The Interaction of Collagen and Acid Mucopolysaccharides

A MODEL FOR CONNECTIVE TISSUE

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(Received 30 November 1964)

1. The interaction of acid mucopolysaccharides of connective tissue with solubilized collagen of native, or near-native, structure was investigated by free solution electrophoresis at pH 7.0. 2. Complex-formation was detected by the appearance of a third peak in the ascending limb only, indicating reversible association. 3. Complex-formation was destroyed by prior heating of solubilized collagen, indicating a probable requirement for high molecular weight or internal structure of the protein. 4. Hyaluronate and chondroitin sulphate of mol.wt. 50000 gave complexes with soluble collagen at I 0.4, whereas heparin and chondroitin sulphate of mol.wt. 15000–18000 did not. All mucopolysaccharides yielded complexes at I 0.1. The stability of the complex appears mainly dependent on electrostatic forces and is increased with increase in chain length of the polysaccharide. 5. Solubilized collagen interacted to yield gels with the 'native' chondroitin sulphate-protein macromolecule from cartilage. 6. A schematic model for the interaction of collagen and chondroitin sulphate-protein macromolecules shows parallel-ordered interaction of collagen fibrils with chondroitin sulphate side chains of the chondroitin sulphate-protein macromolecule. The biological implications of this model are discussed, particularly in relation to the ordered structures and the ionic-network properties of the intercellular components of connective tissue.

The nature and physiological significance of the interaction between the proteins and the acid mucopolysaccharides of animal connective tissues has been a subject of major interest. As indicated in a review by Bettelheim-Jevons (1958), it appeared likely to most early workers that, in the native state, acid mucopolysaccharides were bound to protein by polar linkages. This conclusion, however, was contradicted by Einbinder & Schubert (1951), who detected no binding of chondroitin sulphate to tendon collagen above pH 7.

More recently it has been asserted by some (Jackson, 1954; Kuhnke, 1962; Elden, 1964) and denied by others (Banga & Baló, 1960; Partington & Wood, 1963) that chondroitin sulphate has a stabilizing role in tendon. However, there is agreement that acid mucopolysaccharide interactions with collagen and other proteins occur in cornea (Loeven, 1956; Hedbys, 1961; Offret, Payrau, Pouliquen, Faure & Hameau, 1962), in vitreous humour (Woodin & Boruchoff, 1955) and in synovial fluid (Pigman, Gramling & Holley, 1960). The probable significance of altered interactions in pathology has been extensively discussed by Hartmann (1959), Schwarz (1960) and Sirsat & Khanolkar (1962).

Acid mucopolysaccharides are reported to influence the precipitation of collagen from solution. Chondroitin sulphate may be incorporated into fibrillar collagen to influence fibrillar diameter, and electron-microscope contrast (Kühn, Grassman & Hofman, 1959; Németh-Csóka, 1960), as well as stability to heat (Delaunay & Bazin, 1957). Wood (1960) found that chondroitin sulphates A and C increased the rate of nucleation but retarded the rate of growth of fibrillar collagen; heparin delayed the formation of fibrils, and chondroitin sulphate B and hyaluronate had no effect. In a parallel study with the same materials in electron microscopy, Keech (1961) noted that the fibres obtained from collagen solutions containing chondroitin sulphate resembled those in unfixed normal adult human dermis and concluded that this acid mucopolysaccharide aids normal fibril formation. The above phenomena were interpreted on the basis of electrostatic interactions between anionic charged groups of the acid mucopolysaccharides and cationic groups of collagen.

Considerable information has also been obtained on the nature of the interaction of acid mucopolysaccharides and their protein complexes with serum proteins (Gorter & Nanninga, 1952, 1953;

Badin & Schubert, 1955; Bernfeld, Donahue & Berkowitz, 1957; Bernfeld, Nisselbaum, Berkeley & Hanson, 1960; Anderson, 1963; Gramling, Niedermeier, Holley & Pigman, 1963; Anderson & Slack, 1964), lysozyme (Schubert & Franklin, 1961) and gelatin (Bungenberg de Jong, 1949; Courts, 1963). Collagen had not been used, mainly because of low solubility and stability. Partridge (1948) interpreted his results of electrophoresis of crude extracts of cartilage as indicating a reversible interaction between chondroitin sulphate and a degradation product of collagen. However, the components of the extract were inadequately characterized.

To investigate more directly the main biological problem at a molecular level, information on the interactions between pure acid mucopolysaccharides and a relatively stable and soluble form of collagen was obtained by electrophoresis. These findings and a theoretical model for interactions in connective tissue are given below.

MATERIALS AND METHODS

The preparations of chondroitin sulphate and protein complexes from bovine cartilage have been previously described: CS-18 and CS-50 had mol.wt. 18000 and 50000 respectively (Mathews, 1956a); a typical preparation of a chondroitin sulphate-protein complex contained 25% of protein and had mol.wt. approx. 30×10^6 (Mathews & Losaityte, 1958). Heparin, a gift from Abbott Laboratories (Chicago, Ill., U.S.A.), was assayed at 134 i.u./mg., and had intrinsic viscosity 0.23, corresponding to mol.wt. 15000, according to Laurent (1961). Sodium hyaluronate from group A *Streptococcus pyogenes* was a gift from Dr Harvey Alburn. This preparation, after purification by zone electrophoresis, possessed intrinsic viscosity 1.75, corresponding to mol.wt. 40000 (Laurent, 1957). Bovine serum albumin (bovine fraction 5) was obtained from Armour Laboratories (Chicago, Ill., U.S.A.).

Solubilized collagen was a gift from Dr Arthur Veis. This preparation, obtained by extraction of purified bovine hide collagen at pH 5.75 and 60° for 1 hr., is closely related to native collagen and can be reconstituted to fibres with the native collagen structure (Veis, Anesey & Cohen, 1961). It consists of highly asymmetric molecules in the mol.wt. range 1×10^6 – 20×10^6 , with an average of nine polypeptide chains in cross-section (Veis, Eggenberger & Cohen, 1955). The isoelectric point (salt-free) is about 9.0. In 0.1M-NaCl solubilized collagen is isoelectric at pH 5.75, binding about 0.2 equiv. of NaCl/1000g. of protein. This collagen was denatured by heating in buffer, pH 7, at 100° for 30 min. Less than 10% of amide N, detectable as NH_3 , was released. The average mol. wt. falls to about 50000 (A. Veis, personal communication).

Neutral-soluble collagen was obtained by low-temperature extraction of skin of foetal calves and of 2-month-old rabbits with 0.15M-phosphate buffer, pH 7.0, according to the method of Gross, Highberger & Schmitt (1955). Solutions of these preparations, which contained about 11% of hydroxyproline in the protein, were not exposed to temperatures exceeding 25°.

Buffers comprised 0.01M-phosphate and NaCl to give the required ionic strength, adjusted to pH 7.0, or 0.02M-veronal and NaCl, adjusted to pH 8.6. Solutions containing 0.67% of polysaccharides were prepared by shaking at room temperature and clarified by centrifugation. The solubilized collagen was dissolved at 2.0% (w/v) concentration at 40° with shaking for 5–10 min. Solutions were diluted with buffer or mixed in proper proportion to yield 0.5% concentrations of each solute, dialysed overnight at 25° against 200 vol. of buffer and clarified at 10000g for 30 min. Solutions of neutral-soluble collagen for electrophoresis contained about 0.25% of protein to which was added dry chondroitin sulphate to give a concentration of 0.5%.

Electrophoresis was performed in a Perkin-Elmer model 38 Tiselius electrophoresis apparatus at 22° to avoid gelation at lower temperatures in solutions of solubilized collagen. The gradient in all experiments was approx. 2.0 v/cm.

RESULTS

Complex-formation. Electrophoretic results are presented in Table 1. Interaction between the two macromolecular components is indicated (Expts. 1–3) by the appearance of a peak due to complex in the ascending electrophoretic pattern that is absent from the descending pattern (shown in Fig. 1). Also, the ascending mobility of free protein appears slightly increased, whereas the mobility of polysaccharide is generally not affected. When complex-formation was not observed, the patterns obtained were those expected for two independently migrating components (e.g. bovine serum albumin and CS-50 at pH 7.0 and I 0.1). The failure of denatured solubilized collagen to interact with CS-50 suggests a requirement for high molecular weight and possibly internal structure in the protein. However, discharge at pH 8.6 of about 10% of the cationic groups present at pH 7 in solubilized collagen (Veis *et al.* 1955) does not prevent interaction with CS-50 (Expts. 5–7).

Effect of molecular chain length. Complex-formation occurs with hyaluronate with one-half the charge density of CS-50 and with about the same degree of polymerization, $P_n = 100$ disaccharide repeating units (Expts. 8 and 9). However, neither CS-18 nor heparin, of equal and greater charge density respectively compared with CS-50, but with only one-third the degree of polymerization of CS-50, shows interaction (Expts. 10–12).

Neutral-soluble collagen. Expts. 13, 13a and 14 indicate that native undegraded collagen extracted from rabbit skin and from ox skin forms complexes similar to those of solubilized collagen. The patterns obtained were similar to those in experiments in which the concentration of solubilized collagen only was reduced to 0.1–0.2%.

Table 1. *Electrophoresis experiments with collagen-polysaccharide mixtures*

Mobilities at 0° towards the anode are given. Mobilities of complexes are underlined. Values in parentheses are uncertain because of the presence of turbidity.

Expt. no.	Collagen	Polysaccharide	Buffer		10 ⁵ × Mobility (cm. ² sec. ⁻¹ v ⁻¹)	
			I	pH	Ascending	Descending
1	Soluble	—	0.4	7.0	0.8	0.8
2	—	CS-50	0.4	7.0	10.3	9.1
3	Soluble	CS-50	0.4	7.0	1.3, 2.7, 10.3	0.7, 9.0
4	Soluble, denatured	CS-50	0.4	7.0	1.8, 10.2	1.2, 9.3
5	—	CS-50	0.4	8.6	9.8	9.0
6	Soluble	—	0.4	8.6	1.0	1.0
7	Soluble	CS-50	0.4	8.6	1.3, 2.2, 9.8	1.0, 8.4
8	—	Hyaluronate	0.4	7.0	6.4	5.5
9	Soluble	Hyaluronate	0.4	7.0	1.1, 2.8, 6.6	0.8, 5.4
10	—	CS-18	0.4	7.0	10.1	9.1
11	Soluble	CS-18	0.4	7.0	1.1, 10.1	0.6, 9.1
12	Soluble	Heparin	0.4	7.0	0.9, 10.2	0.9, 9.1
13	Neutral (rabbit)	CS-50	0.4	7.0	0.5, 2.7, 9.6	0.4, 8.7
13a	Neutral (rabbit)	—	0.4	7.0	0.4	0.4
14	Neutral (ox)	CS-50	0.4	7.0	0.7, 2.9, 9.7	0.7, 8.9
15	Soluble	—	0.1	7.0	0.2	0.2
16	Soluble	CS-18	0.1	7.0	1.0, 3.8, 16.2	0, 11.5
17	Soluble	CS-18	0.1	8.6	1.0, 3.0, 16.2	0.2, 11.7
18	—	CS-50	0.1	7.0	16.5	12.1
19	Soluble	CS-50	0.1	7.0	(0), 7.5, 15.8	(0), 10.4
20	Soluble	Heparin	0.1	7.0	(5.4), 15.9	—

Fig. 1. Electrophoretic patterns of Expt. 3: solubilized collagen and CS-50 in buffer, pH 7.0 and *I* 0.4, after 200 min.

Fig. 2. Boundary conditions at start of electrophoresis of solubilized collagen (Col.) and CS-50 with a solution of CS-50 replacing buffer in one section of the cell. Boundaries were at *A*, *B* and *C*.

Effect of ionic strength. At *I* 0.1 both CS-18 and heparin now show complex-formation, as does CS-50 (Expts. 15–20). However, mobility differences between the complexes are apparent. Also, turbidity appears during electrophoresis, particularly on the descending side of experiments with CS-50 and heparin, making identification of some boundaries difficult (Expts. 19 and 20).

Chondroitin sulphate-protein. It appeared particularly desirable to use chondroitin sulphate in its 'native' form, covalently bound to protein. However, mixtures of solubilized collagen and preparations of chondroitin sulphate-protein com-

plex could not be used in electrophoresis owing to gel formation.

Reversibility of complex-formation. The appearance of a peak due to complex on the ascending side, but not on the descending side, suggested that the interaction was reversible. Thus the absence of a peak due to complex on the descending side could be due to the comparatively rapid electrophoretic separation of the two components. This separation might be avoided by replacing buffer overlaying the descending limb with a solution of acid mucopolysaccharide, as illustrated in Fig. 2. The resulting patterns shown in Fig. 3 are the predicted ones.

Fig. 3. Electrophoretic patterns of solubilized collagen and CS-50 with a solution of CS-50 replacing buffer in one section of the cell in buffer, pH 7.0 and I 0.4, after 185 min. On the ascending side (starting boundary at position *C* of Fig. 2), the CS-50 boundary has migrated out of view to the left. On the descending side, the CS-50 boundary (initially at position *A* of Fig. 2) is shown migrating behind the boundaries for collagen and the complex (initially at position *B* of Fig. 2). Boundaries indicating complex-formation are now found in both limbs of the cell.

DISCUSSION

Nature of the interaction. The appearance of a different number of boundaries in the two limbs of the electrophoresis cell appears to be a common phenomenon and has been reported also for mixtures of ovomucoid with nucleic acid (Longworth & MacInnes, 1942), for serum albumin with heparin (Chargaff, Ziff & Moore, 1941), with chondroitin sulphate (Gorter & Nanninga, 1952) or with sulphated dextran (Thompson & McKernan, 1961), and for lipoprotein with heparin (Bernfeld *et al.* 1957). Although a general theory for interpretation of moving-boundary patterns in interacting systems that re-equilibrate is not available, a limited theory for such a model system has been presented for certain special cases by Gilbert & Jenkins (1959). This theory was derived for a 1:1 complex of each interactant and in several cases directly predicts three moving boundaries for the ascending limb and two moving boundaries for the descending limb. However, as a consequence of equilibration, the complex cannot be identified with any boundary, i.e. no boundary has a mobility corresponding to the mobility that would be shown by the complex in the absence of re-equilibration. Thus it is likely that the ascending mobilities listed in Table 1 are too low for the complex and too high for the protein, whereas descending mobilities of the polyanion may be lowered in the presence of protein.

Although entropic factors may have a significant influence, the effect of ionic strength suggests that electrostatic interactions between anionic charges on the polysaccharide and cationic charges on the protein are probably paramount in the formation and stabilization of the complex. Thus stabilization

of the complex will depend largely on the kind of charged groups, the number and distributions of interacting groups, the sizes and conformation of the macromolecules, and effects of micro-ions in competitive binding and on electrostatic shielding. The failure of CS-18 and heparin, although of higher linear charge density than hyaluronate, to form a complex at I 0.4 indicates that chain length, as well as numbers of interacting charged groups/molecule, is important.

The cationic charged groups of collagen are longitudinally distributed in discrete clusters, as indicated by fibril staining with phosphotungstic acid (Nemetschek, Grassman & Hofman, 1955; Hodge & Schmitt, 1960). Many more of these groups are available for interaction to a molecule like hyaluronate capable of extending up to 1000 Å compared with molecules whose maximum extension does not exceed 400 Å. Also, a high linear density of cationic groups, resulting in high local electrostatic potential, occurs in regions of the collagen fibrils with a repeat period of about 700 Å. Though short polyanions can interact with only one such region, polyanions of sufficient length may interact with two cationic clusters with a resultant gain in electrostatic interaction energy/molecule. On reduction of the ionic strength to I 0.1, the increased electrostatic energy of interaction for each charged pair of groups and the decreased competition by Cl^- for binding sites help to stabilize the complex with CS-18 and with heparin. The systems with CS-50 and with heparin (Expts. 19 and 20), however, are unstable and tend to aggregate.

The observed electrophoretic patterns may be due to mutual frictional interactions between migrating components rather than to formation of re-equilibrating complexes. Such an interpretation was suggested by Davies, Nichol & Ogston (1963) for the electrophoretic patterns at pH 7.4 of mixtures of hyaluronic acid and serum albumin obtained by Gramling *et al.* (1963).

Although the electrophoretic results at I 0.4 are consistent with either explanation, the observed effects of ionic strength support an electrostatic interpretation of complex-formation. Electrostatic interaction at pH values well below the isoelectric pH 9.0 would be expected from the known high affinity of solubilized collagen for anions. Thus Wood (1960) found that soluble collagen precipitated from very dilute solutions of acid mucopolysaccharides at pH 7.1 and I 0.23 could still incorporate up to 0.4% by weight of chondroitin sulphate and up to 1.5% of heparin. Also, in Expts. 19 and 20 (Table 1), the solubilized collagen precipitates contained acid mucopolysaccharide that could not be removed by repeated washing. These partly fibrillar precipitates stained metachromatically

with toluidine blue. In polarized light the fibres showed pleochromism, appearing metachromatic when the plane of vibration of the light was perpendicular to the fibre axis and pale blue when the plane of vibration was parallel to the fibre axis. A similar pleochromism was reported by Vidal (1963) for tendon fibres stained with toluidine blue.

It appears that electrostatic factors are of major importance. However, the extent of the contributions of molecular entanglement and of excluded volume effects on the interaction between collagen and acid mucopolysaccharides in various experimental conditions remains to be determined.

Molecular organization of tissue. The above electrophoretic experiments with components that are only moderately altered from their native states are probably relevant to the structure of living tissue. This conclusion is supported by the strikingly similar patterns obtained by Partridge (1948) in electrophoresis of extracts prepared by heating cartilage for 30 min. at 60° in buffer at pH 7. A peak due to complex was likewise observed only in the ascending limb and complex-formation in the extracts was destroyed by heating at 100° for 30 min.

Many workers have suggested in a general fashion that the properties of living connective tissue depend in part on the interaction of collagen and acid mucopolysaccharides. Elucidation of the main chemical and structural features of native chondroitin sulphate-protein macromolecules in connective tissues of mammals (Mathews & Losaityte, 1958), of Chondrichthyes (Mathews, 1962) and of members of other vertebrate classes (Mathews, 1964) now makes possible a more detailed molecular interpretation of experimental findings.

The proposed model in Fig. 4 illustrates the initial stages of one type of organization of interactants that may occur in connective tissues. The basic unit of the chondroitin sulphate-protein molecule of adult bovine cartilage consists of a protein core approx. 4000 Å long to which are covalently bound about 60 chondroitin sulphate chains of mol. wt. 50 000 and contour length approx. 1000 Å. The observed influence of molecular weight on complex-formation suggests that the chondroitin sulphate moiety is aligned in parallel with the fibril. This orientation is also indicated by polarized-light studies of the pleochromism of connective tissues (Romhányi, 1958; Vidal, 1963) and of collagen precipitates containing hyaluronate, heparin and chondroitin sulphate (Németh-Csóka, 1960, 1961) after staining with basic dyes.

In dilute solution, the requirement for parallel alignment of the chondroitin sulphate moieties would lead to maximum interaction under conditions where the protein core of the chondroitin

Fig. 4. Schematic representation of interaction between collagen and basic unit of chondroitin sulphate-protein macromolecules of bovine cartilage. For purposes of clarity, only one-fifth of the chondroitin sulphate (CS) side chains of the chondroitin sulphate-protein molecule are shown. Collagen molecules are organized by 'quarter-stagger' arrangement (Hodge & Schmitt, 1960) in the fibril.

sulphate-protein molecule is also aligned parallel to the collagen fibril. Further growth of the complex may occur by accretion of additional fibrils, collagen molecules, or chondroitin sulphate-protein molecules combining by a similar mechanism. Also, collagen molecules may add to the fibrillar region of the complex by the processes responsible for growth of fibrillar collagen in the absence of acid mucopolysaccharide. Finally, chondroitin sulphate-protein molecules themselves may form self-aggregates by lateral association with the probable participation of additional non-collagenous protein (Mathews & Losaityte, 1958).

The character of the interaction will depend on structural details in the chondroitin sulphate-protein molecule that change with type of tissue, stage of development and vertebrate species. Thus variations, with retention of the central structural feature of the molecule, may occur in the length of the protein moiety, the number and size of the carbohydrate chains, the linear density and specific sites of sulphation etc. (Mathews, 1964).

An extreme condition may occur within tissues

when the mucopolysaccharide exists as carbohydrate chains to which little polypeptide is bound, owing to a defect in biosynthesis or to local proteolytic action. The consequence is a relatively low degree of interaction between collagen and chondroitin sulphate with increased likelihood of loss by diffusion of the mucopolysaccharide from the tissue (see especially Thomas, 1964; Dorfman, 1964). The ready reversibility of interaction between chondroitin sulphate and collagen may also explain the failure of Einbinder & Schubert (1951) to detect absorption of chondroitin sulphate from dilute solution by solid collagen at pH 7 and above.

Hyaluronate occurs naturally linked to little or no polypeptide. As a single-chain acid mucopolysaccharide its capacity for interaction with proteins is less than that of chondroitin sulphate-protein. It therefore is not surprising to find hyaluronate as the sole, or major, acid mucopolysaccharide in highly hydrated and semi-fluid or gel-like connective tissues such as vitreous humour, synovial fluid, Wharton's jelly and sex skin of baboons. Many physiological functions for hyaluronate have been suggested (Rogers, 1961) that depend on the swelling properties and resistance to compression of hyaluronate-protein networks. Woodin & Boruchoff (1955) suggested that the network properties depend on weak electrostatic interactions between hyaluronate and collagen. The model experiments of Fessler (1960) indicate that a similar conclusion may apply to Wharton's jelly.

The collagen fibrils in amphibian skin are formed into a microstructural fabric of high geometric regularity. Weiss (1956) speculated that the electron-optically structureless ground substance in which the fibres are imbedded could play a major part in the ordering process. An ordering role for pure acid polysaccharides was suggested by Sylvén & Ambrose (1955) and by Thiele & Langmaach (1957).

Partridge (1948) suggested that chondroitin sulphate might control the organization of collagen fibrils into parallel-ordered bundles in cartilage. However, all, or nearly all, of the chondroitin sulphate in adult cartilage is contained in chondroitin sulphate-protein molecules, which are well adapted to the formation of ordered structures. Thus the long-range order of large collagen fibrils may rest on the highly ordered interacting molecules of 'structureless' ground substance, as described in Fig. 4.

This work, in part reported in an abstract (Mathews, 1956b), was supported by grants from the Chicago Heart Association, the American Heart Association, the National Foundation and the U.S. Public Health Service. I am grateful to Dr A. G. Ogston and Dr T. C. Laurent for helpful

discussions and to Dr H. J. Rogers for his criticism of the manuscript.

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