each other (119) and are able to bind hyaluronate in the absence of proteoglycan (119). In the native aggregates, hyaluronate, thus presumably binds to three protein components, the two link proteins, and the hyaluronate binding region of the proteoglycan core protein (Fig. 1).

The current model for the structure of proteoglycan monomer molecules isolated from hyaline cartilages is that of central protein core, to which approximately 100 chondroitin sulphate and 50 keratan sulphate side chains
Schematic representation of cartilage proteoglycans and their aggregation by binding to hyaluronate (labelled as HA in the figure). Link a and Link b refer to the link proteins that stabilize the interaction between hyaluronate and the proteoglycan core-protein (Figure from Ann. Rev. of Biochemistry 47, 385, 1978).
are covalently attached\(^{(41,120)}\). About one third of the protein, located at one end of the core contains few or no polysaccharide chains\(^{(119)}\). This portion of the protein (the hyaluronic acid binding region) exhibits a specific, nonvocalent interaction with hyaluronic acid \(^{(116-118)}\). This interaction is essential for the formation of proteoglycan aggregate structures, such aggregate appears to be the predominant way in which the proteoglycans are organized in cartilage extracellular matrices\(^{(13)}\).

The interaction between proteoglycan and hyaluronate is highly specific as other polyanions, including chondroitin, in which galactosamine replaces glucosamine do not complex with proteoglycan\(^{(114,116)}\). The isolation of core fragments with molecular weights in order of 60,000-70,000 which contain hyaluronic acid binding region was done after the mild proteolytic digestion of the aggregates\(^{(13,42,121)}\). Several lines of evidence indicate that the hyaluronate binding region is located at one end to the proteoglycan core protein molecules \(^{(11,16)}\). The interaction between hyaluronate and hyaluronate binding region has been studied in detail, suggesting lock and key rather than cooperative electrostatic binding. They further showed that polyelectrolytes such as dextran sulphate, chondroitin sulphate, sodium alginate, or DNA did not interact with proteoglycan\(^{(114)}\).

Competitive binding experiments with hyaluronate oligosaccharides showed that good binding was with a decassacharide containing a reducing N-acetylglucosamine residue, and larger oligosaccharides\(^{(115,116)}\), further
nonasaccharide with glucuronic acid in reducing terminal position is the smallest fragment capable of strong interaction with the hyaluronate binding site on the proteoglycan core protein (122). In addition, one or more of the N-acetylglucosamine residues must be specifically involved in binding because chondroitin, which differs from hyaluronate only in the configuration of C_4 at the hexosamine residues has no binding ability.

Reduction and alkylation of the core protein in proteoglycan monomers prevent subsequent aggregate formation (123) and interaction with hyaluronate (124) which suggests that the confirmation of the hyaluronate binding region is critical for the binding process. The dansylation and sequential acetylation-dansylation protocols indicate that the HA binding region molecules contain reactive amino group(s) (most probably -amino groups on lysine residues) in or near the active binding site to hyaluronic acid which when dansylated prevent interaction with hyaluronic acid but when acetylated do not. This suggests that these amino group(s) are not essential for the contact interactions which bind hyaluronic acid into the active site but are near enough that substitution with the bulky dansyl moiety sterically blocks access of the hyaluronic acid. While these amino groups do not appear to be critical for binding they may contribute to the stability of the interaction (125).

HYALURONATE - INTERACTION WITH CELL SURFACE COMPONENTS AND AGGREGATION

Numerous studies have shown that GAG are associated with the surface of many different types of cells and that the level of these GAG vary under different physio-
logical conditions *in vitro*. Initially Kraemer(126-128) showed that heparan sulphate is tightly associated with the surface of many different cultured cell lines.

Apart from that, interaction of hyaluronate with the cell surface has recently been studied, as it was shown to be important factor in morphogenesis. Earlier, several investigators have shown that hyaluronate partially inhibits chondroitin sulphate synthesis by cartilage forming cells *in vitro*(105,108,129,130) probably by interacting with the surface of cartilage forming cells, rather than by entering the cells.

The interaction of hyaluronate with the surface of SV-40 transformed 3T3 cells have been studied recently by Underhill and Toole(131). They report that reproducible binding of H₃-hyaluronate with SV-40-3T3 cells can be measured. Extent of binding is a function of H₃-HA and cell number. The nature of cell surface binding sites has been examined further using H₃-hyaluronate as a ligand(132,133). These studies have shown that the binding of H₃-hyaluronate to SV-40 transformed 3T3 cells is saturable of high affinity and optimal at neutral pH. Furthermore, the binding of H₃-hyaluronate is blocked by unlabelled hyaluronate and by closely related carbohydrates, but not by sulphated GAG's. The binding characteristics of hyaluronate are influenced markedly by its molecular weight. The binding of hyaluronate to SV-3T3 cells is enhanced by increasing the ionic strength or by decreasing the temperature(133). It has also been suggested that high affinity of HA to SV-3T3 cells may be due to interaction of single molecule of HA with multiple receptors.
Recently hyaluronate binding receptors on cell surfaces have also been studied in certain types of transformed lymphocytes (8,9) and alveolar macrophages (6,7). The interaction of hyaluronate with cell surface receptors may be important in a variety of phenomena, e.g., during embryonic morphogenesis (92), tissue remodelling (38-40) and tumor growth (4). This multiple receptor occupancy model for hyaluronate has been supported by several studies of cultured cell lines (134), transformed lymphocytes (8,9) and macrophages (6,7) in the latter studies, small amounts of HA induced their aggregation.

The nature of above HA Binding is thought to be firstly, a noncovalent reaction between a protein receptor and the carbohydrate moieties of hyaluronate. Secondly of a type which retains newly synthesized hyaluronate at the cell surface and which involves a protein covalently bound to hyaluronate (20).

The physiological function of these two types of HA binding is not well established, but possibly HA covalently bound to protein is involved in mediating synthesis, transport and secretion of HA. It may act as a primer for elongation of hyaluronate. The binding of exogeneous hyaluronate to cell surface receptors may mediate endocytosis of extracellular hyaluronate leading to its degradation by lysosomal hyaluronidase (135, 136). Thus, these two binding phenomena may be intimately associated with the regulation of hyaluronate metabolism.

Hyaluronate have been implicated in adhesion phenomena that may require binding of the polysaccharide to cell surface structures. Hyaluronate was also found to
be the material deposited on plastic dishes (137). Further, it was noted that a variety of cells in culture produced a factor identified as hyaluronate, that could aggregate certain lymphoma cells (8, 9). It was also found that hyaluronate lost its aggregating properties on partial depolymerization and could not be substituted for any other GAG, on the other hand oligosaccharides derived from either hyaluronate or chondroitin sulphate were able to abolish aggregation induced by polymeric hyaluronate (9). It has been interpreted that only hyaluronate, due to its chain length is able to span the intercellular space and induce aggregation. Apparently, only a limited number of cell lines posses the receptor, as no other cells tested, including lymphocytes and human lymphosarcoma cells were aggregated by hyaluronate.

Recently, Underhill and Colleagues, have attempted a more systematic approach to the effect of hyaluronate on cell aggregation. Underhill and Dorfmann (138) have shown that divalent cation independent aggregation of some transformed cell lines is dependent on endogenous cell surface hyaluronate and this dependence has very similar characteristics to the binding of exogenous hyaluronate. It is hypothesized that endogenous cell surface hyaluronate on one cell binds to free receptor sites on an adjacent cell (Fig. 2A). In support of this idea, Underhill and Dorfmann (138) showed not only that enzymatic degradation of cell surface bound hyaluronate inhibited this aggregation (Fig. 2B), but also the addition of a large excess of exogenous hyaluronate inhibited aggregation (Fig. 2C).
Fig. 2  Hyaluronate mediated aggregation of cell lines in Suspension as suggested by Underhill et al. A. Endogenous cell-surface-associated hyaluronate cross-bridges by binding to receptors on adjacent cells. B. Hyaluronidase treatment degrades the hyaluronate, inhibiting aggregation. C. Addition of excess exogenous hyaluronate blocks all the receptors, inhibiting aggregation.
EVIDENCE FOR THE PRESENCE OF HYALURONIC ACID BINDING PROTEIN:

The first important clue to the organization of cartilage proteoglycan bound to hyalurionate and in turn linked to a common core protein came through the work of Hascall & Sajdera(12). Aggregates are formed by the interaction of proteoglycan and hyaluronic acid, but is apparently stabilized by two link proteins that form the part of the native aggregates. The link proteins are structurally closely related to each other(118) and are able to bind hyaluronate in the absence of proteoglycan (119). Hyaluronate, in the aggregate thus binds to two link proteins and the hyaluronate binding region of the proteoglycan core protein. Core fragments with molecular weights in the order of 60,000-70,000, which contain the hyaluronate binding region, could be isolated after mild proteolytic digestion of aggregates(13,42,121). Several lines of evidence indicates that the hyaluronate binding region is located at one end of the proteoglycan core protein molecules.

The interaction between hyaluronic acid and hyaluronic acid binding region, has been studied in considerable detail, mostly using gel chromatography, viscometry, density gradient centrifugation and molecular sieve chromatography. In the absence of the link protein, the HA binding region of monomers is sensitive to trypsin, even in the presence of HA, the HA binding region can no longer be recovered in a form which can bind to HA(119). Therefore, the link protein is required to protect the HA Binding region from proteolysis. Similar extensive studies have been done on proteoglycan aggregates from various sources(13,116).
The first report of hyaluronic acid binding protein came from Delpech et al. (17) from human nodes of Ranvier and they coined the name "Hyaluronectin" for this brain glycoprotein. They observed that hyaluronectin binds to the immobilized hyaluronic acid. The binding was reversible and gave pure 99% antigen in high yields as much as 80%. They also found that the binding of hyaluronectin was suppressed by incubating the affinosorbent with hyaluronidase and the presence of GAG other than HA in the sample did not inhibit the binding.

Later the same group, Delpech et al. (18) further studied this glycoprotein and reported the hyaluronectin from human brain using affinity chromatography and enzyme labelled immunosorbent assays (ELISA) combined with affinity adsorption. They isolated the hyaluronectin by homogenizing the brain first in PBS and later in glycine-HCl buffer pH 2.2 followed by centrifugation. In their experiments they were not able to show the purified hyaluronectin as a single band in polyacrylamide gel electrophoresis but used immudiffusion in slices of the gel. Here also the diffusion was not shown and discussed as specific to antiserum of protein. The molecular weight determination as measured by SDS-PAGE, gives an account that mole wt. ranged in the zone 40 K to 100K. Its main band indicated a molecular weight of 68 K.

Furthermore, they report that hyaluronectin binding to hyaluronic acid was inhibited by hyaluronic acid and by the end products of hydrolysis of HA by hyaluronidase from bovine testes, but was not inhibited by other GAG or by monosaccharides. Finally, they reluctantly conclude, that GAG's other than hyaluronate are in the form of proteoglycans, and hyaluronate, in association with proteins, would play a role as a linking agent.
for proteoglycans (139). Thus, Hyaluronectin, could be one of the proteins involved in that linkage.

Girard, et al. (19) reported the hyaluronectin in human tumor heterografts in the nude mice. The histopathology and the hyaluronectin staining were similar to those of the tumors of the origin. In their adsorption experiments they were able to show that one of the three human grafted tumors contain a human type hyaluronectin in contrast to the other two. This tumor can be used to test the production of human hyaluronectin in vitro and in vivo. Contrary to their earlier report they say that maximum purified protein was recovered from PBS extract, but in earlier report (18) eighty percent of the brain glycoprotein hyaluronectin was recovered from the glycine-HCl extract. This discrepancy in their methodology of the purification of hyaluronectin provoked us to purify hyaluronic acid binding protein by the steps, different from these known investigators.

Simultaneously, the independent works of Underhill (21) and Turley (22) ushered a line for deeper investigation of hyaluronic acid binding protein. Underhill (21) reported that in horse serum a natural antibodies exist which bind to hyaluronate. They purified the antibody by ammonium sulphate précipitation, ion-exchange and molecular sieve chromatography as well as affinity chromatography. The binding activity of antibody associated with horse serum was found saturable, of relative high affinity and specific for hyaluronate. The molecular weight of the purified protein as analysed on SDS-PAGE was found to be similar to that of IgG with a heavy and light chain of molecular weight of 54,000 and
24,000 respectively. Here, they differentiated the purified antibody of horse serum from IgG from the fact that the purified antibody peak fraction did not stick to DEAE-cellulose, which is one of the characteristics of IgG(140).

On the other hand, Turley(22) purified partially hyaluronate acid binding protein from chick fibroblast culture's media by ultracentrifugation and using Dowex-hyaluronate affinity chromatography. The principal hyaluronate binding protein in the affinity purified fraction has a molecular weight of 60,000-63,000 daltons. The binding of purified protein was very specific to hyaluronate and the other GAG's like chondroitin sulphate or heparin did not inhibit the binding of this protein to hyaluronate. One interesting finding about the biological effect of hyaluronic acid binding protein came from them that when this purified protein was inserted into the cell layer of urea-pretreated fibroblast monolayers, it specifically increased the amount of exogenous hyaluronate, not other sulphated GAG's and this interaction promoted the culture morphology and nuclear overlap ratio similar to virally transformed cells.

Hyaluronate-protein complex purification of Rous sarcoma virus-transformed chick embryo fibroblast by Toole et al.(20) have also given a good base to compare and analyse our purified protein in detail. They have been able to label the HA-protein from cultured RSV-fibroblast, and partially characterized this protein. The preparations were purified by Bio-Gel A-15 m gel filtration as CsCl density gradient ultracentrifugation under dissociative conditions followed by acid agarose
gel electrophoresis in the presence of 0.1% Nonidet P-40. The cell derived hyaluronate protein was resistant to pronase but susceptible to proteinase-K in the presence of SDS. The $H^3$-labelled protein gave a broad band corresponding to $M_r 12,000$ on SDS-urea polyacrylamide gel electrophoresis. Finally, they propose that the cell-associated hyaluronate protein may be bound to the cell surface and the protein may be wholly or partly be lost on secretion.

Recently, in 1984(24), the report of the expression of hyaluronic acid-binding glycoprotein, hyaluronectin, in the developing rat embryo, gives a powerful information for probing the biological role of hyaluronate and its binding protein. They studied the distribution of hyaluronic binding protein in developing rat embryo and found that this protein was absent in morula and blastula, whereas it was first detected in the mesenchyme bordering the neural lobe that is, at the time when hyaluronic acid is already widely present. They also suggest that hyaluronectin could play a role in association with fibronectin and hyaluronic acid in the physiology of embryonic extracellular matrix. Since hyaluronectin appeared at late stage in the embryonic nervous tissue, its distribution was extracellular in areas where both cell migration and proliferation occur.

In the last decade though the research on several biomolecules capable of binding to GAG's have been reported, but, very little is known about hyaluronate acid binding protein. Since recent evidence suggests that hyaluronate and may be hyaluronectin play an important role in connective tissue structure as well as in
cell behaviour(141), it has become urgent to acquire a better knowledge of the regulation of HA metabolism. Thus, it was of our interest to purify and characterize the hyaluronic acid binding protein from brain and liver tissues and to examine the specificity of hyaluronic acid binding and to study the interaction of this protein with other GAG and monosaccharide. An attempt has also been made to determine the level of this protein in different tissue as well as in newborn young and adult tissues.