INTERCELLULAR MACROMOLECULES CONTAINING POLYSACCHARIDES

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ABSTRACT A survey is presented of the carbohydrate of intercellular spaces that is known to exist in the form of polysaccharide. This amounts to considering the polysaccharides made by connective tissue cells. The chemical structures of these polysaccharides are discussed in the cases in which they are known and regularities in patterns are noted. Such regularities make of these polysaccharides a natural group that may be related to the natural group of connective tissue cells that produce them. Viscosity is discussed as an important property of hyaluronate. The polyelectrolyte properties of these polysaccharides are considered in detail mainly from the point of view of their use in studying the polysaccharides, fractionating them, and accounting for their behavior in tissues. Polyelectrolyte properties are considered in connection with simple cations, micellar detergent cations, metachromatic dye cations, and protein cations. The existence of the polysaccharides in native tissues as compounds with proteins is discussed in detail, and with particular reference to the two cases most thoroughly studied, the chondroitin sulfate of cartilage and the hyaluronate of synovial fluid. A sharp distinction is drawn between compact and diffuse macromolecules by a contrast of tropocollagen and hyaluronate. The known proteinpolysaccharides of intercellular space are diffuse molecules. Experiments are described to show how hyaluronate hinders sedimentation, excludes compact macromolecules, or entangles with fibrils. Binding of water by connective tissue and elasticity of cartilage may be related to the tissue content of such diffuse macromolecules.

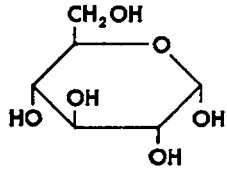
The foregoing discussions have been concerned with collagen, the protein that forms water-insoluble fibers whose main function seems to be to create the mechanical structure which is the gross outwardly recognizable animal body together with all its internal pockets and furnishings. Distributed throughout this structure, the cells of the animal live and carry out their biochemical processes. Their raw materials and their metabolic products are carried to and from them by flow or diffusion in solution. They live in an aqueous environment, somewhat similar to plasma, but containing, in addition to plasma components, another group of intercellular macromolecules which are generally water-soluble and which can be distinguished by the

fact that they contain carbohydrate. The carbohydrate may consist of small units attached to a large protein molecule, or the carbohydrate may itself be a macromolecule in which case it is called a polysaccharide. Macromolecules of polysaccharides may also be attached to macromolecules of protein. Intercellular macromolecules that contain carbohydrate are formed by cells of both epithelial and connective tissues. Because of the mucilaginous nature and feel of the secretions of many epithelial tissues the products they elaborate came to be called mucins and from this root there grew a whole family of words such as mucoids, mucopolysaccharides, mucoproteins, and mucinases. Because of the indiscriminate usage of many of these words in areas in which characterization of the substances referred to has been extremely difficult, these words have come to have ill-defined meanings and it may be best not to use them.

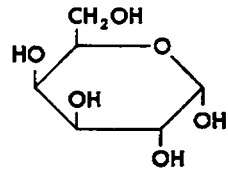
The present discussion will be focused on those intercellular macromolecules in which the carbohydrate is known to be in the form of a polysaccharide. This excludes many of the products derived from epithelial cells since in most cases we have no knowledge of the size of the saccharide chains in these products, though in some cases, as in submaxillary mucoid, the carbohydrate seems to be present as a large number of oligosaccharide units separately attached to protein. In our present state of ignorance this restricts our attention to the polysaccharides produced by cells of the connective tissue. There are tissues of the body that are almost entirely composed of connective tissue such as tendons, cartilage, and the derma of the skin. Many other tissues, such as vessel walls or intestinal walls, are more or less intimate associations of connective tissue with other kinds of tissue. Since connective tissues and their cells exist so widely spread throughout the body, so do the products of their metabolism. Among these products are the polysaccharides which are our present concern.

THE POLYSACCHARIDES

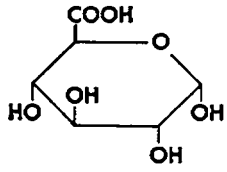
A polysaccharide is a macromolecule made up of many saccharide units. These units may be all alike or they may be different. But even when different there are usually only a few different kinds in any one polysaccharide, and they seem not to have the apparently random distribution of the amino acid units in proteins. The known polysaccharides of connective tissue have molecular weights that lie between 15,000 and 10,000,000, and the number of saccharide units in a molecule lies between 50 and 50,000. The most common saccharide unit in the world is glucose, and the most common polysaccharide in the world is cellulose which is made up entirely of glucose units in unbranched chains. The polysaccharides of connective tissue are made up of saccharide units different from glucose but closely related to it and metabolically easily derived from it. These saccharide units and their relation to glucose are shown in the structures:



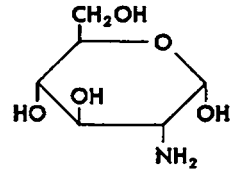
D-glucose



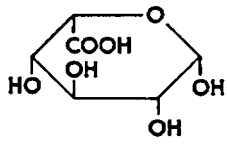
D-galactose



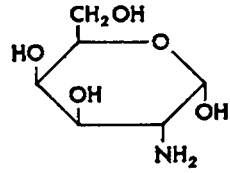
D-glucuronic acid



D-glucosamine



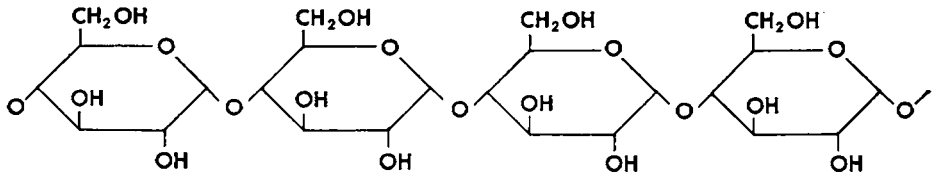
L-iduronic acid



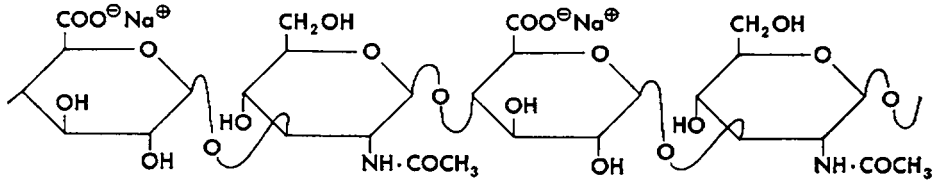
D-galactosamine

In a polysaccharide, the saccharide units are united by the formation of an ether link between the OH on C_1 of one saccharide unit and an OH on a carbon atom other than C_1 of a second saccharide unit. As an example there is shown a short segment of a starch molecule in which adjacent glucose molecules are linked by ether bonds from C_1 to C_4.

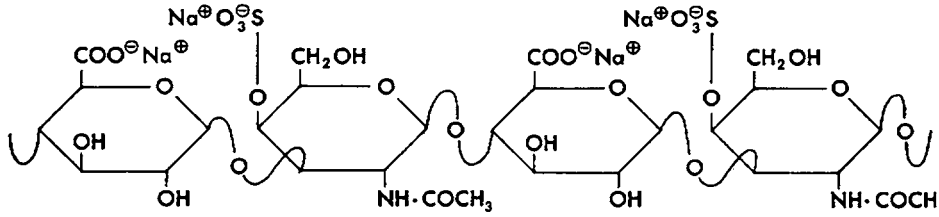
It should be noticed that at C_1 there are generally two ether bonds, one within a single glucose unit, the other to the adjacent glucose unit. Where such double ether links occur at a single carbon atom they have special properties and so they have a special name; they are easily hydrolyzed by acids and they are called acetals. Also shown are segments of the best known of the connective tissue polysaccharides, sodium hyaluronate and sodium chondroitin sulfate. They are composed of a regular alternation of two different saccharide units, and there is also a regular alternation of C_1 to C_4 and C_1 to C_3 links as shown. Because of the regularity of these alternations both the hyaluronate molecule and the chondroitin sulfate molecule can be considered as long chains of similar disaccharide units, called repeating units or periods. The determination of the structural details of this and of most of the other polysaccharides of connective tissue is very largely due to the painstaking work of Karl Meyer (1–3). The other polysaccharides of connective tissue whose structures are known at least partly are listed in Table I. This shows the component saccharide units and the



Starch segment



Sodium hyaluronate segment



Sodium chondroitin sulfate segment

occurrence of ester sulfate or amide sulfate as an additional repeating component. Without going into details of the structure of each polysaccharide certain generalizations seem true of all of them. Each polysaccharide is composed of two different saccharide units which alternate regularly along a chain in which so far no branching has been proved to occur. In all cases one of the saccharide units is a hexosamine,

TABLE I

THE COMPONENTS OF SOME INTERCELLULAR POLYSACCHARIDES PRODUCED BY CONNECTIVE TISSUE CELLS

Polysaccharide	Hexosamine	Hexuronate	Hexose	Sulfates/Period	
				Ester	Amide
Hyaluronate	Glucosamine	Glucuronate	—	—	—
Chondroitin-4-sulfate	Galactosamine	Glucuronate	—	1.0	—
Chondroitin-6-sulfate	Galactosamine	Glucuronate	—	1.0	—
Dermatan-4-sulfate	Galactosamine	Iduronate	—	1.0	—
Heparin	Glucosamine	Glucuronate	—	1.5	1.0
Heparitin sulfate	Glucosamine	Glucuronate	—	0.5	0.5
Keratan sulfate	Glucosamine	—	Galactose	1.0	—

either D-glucosamine of D-galactosamine. The other saccharide unit in most cases is a uronic acid, either D-glucuronic or L-iduronic acid, but in one case is D-galactose. The amino group of the hexosamine seems never to be free; it is either acetylated or it is sulfated. The result of this is that it is never basic, and at the pH of tissues these amide groups do not attach a proton and cannot become positively charged or cationic. On the other hand each of these polysaccharides has anionic groups; carboxylate, ester sulfate, or amide sulfate, which at the pH of tissues, exist as a negatively charged or anionic group, each associated with a small cation such as sodium. This may be their most important characteristic. Each molecule of any one of these connective tissue polysaccharides carries a large number of negative charges attached to and regularly spaced along the polysaccharide chain. To this we will return later.

These polysaccharides form a natural group for several reasons; they seem to have a set of structural similarities in common, they all seem to be produced by connective tissue cells of one kind or another, and they all are regularly repeating polyanions. But there are different kinds of connective tissue and they contain cells with different patterns of polysaccharide production. These different kinds of connective tissue cells are all thought to be derived from a single kind of ancestral cell called the primitive mesenchymal cell which is itself derived from the mesoderm of the embryo. In further development the primitive mesenchymal cell seems to be able to change to one of a number of specialized forms only a few of which have specialized names as indicated in Table II. Perhaps the least specialized is the fibroblast, but this

TABLE II

SOME PRODUCTS FORMED BY A FEW TYPES OF CONNECTIVE TISSUE CELLS

Mesoderm

↓ Muscle

↓ Undifferentiated mesenchymal cell

	Fibroblast	Synovial living cell	Mast cell	Chondroblast	Osteoblast
Collagen	+	−	−	+	+
Hyaluronate	+	+	−	−	−
Chondroitin sulfate	+	−	−	+	+
Heparin	−	−	+	−	−

may only be because we have not yet learned to distinguish different kinds of fibroblasts, for certainly in different areas of the body cells called fibroblasts produce different kinds of tissue. Tendon and derma are obviously and chemically different tissues yet the cells that produce them are both called fibroblasts. We do not at

present know in enough detail the kinds and proportions of polysaccharides that are produced by each kind of connective tissue cell. Table II gives only a gross summary which shows that there are indeed great differences in the products produced.

Besides the different polysaccharides produced by connective tissue cells in mammals listed in Table II there are others related to these but less well defined. There is for instance one found in corneal tissue that seems to be similar to chondroitin sulfate but that contains very little sulfate and so is called chondroitin (4). The study of connective tissues in animals other than mammals promises to open up a vast area for the study of the evolution of connective tissue polysaccharides as related to the evolution of the animal forms that carry them. At present only a few such forms have been studied. In shark, horseshoe crab, and squid there were found forms of chondroitin-6-sulfate and keratan sulfate that contain between one and two ester sulfates per period (5, 6). A marine snail has a tissue resembling cartilage that was found to contain a very different, yet still related polysaccharide, a polyglucose sulfate (7).

Of these polysaccharides the two most thoroughly studied are hyaluronate and chondroitin sulfate. The structure of hyaluronate shows the regular alternation of glucosamine and glucuronate links, and the alternation of $1 \rightarrow 3$ and $1 \rightarrow 4$ links. These links are of very unequal stability with respect to acid hydrolysis, the glucosaminidic link is split much more readily than the glucuronidic link with the result that rather good yields of the disaccharide, hyalobiuronic acid, can be obtained after mild acid hydrolysis.

There are several enzymes called hyaluronidases capable of hydrolyzing hyaluronate. A second area of Karl Meyer's achievements lies in working out some of the details of the action of hyaluronidases from different sources (8). The action of the first, found in mammalian testicular tissue and in snake venom, is to split the hexosaminidic bond, and to yield as the main product a tetrasaccharide having the glucosamine at the reducing end of the molecule. The action of the second, found in the head of the medicinal leech, is to split the hexuronidic link, and to yield as the main product a tetrasaccharide having the glucuronate at the reducing end of the molecule. The action of the third, found in bacterial extracts, is radically different in character. It splits the hyaluronate at the hexosaminidic link but the final product is a disaccharide of a new kind, having a double bond between C_4 and C_5 of the hexuronate ring. This disaccharide has the unusual property of absorbing ultraviolet light at 230 mμ. The only clear evidence for the existence of hyaluronidase in mammalian tissues is that for testicular tissue.

The molecular weight of hyaluronate depends on its source (9), that from vitreous humor is of the order of 200,000 to 500,000 but that from synovial fluid, umbilical cord, or rooster comb lies in the range 1 to 5×10^6. Hyaluronate of molecular weight 10^6 would have 2500 periods. Since there is no evidence of branching, the length of the chain would be about 25,000 A or 2.5 μ. Stretched out, its length would

be one-third the diameter of a red cell. In the absence of constraints to hold it in this position a molecule in solution could not remain completely stretched out. Such a configuration is quite improbable. In very dilute solution the size, or rather the space, through which such a molecule extends has been estimated to be 4000 A, and light-scattering results have been interpreted to indicate a spherical shape. A molecule of molecular weight 10^6 in solution in a spherical volume of 4000 A diameter would correspond to a solution concentration of about 0.01 per cent.

One of the most interesting properties of hyaluronate is the high viscosity of its aqueous solutions. This is such a prominent property that it has been thought to be related to its functions in tissues. Its occurrence in the synovial fluid of joints has suggested that it has a lubricating function. In other tissues such as derma it might have a lubricating function at a molecular level, perhaps acting as a plasticizer to diminish friction and wear between collagen fibrils. Another function has been thought to be as a retardant to invasion of tissues by bacteria. In this connection it has been thought that bacteria which produce their own hyaluronidase would thereby have an advantage in invading tissues.

Viscosity is a measure of the drag or friction exerted by adjacent layers of fluid that are moving past each other. In a solution this depends on the sizes and shapes of both solvent and solute molecules. The unusually high viscosity of hyaluronate solutions is related to the unusually large volume or domain these molecules occupy in solution. What is the condition in a very dilute solution, of a molecule that spreads throughout so large a volume of space that it is equivalent to a 0.01 per cent solution? It is not a compact molecule but a highly diffuse, spreading molecule. There is no evidence of any significant or stable cross-linking of the long hyaluronate chain. It seems to exist in solution as a very loosely coiled, somewhat stiff, randomly distributed, unbranched chain. The hyaluronate molecule does not occupy all the space, the domain, through which it is spread. Molecules of water and small solute molecules can drift freely through this domain. In solutions of hyaluronate below 0.01 per cent the molecular domains will tend to form separate islands. In human synovial fluid the concentration of hyaluronate is about 0.4 per cent. In such a solution as a whole hyaluronate is more concentrated than it is in the domain occupied by each hyaluronate molecule in a very dilute solution. When the molecules become so crowded together they may occupy much smaller domains, or they may interpenetrate. When adjacent layers of such a solution move relatively to each other, there could be expected to be a drag on the partially flexible hyaluronate chains by the flow of water past them, a drag of adjacent diffuse hyaluronate molecules against each other, deforming each other, and a drag of adjacent molecules that may have interpenetrated so that their chains have become to some extent entangled.

Hyaluronate is the only one of the connective tissue polysaccharides whose solutions have such a pronounced viscosity. The others have by comparison only negligible viscosities. The others also have, as polysaccharides, much lower molec-

ular weights and could not extend throughout so large a domain in solution. The high viscosity of hyaluronate solutions makes it easy to observe hydrolytic effects more subtle than can be detected by chemical means. For example the very earliest stages of hyaluronidase action may produce striking changes in viscosity when so few glycosidic bonds are broken that they are scarcely detectable by chemical measure of the increased number of reducing end groups present. This may account for the observations of Pigman (10) that hyaluronate solutions in the presence of cysteine, hydroquinone, or ferrous salts may show marked loss of viscosity.

POLYELECTROLYTE PROPERTIES

All the polysaccharides of the group under discussion have one property in common that is probably of importance in their functioning in the tissue and is certainly of importance in studying their behavior in solution and in separating them from other connective tissue components and from each other; they all carry a large number of negatively charged groups. There is at least one and sometimes more than two for each repeating disaccharide unit, so the charges are rather evenly distributed along the chains, but their distribution along chains may not always be strictly regular. These polysaccharides are therefore also polyanionic and are always associated with an equivalent number of cations, called counterions. In tissues the most common cations of extracellular spaces are sodium ions so that the polysaccharides occur in tissues almost entirely as sodium salts. However, other cations can readily be exchanged and the extent to which this occurs is determined mainly by their concentration in solution and their valence. In tissues potassium, calcium, and magnesium ions will also be associated with the polysaccharides though in amounts much smaller than sodium. These polyanionic polysaccharides behave as weak electrolytes; they behave as if a considerable fraction of the cations were not altogether free. Cations can all be freely exchanged by other cations in the solution, but they are not altogether freely mobile, a fraction behaves as if bound by the polyanion. This fraction in the case of chondroitin sulfate has been shown to amount to about 50 per cent of the counterions associated with chondroitin sulfate when the counterions are sodium or potassium, or 70 per cent of the counterions when these are calcium (11, 12). Counterions of higher valence are bound to an even higher degree. The trivalent cation containing cobalt associated with six neutral ammonia molecules, precipitates chondroitin sulfate and heparin directly from dilute aqueous solution, and the hexavalent cobalt cation of hexol chloride even precipitates hyaluronate directly from very dilute aqueous solution (13, 14). An important aspect of this binding of simple cations of higher valence is that even these more firmly bound polyvalent cations can be displaced from the polyanionic chain by univalent cations provided there are enough of the univalent cations in the neighborhood; that is if their concentration is sufficiently high. A practical result of this is that a precipitate of the trivalent hexamminecobaltic cation with chondroitin sulfate can easily be redissolved by addition of

sodium chloride to a concentration of 0.05 M. This binding or fixing of simple polyvalent cations is used in histochemical studies to locate in tissue sections the positions of polyanionic polysaccharides. In the Hale stain a trivalent ferric cation complex with manitol is used, and the locations where it has been fixed are then made visible by the addition of ferrocyanide producing a precipitate of Prussian blue at these sites. More recently a dye, Alcian blue, has come into use for the same purpose. This is a dye with a tetravalent cation that directly stains areas containing polyanionic polysaccharides. The finding of hyaluronate in the electric organ of the electric eel has raised a question whether the effect of curare on this organ might be related in some way to the observed binding of divalent curare cations to hyaluronate polyanions (15).

Another area in which the polyanionic properties of connective tissue polysaccharides are of use lies in their behavior with cationic detergents. The ionic detergents or soaps are generally molecules with a long hydrocarbon chain having at one end a fixed ionic group. The hydrocarbon chain of the molecule is hydrophobic or lipophilic, like paraffin; the ionic part is hydrophilic. Such molecules act as if they had a dual personality, they might be called schizophilic. In water such molecules tend to collect at surfaces with their hydrophobic parts being almost literally pushed out of the water. Deep in the water such molecules tend to associate with each other and form aggregates with their hydrophobic parts pressed together offering least total exposure to the water. Such aggregates are called micelles. Since one end of each molecule carries a fixed charge, the micelle as a whole carries a large number of fixed charges, perhaps 50 to 100. A cationic detergent, such as cetyl pyridinium chloride, in solution in water forms micelles that act as polyvalent cations with a high charge. As might be expected from what has been said of simple polyvalent cations, such cationic micelles associate with polyanionic polysaccharides and precipitate them from solution. As might also be expected, such precipitates can generally be redissolved by the addition of neutral salts such as sodium chloride. The salt concentration required just to prevent such precipitation, or to redissolve precipitates already formed, is different for different anionic polysaccharides. This set of properties forms the basis for a method of fractionating anionic polysaccharides which in practice can be carried out in different ways (16–18).

Still another area in which use is made of the polyanionic properties of connective tissue polysaccharides lies in the histochemical application of metachromatic staining. In this peculiar type of staining a violet to red color results in those parts of tissue sections that contain anionic polysaccharides when they are stained with a blue solution of toluidine blue. The spectral changes that occur in dilute solutions of toluidine blue or in solutions of other metachromatic dyes on addition of chondroitin sulfate or hyaluronate have been extensively studied (19, 20). The essentials needed to produce the metachromatic color are: a cationic dye that does not obey Beer's law, that is to say, a dye whose spectrum changes with concentration; a polyanion

in solution; and a low salt concentration, all in aqueous solution. There is now evidence that the metachromatic color is due to the formation in solution of a single compound composed of equivalent amounts of appropriate cationic dye and polyanion. Such compounds can be rather specifically removed from solution by high speed centrifugation, by adsorption to $CaHPO_4$, or by shaking their solutions with an immiscible organic solvent (21). In the last case the metachromatic compound collects at the interface and does not at all enter the organic solvent. Analysis of the products removed in all these cases showed them to contain dye and polyanion in equivalent amounts even when the polyanion present in the original solution was in a tenfold excess over the dye. The spectral changes that occur on the addition of a salt of a polyanion to a solution containing the dye cation have been interpreted as due to the formation of a single salt-like compound of dye and polyanion stabilized by the sharing of π-electrons between adjacent dye molecules fixed at regular intervals along the polyanionic chain. The sharing of π-electrons between adjacent dye molecules is presumably the cause of the color change of the dye associated with the polyanion. The stability of metachromatic compounds varies widely for different combinations of dye and polyanion, and depends not only on the π-electron bonds but also on the electrostatic bonds. A method has been described to measure relative stabilities of different metachromatic compounds in terms of the ethanol concentration at which the main band (α-band) of the free dye has returned half-way to its value when the dye is alone in ethanol (22).

Another kind of electrostatic interaction with connective tissue polysaccharides, that may be of physiological importance and that has also been found of use in laboratory studies, is that with proteins. This takes its simplest form under conditions such that the protein is dominantly cationic. An example is the mucin clot formed in synovial fluid on the addition of acetic acid. In the acid medium the serum albumin, which is the protein present in largest amount in normal synovial fluid, becomes cationic and precipitates with the anionic hyaluronate. Progressive digestion of hyaluronate by hyaluronidase can be followed by progressive loss of the turbidity produced by the addition of serum and acetic acid to samples taken from the reaction mixture. Interaction between proteins and anionic polysaccharides is, however, not confined to immediate precipitation. Fig. 1 shows some results of a study (23) of the interaction between chondroitin sulfate and lysozyme, a protein of molecular weight about 16,000 and having a net positive charge of about 8 at pH 7. As long as lysozyme is in excess and salt concentration is low (<0.06 M KCl) the amount of lysozyme removed from solution is exactly proportional to the amount of chondroitin sulfate added. At exact equivalence there is practically no lysozyme left in solution and up to this point the product, apparently a simple salt of lysozyme and chondroitin sulfate containing equivalent amounts of each, can be centrifuged off completely at low centrifugal speed. In the absence of salt a small excess of chondroitin sulfate causes abrupt and striking changes. The product can be only partly centrifuged off

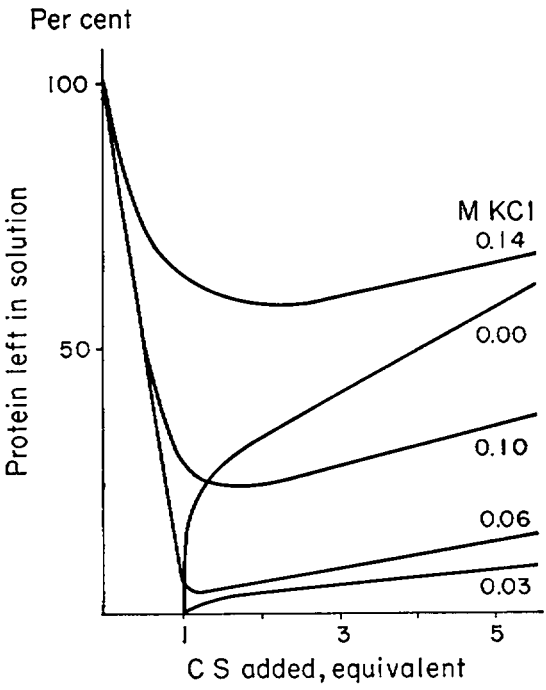


FIG. 1 The amount of lysozyme left in solution (ordinate) after addition of the amount (in equivalents) of chondroitin sulfate indicate on the abscissa, and centrifuging at 25,000 × *g*. Each curve shows the results at the concentration of KCl indicated.

at low speed leaving an opalescent solution. Centrifuged at higher speed, over 30,000 RPM, a clear supernatant solution results but contains 25 to 60 per cent of the initial lysozyme as the chondroitin sulfate equivalence is raised from 1.5 to 6.0. At a low salt concentration (0.03 to 0.06 M KCl) the situation again changes and even in the presence of excess chondroitin sulfate up to fivefold nearly but not quite all of the lysozyme can be removed. A further set of changes occurs at progressively higher salt concentrations up to the physiological range; progressively less lysozyme is removed from solution, and this occurs in either lysozyme or chondroitin sulfate excess. Analysis of the products removed from solution in the presence of 0.15 M KCl by this centrifugal procedure shows that in the presence of progressively greater excess of chondroitin sulfate the products contain progressively more chondroitin sulfate for each equivalent of lysozyme. In other words, not all the anionic groups of chondroitin sulfate are associated with an equivalent of lysozyme cation. Somewhat parallel findings have been reported for several other proteins, particularly serum globulins, but the results cannot be so clearly analyzed in terms of equivalents because the effective cationic charge per mol of a mixture of globulins cannot be so sharply calculated (24).

Considerable emphasis has been put on discussion of the polyelectrolyte properties of connective tissue polysaccharides because these are among their most outstanding characteristics. These properties are not only of value in the development of labora-

tory methods to fractionate and to study them, but also are probably fundamental to their functions in the extracellular spaces of the living organism.

COMPOUNDS OF PROTEIN AND POLYSACCHARIDE

Krukenberg (25) in 1884 was the first to isolate one of the polysaccharides of this group. By extraction of cartilage with 5 to 10 per cent sodium hydroxide for 2 to 3 days he isolated in a recognizable form what we now call chondroitin-4-sulfate. He actually called it chondroitic acid reviving an earlier name bestowed by Boedeker before 1861. Boedeker used this name for a component of cartilage which he had never isolated but which he recognized as being carbohydrate in nature. Krukenberg clearly stated that, since water alone extracted only traces of chondroitin sulfate, and the much more drastic treatment with sodium hydroxide was necessary for its separation, the chondroitin sulfate could not exist as such in the native cartilage. A few years later in 1891 Schmiedeberg (26) came to the conclusion that the chondroitin sulfate of cartilage existed in the native state as compounds with proteins. The products then known all contained 12 to 15 per cent nitrogen or 75 to 90 per cent protein, much of which was gelatin formed from the collagen of cartilage during the rather drastic extraction procedure. More recently it has been found possible to isolate from cartilage about 80 per cent of its total content of chondroitin sulfate by extraction with water or dilute solutions of neutral salts provided the tissue structure is mechanically disintegrated sufficiently. The product isolated by such procedures behaved as a compound of chondroitin sulfate and protein. For instance, attempts to fractionate it by graded precipitation of its salts with alcohol were unsuccessful, protein could not be selectively removed by the use of protein adsorbants, and electrophoretically it migrated as a single component, with a mobility that was constant over the range of pH from 5 to 10 and practically equal to the mobility of chondroitin sulfate. This product was called chondromucoprotein (27). In a later study (28) of its centrifugal properties it was found possible to separate it into two distinct fractions, one of which sedimented readily at 50,000 $\times$ g, and the second of which did not sediment readily at 100,000 $\times$ g. Both products consisted mainly of chondroitin sulfate and protein. From bovine nasal cartilage the less easily sedimentable fraction constituted about 80 per cent of the total chondromucoprotein isolated from cartilage, and it consisted of about 85 per cent chondroitin sulfate and 15 per cent protein. About this time there were being raised rather valid objections against the use of the words mucopolysaccharides and mucoproteins, so these products were referred to as proteinpolysaccharides (in analogy with lipoprotein and lipopolysaccharide). This emphasized the fact that these compounds contained carbohydrate in the form of a polysaccharide, as distinct from glycoproteins, in many of which the carbohydrate appears to exist as oligosaccharides. So the new products were called PP-H and PP-L, heavy and light referring to their ease of sedimentability. A more specific name than proteinpolysaccharide could be proteinchondroitin-4-

sulfate. Molecular weight estimation on products made by similar methods by Mathews and Lozaityte (29) gave values in the range 4×10^6 to 50×10^6. Sedimentation and zone electrophoresis study of PP-L showed that it behaved as a single entity. Similar studies could not be made with PP-H because of its easy sedimentability and its immobility in zone electrophoresis. Both products on treatment with sodium hydroxide (0.15 N, 16 hours, 25°) yielded chondroitin-4-sulfate in the amounts expected. This chondroitin sulfate persistently contained about 5 per cent protein. Further analysis in several laboratories showed that both PP-L and PP-H contained small amounts of glucosamine and galactose (30) as well as sialic acid (31). Among the alkaline hydrolysis products of both PP-L and PP-H small amounts of keratan sulfate have been found. It is with the keratan sulfate that the sialic acid seems to be associated. PP-L can be degraded to give chondroitin sulfate not only by alkali but by trypsin. The chondroitin sulfate produced by trypsin degradation is similar to that produced by alkaline degradation but differs in one important respect, its chain weight is higher. Chain weight is a valuable tool developed by Partridge, Davis, and Adair (32) to measure the number of reducing end groups per gram of material. Since each polysaccharide chain has only one reducing end group per chain, the larger the number of end groups per gram the shorter the chain length. Chondroitin sulfate made by alkaline digestion has a chain length about half that made by trypsin digestion. Furthermore that made by trypsin digestion is about the same as that found in PP-L itself. In other words, PP-L consists of protein and a number (about 15 to 20) of independent chains of chondroitin sulfate, each with its own reducing end group, for a molecular weight of 10^6. PP-L is a highly branched molecule.

Human cartilage, both articular and costal, differs in several respects from bovine nasal cartilage. It contains less total polysaccharide, it contains a larger fraction of PP-H and a smaller fraction of PP-L, and both PP-H and PP-L contain a larger fraction of keratan sulfate and a smaller fraction of chondroitin sulfate. A further difference is that while PP-L from bovine nasal cartilage behaves in free electrophoresis as a single entity, PP-L from human costal cartilage shows two distinct peaks.

These compounds of protein and chondroitin sulfate seem to be catabolized (33) and synthesized (34) as a unit.

Work on connective tissues other than cartilage, aimed at showing the existence of compounds of protein and the anionic polysaccharides, involves several additional complications. Most other connective tissues contain much smaller percentages of total polysaccharides, there are frequently at least three or four different polysaccharides present, and finally the tissues are more commonly interpenetrated by other more or less unrelated tissues and are richer in content of cells. Examples of such kinds of connective tissue are derma, tendons, heart valves, and arterial vessels. In aorta there is evidence for the presence of at least four polysaccharides; hyaluronate, chondroitin-6-sulfate, dermatan-4-sulfate, and heparitin sulfate. Extraction of hu-

man aorta with water during appropriate disintegration of the whole tissue, followed by high speed centrifugal fractionation, and purification of the not readily sedimentable material on a cellulose column by the use of a cetylpyridinium salt by the principles discussed earlier led to the isolation of a compound of protein and chondroitin-6-sulfate apparently of the same general composition and properties as the PP-L from bovine nasal cartilage (35).

There have been a few reports of mild experimental procedures for extraction of connective tissues, particularly of cartilage, in which the claim has been made that a polysaccharide free of protein could be isolated. In the cases of those methods which have been rechecked no free polysaccharide has been found. While it is not possible to say at present that all connective tissue polysaccharides occur in the native state bound to protein, there is no clear case in which a polysaccharide free of protein is known to occur. There is a thought, however, which suggests that there might be small amounts of polysaccharide relatively free of protein in connective tissue. Where measurements have been made it seems that the half-life of these polysaccharides in the tissues is of the order of a week. It is possible that in their normal turnover the first step could be breakdown of the native protein polysaccharide compounds releasing polysaccharides relatively free of protein as mentioned above in the action of trypsin on PP-L. Such small fragments could diffuse out of the tissue rapidly.

A case of special interest is hyaluronate. From vitreous humor it has been isolated in a form thought to be free of protein (36) yet the data presented indicate a protein content of 2 per cent. From synovial fluid the material isolable has been the subject of considerable controversy. The mucin clot produced by acidification of diluted synovial fluid with acetic acid is mainly a salt of cationic albumin and anionic hyaluronate. A major advance in the purification of synovial fluid hyaluronate was made when it was found (37) that hyaluronate molecules in solution could literally be filtered out of the solution by passage through fine fritted glass filters having an average pore diameter of about 600 mμ. A large fraction of the albumin and globulin content of synovial fluid easily passes through such filters while all the hyaluronate is retained. This is another consequence of the large domain occupied in solution by the hyaluronate molecule, discussed in connection with the viscosity of hyaluronate solutions. Washing of the hyaluronate retained by stirring with dilute salt solutions and continuing the filtration was repeated up to 20 and even 30 times during a total time of over a month. The product left in solution on the filter was considered to be a compound of hyaluronate and protein containing about 25 per cent protein. In more recent work (38) millipore filters with a pore diameter of 100 mμ have been used instead of the glass filters, and the retained hyaluronate dissolved in dilute salt solution passed through a column packed with a mixture of IRC-50 and hydroxylapatite. The procedure is far more rapid and yields a product with a much lower protein content, about 2 per cent. That this protein was bound to the hyaluronate

was shown in several ways. Perhaps the most convincing was to label it with iodine-131 and show that on zone electrophoresis at several pH values the uronic acid and I^{131} traveled together.

It seems to be true that hyaluronate and all the sulfated connective tissue polysaccharides in their native state in tissues are bound to protein. In two cases which have been studied in some detail, hyaluronate and chondroitin sulfate, there is evidence of a marked difference in the structure of these compounds of protein and polysaccharide. In the case of hyaluronate the polysaccharide itself appears to be a single long unbranched chain with a molecular weight about 10^6. The protein chain to which it is attached, about 2 per cent of the whole molecule, could not have a weight over 20,000. The properties of the polysaccharide dominate the properties of the proteinpolysaccharide, and nibbling at the protein with a protease has no observable effect on the domain occupied by the molecule as expressed in its viscosity properties. The relation between protein and polysaccharide in the native form of chondroitin sulfate is quite different. In the first place, there seem to be at least two forms of association of chondroitin sulfate with protein, those called PP-H and PP-L. Of these PP-H cannot yet be purified sufficiently to be able to characterize it as a single molecular entity. It is even difficult, because it can be so easily sedimented from its very opalescent solution, to make an intelligible statement about its solubility. In this respect PP-L is far more tractable, gives solutions clear enough so that optical rotation can be determined, sediments as a single entity, and no attempts at fractionation have yet given clearly separable components. PP-L is similar to synovial fluid hyaluronate in having a molecular weight about 10^6, but it contains 15 per cent protein. In this molecule the chondroitin sulfate is not a single continuous polysaccharide chain as is the case in the hyaluronate compound with protein. Reducing end group assay combined with molecular weight determination indicates that in a molecule of PP-L of molecular weight 10^6 there are about 17 chondroitin sulfate chains with an average weight of 50,000 each. Filtration experiments similar to those described for hyaluronate, as well as viscosity measurements, also indicate that PP-L occupies a large domain, though not so large as hyaluronate. This would be expected since at least 17 constraints have been imposed on the spreading of the PP-L molecule to a large domain in solution by the separate binding of each chondroitin sulfate chain. The PP-L molecule is a relatively highly branched molecule compared to hyaluronate. This difference is strikingly reflected in the contrast of the effects of trypsin. On hyaluronate with its single long polysaccharide chain and its 2 per cent protein, trypsin has no effect on the viscosity of the solution nor on the domain of the molecule. On PP-L with its multiple shorter polysaccharide chains and its 15 per cent protein, trypsin produces a sudden and large drop in viscosity, the domain occupied by the PP-L has collapsed, the separate chondroitin sulfate chains occupy rather small domains and become easily filterable through millipore filters of 100 $m\mu$ pore size.

The nature of the binding of the chondroitin sulfate chains to protein in PP-L has been a subject of speculation since before PP-L was known because it was generally assumed that in native cartilage the chondroitin sulfate must be attached to some protein. The ease with which chondroitin sulfate can be extracted from cartilage by the use of dilute alkali naturally led to the suggestion of an ester bond. Treatment of PP-L with hydroxylamine at pH 9-10 has yielded no product characterizable as chondroitin sulfate.

PROTEINPOLYSACCHARIDES AS DIFFUSE MOLECULES

The polysaccharides of connective tissues exist mainly in that space between cells and fibers called ground substance. In this area the known forms seem to exist in solution as compounds with protein. Though they are generally water-soluble there are also some forms, as PP-H, whose condition as regards solubility in water when isolated from the more soluble proteinpolysaccharides, is not clear. Of the more soluble forms, hyaluronate and PP-L have been discussed in particular. They have some striking properties that may account for much of their physiological function. Two of these properties considered in some detail are their properties as polyanions, and their existence in solution not as compact molecules but as highly diffuse molecules that extend throughout a volume of the solution, called their domain, that is large in comparison to the weight of the molecule, such as 1 to 10 liters per gm. Consider for example a comparison of the volumes of the space in solution occupied by equal weights of tropocollagen and hyaluronate. The hyaluronate molecule, MW 10^6, occupies a sphere of diameter 4000 A or a volume of $330{,}000 \times 10^{-19}$ ml. A molecule of soluble collagen, MW 345,000, occupies a rigid cylinder of diameter 14 A and length 2800 A, or a volume of 4.3×10^{-19} ml. Three such collagen molecules would weigh as much as a hyaluronate molecule, but the hyaluronate molecule occupies 25,000 times as much space as the three collagen molecules. This illustrates the difference between the diffuse hyaluronate and the compact collagen molecules. The domain occupied by a diffuse molecule in solution may be likened to the space occupied by a hedge, a piece of foam rubber, or a mass of sephadex gel in a column. Such a domain has different degrees of porosity to molecules of different sizes and it offers different degrees of resistance to the passage of other molecules through it, depending more on their size and shape than on the chemical identity of their component atoms. Several kinds of experiments have been reported in recent years on methods with which measurements can be made of these properties.

A study was made by Laurent and Pietruszkiewicz (39) of the effect of hyaluronate in solution on the rate of sedimentation in a centrifugal field of a series of particles varying in size from serum albumin, through virus particles, to polystyrene latex particles. The results showed that only a small effect of hyaluronate was noticeable in slowing up the sedimentation of serum albumin (40×150 A) but that the

retarding effects on the sedimentation of larger particles increased very rapidly as the sizes of the particles increased from 300 to 3000 A. Increasing hyaluronate concentration also decreased the sedimentation rates of all these particles but the decrease was always greater the larger the particle. These results were interpreted in terms of a random three dimensional network of hyaluronate chains extended throughout the solution acting as a brake to slow up the rate of fall of the particles, and slowing them up more markedly the larger the particles and therefore the greater the probability of their encountering chains across their paths. The effects noted here are reminiscent of the interpretation of the action of spreading factors noted years ago when testicular extract containing hyaluronidase was found to increase the rate of spread of large colloidal particles through rabbit skin.

There is another kind of effect of hyaluronate in solution on large molecules that is different from the one just described because it measures an effect on an equilibrium condition and not on the rate of a process. This is described by Ogston and Phelps (40) and appears to be a further outcome of the work of that laboratory on the use of filters of small pore size. In this work the millipore filter was used as a diffusion membrane, impermeable to hyaluronate but permeable to molecules of the size of serum globulins or serum albumins. With such a system equilibrium dialysis experiments were set up. Hyaluronate in buffer was dialyzed against inulin in buffer. When the system came to equilibrium there was markedly less inulin in the solution on the side of the membrane where there was hyaluronate than on the side where there was no hyaluronate. The same effect resulted if at the start of the experiment the inulin was put in the same solution as the hyaluronate. On equilibration less inulin stayed on the side of the membrane with hyaluronate than on the side with no hyaluronate. Similar results were found when a variety of other large molecules, all of which were small enough to be able to diffuse through the millipore filter, were dialyzed with hyaluronate on only one side of the membrane. These other molecules included serum albumin, carbon monoxide hemoglobin, dinitrophenyl derivatives of several proteins, and several forms of polyglucose. All these substances dialyzed against hyaluronate showed the same effect at equilibrium. Their concentration on the side of the membrane containing hyaluronate was less than their concentration on the side containing no hyaluronate. On the other hand, small molecules came to equilibrium with equal concentrations on the sides with and without hyaluronate. The effect of the hyaluronate appeared to be to exclude large solutes from a part of the solution it occupied. The fraction of the hyaluronate solution from which large solutes appeared to be excluded could be called the excluded volume with respect to a particular solute such as albumin. The apparent excluded volume became larger the higher the concentration of hyaluronate, and the larger the molecular size of the solute excluded.

In this case, as in the work on sedimentation, the concept of a random three dimensional network of hyaluronate chains is used as the basis to explain the re-

sults. Statistically these chains will often form regions of high chain densities into which large molecules cannot penetrate, and chain stiffness does not allow rapid adjustment of the chains to admit intruding large molecules. The volume actually occupied by the hyaluronate chains may include a hydration zone of fixed water of unknown size. This phenomenon suggests some interesting speculation concerning the plasma protein content of normal and arthritic synovial fluid. Normal plasma contains about 7 per cent protein, normal synovial fluid about 2 per cent protein. This is, at least as regards order of magnitude, what one might expect in a millipore dialysis system with a concentration of hyaluronate of 0.3 to 0.4 per cent on one side and a concentration of total plasma protein at equilibrium of 7 per cent on the other side. In arthritic fluid where the hyaluronate concentration is about half that in normal fluid, the amount of plasma protein could be expected approximately to double, and in arthritics the protein content of the synovial fluid averages about 4 per cent.

Still another kind of effect of hyaluronate in solution has been studied. This came from a consideration of how these molecules, which occupy such an extensive domain, might interact with insoluble fibrils. This is an attempt to approach the real conditions existing in native connective tissues where a fibrillar network of collagen fibrils traverses areas of ground substance. A study of this type was made by Fessler (41) using a rather simple procedure with interesting results. A solution of collagen containing 2.5 mg per ml was made by extraction of tissues with cold sodium chloride solution. On being heated to 37° it yielded a stiff gel of precipitated collagen fibrils. When this gel was centrifuged it produced a small pellet of sedimented collagen fibrils with water trapped between them. The net weight of such a pellet amounted to 10 mg per mg of collagen taken in the initial solution of collagen. The effect of dissolved hyaluronate on this system was then studied. Before precipitation of the collagen fibrils, hyaluronate to the extent of 0.06 per cent was added to the cold collagen solution, and the combined solution of collagen and hyaluronate was heated to 37° as before. This again resulted in precipitation of the collagen fibrils in the form of a gel. On centrifugation of this gel a pellet was again produced but this time weighed five times as much as the control pellet formed in the absence of hyaluronate. Of course, this increase in the pellet weight is due to the increased amount of water trapped among the fibrils. A small extra amount of hyaluronate was also trapped among the pellet fibrils as was shown by the decrease in concentration of the hyaluronate of the supernatant solution. The pellets formed in the absence and in the presence of hyaluronate contain the same amount of collagen and the weight of hyaluronate trapped in the experimental pellet is completely negligible. Why then does the pellet formed from the very diulte hyaluronate solution weigh so much more than the control pellet? Both were formed in a centrifugal field of the same magnitude. The fibrils formed in the hyaluronate solution resisted compression more than those formed in the absence of hyaluronate. It is as if the fibrils sedimented in the presence of hyaluronate had springs interposed between them opposing the force

of the centrifugal field tending to compress them. This thought agreed with the observation that the hyaluronate pellet differed markedly from the control pellet in having a firm elastic consistency and that this elastic property was lost when the pellet was incubated with hyaluronidase. Control pellets did not have this elastic property and were unaffected by hyaluronidase.

The picture drawn from these experiments is that collagen fibrils formed in hyaluronate solution penetrate through the domains of hyaluronate molecules, that hyaluronate molecules not only become entangled with each other but with insoluble collagen fibrils. The dimensions of collagen fibrils (about 500 A in diameter) and of hyaluronate domains (4000 A in diameter) allow this possibility. Resistance of collagen fibrils in hyaluronate solution to compression in a centrifugal field is then a reflection of the resistance of the hyaluronate molecule to compression of its domain. This is the effect of the repeating anionic charge along the chain, the stiffness of the chain, its great length, and its tendency to extend throughout a large domain of solution.

While the experiments on hindered sedimentation, excluded volume, and entanglement with fibrils have all dealt with hyaluronate, they must be applied to other connective tissue polysaccharides in the form as they exist in tissues, as compounds with protein. The PP-L of cartilage also appears to be a diffuse molecule, though not so diffuse as hyaluronate, because it is multiply branched. It gives solutions of considerable viscosity, though not so viscous as hyaluronate. A major difference from hyaluronate is that the viscosity of solutions of PP-L is destroyed in a few minutes by treatment with trypsin.

There are several areas in which the concept of the entanglement of diffuse soluble molecules with each other or with insoluble fibrils may help in handling connective tissues in the laboratory or in explaining their properties in the animal. The difficulty usually found in extracting the native compounds of protein and polysaccharide from connective tissue is particularly obvious in cartilage because there is so much in this tissue. If this were due to entanglement of PP-L with the fine fibrils of cartilage, one could understand why destruction of the fibrillar network yields PP-L in solution in large amounts. There is even some difficulty in separating PP-L from PP-H since several high speed centrifugings seem needed to effect good separation, though once separated PP-H sediments much more easily even at lower centrifugal speeds.

Two further properties of some connective tissues that may be related to entanglement of diffuse water-soluble molecules with fine insoluble fibrils are the binding of water in tissues and the elasticity of cartilage. The basis on which these properties are dependent is most clearly suggested in the experiments of Fessler described above in which collagen fibrils freshly precipitated in hyaluronate solution and then sedimented yielded elastic pellets with high water content.

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