The Effect of Acid Mucopolysaccharides and Acid Mucopolysaccharide-Proteins on Fibril Formation from Collagen Solutions

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1. The effects of acid mucopolysaccharides and acid mucopolysaccharide-proteins on the size and rate of formation of fibril aggregates from collagen solutions in pH 7.6 buffers were studied by turbidimetric and light-scattering methods. 2. Serum albumin, orosomucoid, methylated cellulose, chondroitin sulphate A and chondroitin sulphate C of molecular weight less than 20 000, and hyaluronate of molecular weight less than 40 000 did not influence rates of fibril formation. Chondroitin sulphate A, chondroitin sulphate C and hyaluronate of high molecular weight retarded the rate of fibril formation. This effect of high-molecular-weight chondroitin sulphate C decreased with increasing ionic strength. Heparin, though of low molecular weight (13 000), was highly effective, as was also heparitin sulphate. The chondroitin sulphate-proteins of very high molecular weight were highly effective, despite the fact that for some preparations the component chondroitin sulphate chains had molecular weights much less than 20 000. 3. Agents that had delayed fibril formation were also effective in producing an increase in degree of aggregation of fibrillar collagen, as indicated by dissymmetry changes observed in light-scattering experiments at low collagen concentrations. Methylated cellulose and heparin at 2.5 $\mu\text{g./ml.}$ were unusual in decreasing aggregation, but heparin at 0.25 $\mu\text{g./ml.}$ increased aggregation. Electron microscopy of gels showed fibrils and fibril aggregates with 'normal' collagen spacing and dimensions consistent with the light-scattering results. 4. The rates of electrical transport of agents and of solvent (electro-osmosis) through collagen gels indicated a contribution of molecular entanglement that increased with increase in molecular size of the agents. Electrostatic binding of heparin to collagen was noted. Binding to collagen during fibril formation was also found for heparitin sulphate and a chondroitin sulphate with extra sulphate groups. 5. Electrostatic binding of acid mucopolysaccharide-proteins to collagen may be an important factor in the organization and functioning of connective tissues at all stages of growth and development. Excluded-volume (molecular-entanglement) effects may also be important. These factors operate simultaneously and interact mutually so that precise assessment of their relative importance is difficult.

A previous study (Mathews, 1965) by electrophoretic methods indicated that collagen in solution interacted reversibly with acid mucopolysaccharides. The stability of the complex formed depended on electrostatic forces and the length of the polysaccharide molecule. However, the extent to which molecular-entanglement and excluded-volume effects contributed to the observations could not be readily assessed.

Gross & Kirk (1958) reported that amino acids, ascorbic acid, urea and other small molecules were effective inhibitors of the rate of fibril formation

from collagen solutions; hyaluronate and CS* were ineffective. However, Wood (1960b) found that CS-A and CS-C increased the rate of nucleation but retarded the rate of growth of collagen fibrils; heparin delayed the formation of fibrils, and CS-B and hyaluronate were ineffective. The molecular sizes of the mucopolysaccharides used were not cited by these authors, but might have been factors contributing to the discordance between the reported results.

* Abbreviations: CS, chondroitin sulphate; CS-A, CS-B and CS-C, chondroitin sulphate A, B and C respectively.

The present study used similar systems that form fibrils to investigate further the interactions of 'native' collagen with highly purified and physically characterized mucopolysaccharides and 'native' mucopolysaccharide-proteins. A preliminary account of this work has been presented (Mathews & Decker, 1967).

MATERIALS AND METHODS

Preparations. Acid mucopolysaccharides and acid mucopolysaccharide-proteins were isolated from a variety of animal connective tissues, extensively purified, and chemically and physically characterized by methods described previously (Mathews & Lozaityte, 1958; Mathews & Inouye, 1961; Mathews, 1958, 1962, 1966a, 1967). Weight-average molecular weights of 30000 and higher were measured by light-scattering methods, and lower molecular weights were calculated from viscosity data and from established relationships between intrinsic viscosity and weight-average molecular weight (Mathews, 1967). Heparin (molar ratio, sulphate: hexosamine 2.46:1.00) and heparitin sulphate (molar proportions, *N*-acetyl: sulphate: hexosamine 0.50:1.09:1.00) were highly purified preparations given by Dr J. A. Cifonelli. A mucoprotein from human urine (Tamm, Bugher & Horsfall, 1955) and a preparation of orosomucoid (Weimer & Winzler, 1955) were given by Dr N. Kefalides. A sialoprotein from cortical bone (Andrews, Herring & Kent, 1967) was a gift from Dr G. M. Herring. Dr A. Veis gave a sample of a partially characterized sialoprotein from bovine tooth dentine, containing phosphoserine residues (Zamosciany & Veis, 1966). Methocel (average degree of polymerization, 220 glucosyl residues) and crystalline bovine serum albumin were given respectively by Dow Chemical Co., Midland, Mich., U.S.A., and by Armour and Co., Chicago, Ill., U.S.A.

Purified soluble tropocollagen was prepared from calf skin, obtained through the courtesy of Dr C. De Fiebre of Wilson Laboratories, by the procedure of Rubin *et al.* (1965); the preparation, corresponding to their fraction 2A, was stored frozen. Analysis on a Technicon AutoAnalyzer showed 329 residues of glycine, 127 residues of proline, 91 residues of hydroxyproline, 8 residues of hydroxylysine, 3 residues of tyrosine and less than 2 residues of hexosamine/1000 residues. The intrinsic viscosity in 0.15M-sodium citrate buffer, pH 3.7, at 20° was 15.8 dl./g. This preparation was thus very similar to the preparations obtained by Rubin *et al.* (1965).

Collagen solutions of 1.5–2 mg./ml. in cold 0.15 M-citrate buffer, pH 3.7, were freshly prepared every week and dialysed against several changes of sodium phosphate buffer, pH 7.6 and *I* 0.13, for 48 hr. in the cold. Solutions were centrifuged for 1 hr. at 3000g, analysed for nitrogen by a colorimetric micro-Kjeldahl procedure and diluted with buffer to a concentration of 0.85 mg. of protein/ml. The protein content was based on an assumed content of 17.6% of nitrogen in tropocollagen (Rubin *et al.* 1965). Dried samples of substances to be tested were dissolved directly in the same phosphate buffer.

Collagen was treated with Pronase (batch no. 53702, grade B; California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) by the procedure of Drake, Davison, Bump & Schmitt (1966) for 12 hr. at 20°, and freed of enzyme

by repeated precipitation. The intrinsic viscosity of 9.5 dl./g. and molecular weight of 280000 from light-scattering agreed well with values obtained by Davison & Drake (1966) from ultracentrifugal analysis. In addition, the light-scattering envelope corresponded to a rod-like conformation of length 2600 Å, in agreement with accepted values for tropocollagen. Pronase removed about 5% of peptide (telopeptide) external to the collagen helix and removed all of the cross-links.

Gelation rate. Studies on the rate of gelation were performed on 2 ml. samples of cold collagen solution to which either 0.5 ml. of cold buffer or 0.5 ml. of cold agent solution were added in round cuvettes (10 mm. internal diam.). The control was run simultaneously with the experimental tube. All glassware used was kept at 4°. At zero time, the two tubes were placed in a water bath at 25 ± 0.01° for 1.5 min., and the extinction was read at 440 mμ 0.5 min. later and thereafter at 2 min. intervals for a total of 40 min. During this period of time the tubes were maintained at 25 ± 0.5°. Plateau values of extinction at 40 min. for solutions containing agents and for control solutions did not differ by more than 10% except when CS-proteins were used at high concentration (0.2 mg./ml.).

The degree of trapping of agent was calculated from analyses of supernatants for uronic acid, hexosamine, or hexose after compression of gels to small pellets by centrifugation at 1000g. Analyses for nitrogen indicated that more than 95% of the protein present was sedimented.

Light-scattering. Experiments were performed with a cylindrical cell in a Brice-Phoenix instrument at 436 mμ by procedures based on those used previously (Mathews & Lozaityte, 1958). Buffer (27 ml.) containing agent was filtered through a Millipore filter, and values of angular intensities were obtained at 0°, 45°, 90° and 135° at a temperature of 25°. The cold collagen solution (3 ml. containing 2.55 mg. of protein), previously centrifuged at 5000g, was then added, and angular scattering was measured over a period of 150–200 min. During this time the temperature of the cell contents rose by 2–3°, a uniform gel formed, and plateau values of scattered-light intensities were reached. The percentage transmission was calculated from measured values of *I*₀ and the *I*₀ of buffer with agent; the dissymmetry, *Z*, was calculated as the ratio *I*₄₅/*I*₁₃₅ after correction of the measured values for the contribution of buffer and agent. Collagen solutions were prepared freshly every 5 days.

Electrokinetics. Experiments were performed with the 6 ml. cell supplied for use with a Perkin-Elmer Tiselius electrophoresis apparatus with the following modifications of the usual procedure. The bottom section of the cell (Fig. 1) was filled with a solution of 0.68 mg. of collagen/ml. in buffer containing a non-ionic dye. This dye, Apalon Yellow, was obtained from Gelman Instrument Co., Ann Arbor, Mich., U.S.A., and diluted 1:40 with buffer before being mixed 1:5 with the collagen solution. When a firm gel had formed (about 60 min.), the cathode limb was filled with a 0.2 mg./ml. solution of acid mucopolysaccharide and the anode limb was filled with buffer. The cell assembly was completed with the top section, buffer vessels and Ag/AgCl electrodes and, after equilibration in a water bath at 25.0 ± 0.1°, a potential (1.72 v/cm.) leading to a current of 10 ma was applied for 2 hr. (3 hr. when sodium hyaluronate was used.) The contents of both limbs of the cell were removed. The gel was compressed by manipulation with

Fig. 1. Diagram of electrophoresis cell and contents in electrokinetic experiments. The arrows indicate the direction of migration of acid mucopolysaccharide, which is opposite to that of electro-osmotic flow of buffer. The anode and cathode limbs each have a volume of 4.5 ml. and a path length of 5.0 cm. The bottom section has a volume of 1.6 ml. and a path length of 1.8 cm.

a spatula to a small pellet to release the free fluid. The extinction of this free fluid as well as that of the original collagen-dye mixture, which was stored at 4°, was measured at 480 m μ . The collagen pellet was heated in 5 ml. of water and the resulting solution, the other fluid samples and the original acid mucopolysaccharide solution were assayed for uronic acid.

The average distance of migration of acid mucopolysaccharide was calculated from the loss of agent from the cathode limb and also from the total gain in agent by the middle section and the anode limb. The two values thus obtained agreed within 10%. The distance of displacement of solvent due to electro-osmosis was calculated from the loss of dye from the middle section. The electrophoretic mobility relative to the solvent was then obtained from the relation:

$$\text{Mobility} = (d - d_{\text{osm.}})/Xt$$

where X is the voltage gradient (1.72 v/cm.) and $t = 7200$ sec. (or 10800 sec. for hyaluronate).

Electron microscopy was very kindly performed by Dr Roger Pearson, University of Chicago, on the gels produced in light-scattering experiments, with a Siemens Elmiskop I instrument. The grids were touched lightly to the surface of the gels, air-dried and negatively stained with phosphotungstic acid.

RESULTS

Rate of gelation. Results of a typical experiment are presented in Fig. 2. The added acid mucopolysaccharide delayed the rise in turbidity of the

Fig. 2. Effect of acid mucopolysaccharides on rate of gelation of collagen. For collagen at 0.68 mg. of protein/ml. (●), $t_1 = 6.0$ min. For collagen at 0.68 mg. of protein/ml. with CS-C (mol. wt. 50000) at 0.2 mg./ml. (○), $t_1 = 12.0$ min.

Fig. 3. Dependence of relative half-time of gelation, R (see the text), on ionic strength. The concentration of CS-C was 0.2 mg./ml.

solution during fibril formation relative to the control, but had little effect on the plateau turbidity value. The measure of effectiveness of an agent was taken as R , the ratio of the half-time of gelation in the presence of agent to the half-time of gelation of control.

Ionic-strength effect. The half-time of gelation of collagen solutions increased from 6.0 min. at I 0.13 to 13.4 min. at I 0.25. However, as shown in Fig. 3, the effect of acid mucopolysaccharide on gelation became greatly decreased with higher ionic strength.

Molecular-weight effect. The dependence of R on molecular weight of the acid mucopolysaccharide is evident from Fig. 4. Hyaluronate had little effect when the molecular weight was below about 40000. CS-A or CS-C had little effect if their molecular weights were less than about 20000, and their effect

Fig. 4. Effect of molecular weight on relative half-time of gelation (R). The preparations of CS-A (○) of molecular weights 14000 and 17000 were obtained from notochord of sturgeon and lamprey respectively; the two higher-molecular-weight preparations were from bovine cartilage each composed of 70% CS-A and 30% CS-C. The preparation of CS-A of molecular weight 50000 consisted of two polysaccharide chains joined by a short peptide sequence (Mathews, 1968). The preparations of CS-C (●), in order of increasing molecular weight, were obtained from lamprey cartilage, human cartilage (Mathews & Glagov, 1966), human umbilical cord (Mathews, 1966b), sturgeon cartilage and human umbilical cord respectively. The preparations of sodium hyaluronate (□), in order of increasing molecular weight, were from rat skin, rabbit skin (Schiller, Mathews, Jefferson, Ludowig & Dorfman, 1954) and umbilical cord after ethanol fractionation (Mathews, 1966b). Hyaluronate (streptococcal) (×) was purified from a preparation from *Streptococcus pyogenes* given by Dr H. Alburn. The CS-B preparation (▲) was from ox lung (Mathews, 1959).

exceeded that of hyaluronate at equal molecular weight. The single point for CS-B fell on the curve for CS-A. However, streptococcal hyaluronate showed an anomalously large effect. This preparation has an unusual content of about 30 amino acid residues per unit of molecular weight 96000, consisting primarily of serine and glutamic acid.

Concentration effect. The effectiveness of acid mucopolysaccharides generally increased with concentration, as shown in Fig. 5. At concentrations of 1.0 mg./ml., high-molecular-weight CS-C and hyaluronate (streptococcal) gave very large values of R , which could not be accurately measured. Also, some agents that were very effective at low concentration (Table 1) produced, at concentrations of 0.2 mg./ml., gels that either contracted and clumped (agents 197, CSP, W-2B, RCSP, RNP and DMP; see Table 1) or were only slightly opaque (heparin at 0.2 mg./ml. and 0.02 mg./ml.).

Polysaccharide-proteins and other macromolecules. Various macromolecules listed in Table 1 were tested at 0.2 mg./ml., or at lower concentrations

Fig. 5. Dependence of relative half-time of gelation on concentration of acid mucopolysaccharide. For CS-C (mol. wt. 50000) (●) and for hyaluronate (streptococcal) (□) at 1.0 mg./ml. the values of R exceed 6. Also shown is the graph for CS-C (mol. wt. 10000) (○).

when unstable gels were produced at the higher concentration. The uncharged polysaccharide, methocel, has a chain contour length near that of CS of molecular weight 50000, but it was entirely without effect on rates of gelation of collagen (however, see the results of the light-scattering experiments). Neither albumin nor sialoproteins of moderate anionic charge were effective. However, both agent DMP and agent BSP, which contain phosphate groups in addition to sialic acid, were sufficiently charged with anionic groups to be effective.

Hyaluronate-peptide, containing about 3% of peptide, had an effectiveness consistent with its high molecular weight (Fig. 4). The difference in molecular weight may account for the difference in effectiveness of the two fractions of sulphated CS-C. Squid CS, heparin and heparitin sulphate were highly effective agents. They were partly trapped within the collagen gel pellet, but none of the preparations indicated in Fig. 4 were similarly trapped.

The CS-proteins were all effective agents. It is noteworthy that the isolated acid mucopolysaccharide chains derived from the CS-proteins obtained from sturgeon notochord (RNP), lamprey notochord (LNP) and lamprey cartilage (LCP) have molecular weights less than 17000, and were

Table 1. *Effect of some macromolecules on the rate of gelation of collagen solutions*

R is half-time of gelation relative to control.

Agent	Type	10 ⁻³ × Mol.wt.	Concn. (mg./ml.)	<i>R</i>	% trapped in collagen gel	Remarks
Methocel	Neutral polysaccharide	41	0.2	1.04	0	Methylated cellulose
Serum albumin	Crystallized protein	80	0.2	1.01	—	
Orosomucoid	Sialoprotein	49	0.2	1.10	—	
MP	Sialoprotein	7000	0.02	0.96	—	From urine; 3.2% sialic acid
DMP	Sialoprotein	—	0.02	1.57	—	From dentine; 6% phosphate
BSP	Sialoprotein	23	0.05	1.36	—	From bone; 20.5% sialic acid, 1.4% phosphate
Hy-5	Hyaluronate-peptide	1500	0.2	1.80	0	From umbilical cord
W-2P1	Sulphated CS-C	35	0.2	1.33	0	From ray cartilage; sulphate: hexosamine ratio 1.25
W-2P2	Sulphated CS-C	56	0.2	1.91	0	From ray cartilage; sulphate: hexosamine ratio 1.25
197	Squid CS	107	0.02	1.62	30	Sulphate: hexosamine ratio 1.22
H120	Heparin	13	0.005	2.12	40	From bovine lung
HS	Heparitin sulphate	35	0.02	1.43	20	From bovine lung
CSP	CS-A-protein	50000	0.02	1.30	100	From bovine cartilage
W-2B	CS-C-protein	20000	0.02	1.52	100	From ray cartilage
RCSP	CS-C-protein	2900	0.02	2.04	50	From sturgeon cartilage
RNP	CS-A-protein	1300	0.02	1.76	10	From sturgeon notochord
LNP	CS-A-protein	500	0.2	1.57	10	From lamprey notochord; <i>R</i> 1.23 at 0.02 mg./ml.
LCP	CS-C-protein	340	0.2	1.73	10	From lamprey cartilage; <i>R</i> 1.45 at 0.02 mg./ml.

Fig. 6. Light scattered during gelation process. The graphs show percentage transmission (▼) and dissymmetry (●) for collagen at 85 μg./ml., and percentage transmission (▽) and dissymmetry (▲) for collagen at 85 μg./ml. with sulphated CS-C at 2.5 μg./ml.

Light-scattering. Results obtained in a representative light-scattering experiment are illustrated in Fig. 6; more extensive results are listed in Table 2. Most of the experiments were conducted at a ratio of collagen to agent of 34:1. This ratio is the same as that used in the earlier gelation experiments in which the agent concentration was 0.02 mg./ml. The eightfold dilution of interactants results in an approximately fourfold increase in the time required to reach plateau values. The decrease in percentage transmission and the increase in dissymmetry with time may be considered, to a first approximation, to represent an increase in the size of scattering elements produced by aggregation of collagen molecules. Thus the effect of an agent on the degree of collagen aggregation is indicated by the difference of these values from the corresponding values of the control when the limiting state is reached.

The agents listed in Table 2 show an order of effectiveness that accords generally with the order of effectiveness indicated by the *R* values given in Fig. 4 and Table 1. However, methocel and heparin at 2.5 μg./ml. decreased the degree of aggregation of collagen. Although the gel produced in the presence of heparin was highly transparent, more than 90% of the collagen was sedimented at 5000*g*. A similar degree of sedimentation of

virtually ineffective agents (see Fig. 4). The degree of trapping of CS-proteins appeared to be correlated with molecular size.

Table 2. *Effects of agents on the formation of fibrils from collagen solutions (85 $\mu\text{g.}/\text{ml.}$) in light-scattering experiments*

Agent	$10^{-3} \times \text{Mol.wt.}$	Concn. ($\mu\text{g.}/\text{ml.}$)	Limiting difference from control	
			Transmission %	Dissymmetry (Z)
Albumin	80	2.5	0	0
Methocel	41	2.5	7	-1.2
CS-A	50	2.5	-8	1.6
CS-A	14	2.5	-3	0.4
CS-C	50	2.5	-3	0.9
CS-C	6	2.5	0	0
Sulphated CS-C	56	2.5	-11	2.1
CS-A-protein (CSP)	50000	2.5	-13	1.5
CS-A-protein (CSP)	50000	0.5	-2	0.3
CS-C-protein (RCSP)	2700	2.5	-18	1.8
CS-C-protein (LCP)	340	2.5	-4	0.8
CS-C-protein (W-2B)	20000	2.5	-17	2.8
CS-C-protein (W-2B)	20000	0.5	-15	2.0
Sialoprotein (BSP)	23	2.5	0	0
Sialoprotein (DMP)	—	2.5	-28	4.6
Heparitin sulphate	35	2.5	-7	1.5
Heparin	13	2.5	39	0
Heparin	13	0.25	-9	1.7

Table 3. *Electrokinetic experiments*

Agent	$10^{-3} \times \text{Mol.wt.}$	$10^5 \times \text{Mobility}$ ($\text{cm.}^2\text{v}^{-1}\text{sec.}^{-1}$)	Electro-osmosis (% of mobility)	Uronic acid of bottom section ($\mu\text{g.}$)	
				Pellet	Fluid
CS-C	6	-25	<10	<4	89
CS-C	50	-25	<10	<4	115
Squid CS	107	-25	20	<4	63
Hyaluronate (streptococcal)	96	-15	10	<4	130
Hyaluronate	300	-15	15	<4	121
Hyaluronate-peptide	1500	-15	25	<4	104
CS-protein	20000	-25	50	60	40
CS-protein	50000	-25	50	44	42
Heparin	13	-28	10	30	78

collagen was obtained in experiments with other agents also.

A solution of Pronase-treated collagen was clarified by centrifugation and filtration through a Millipore filter and placed in the light-scattering cell at 25°. The intensities of scattered light remained constant for 3 hr. On addition of a solution CS-protein (agent RCSP) at a final concentration of 2.5 $\mu\text{g.}/\text{ml.}$, the light-intensities became approximately additive for the two components and remained unchanged for 2 hr., indicating that aggregation of collagen did not occur.

Electron microscopy. At high magnification, all the gels, with the exception of those produced in the presence of 2.5 $\mu\text{g.}$ of heparin/ml., showed the characteristic cross-striations of normal collagen fibrils with an average period near 650 Å. Heparin

produced a dense mat of fibrils of very small diameter that were not clearly resolved.

The gel samples were examined at a magnification of $\times 3500$ in an attempt to correlate the light-scattering results with visual appearance. The sampling problem was such that a quantitative analysis of fibril diameter and degree of fibril association did not appear feasible. However, in those instances in which an agent produced a large effect on the light-scattering characteristics of a gel, a qualitative difference from the control collagen gel was readily evident. Samples of typical fields in Plate 1 show a small decrease in average fibril diameter from control for gels formed with methocel present and a large increase over the control in the average size of fibril aggregates formed in the presence of a CS-protein.

EXPLANATION OF PLATE 1

Electron micrographs of collagen gels showing typical fields obtained with (a) methocel, (b) no additions, and (c) RCSP (see Table 1).

Electrokinetics. The electrophoretic transport of various agents through a collagen gel (see Fig. 1) was studied in an attempt to evaluate the relative importance of electrostatic binding and steric factors. The results are given in Table 3. It should be noted that the mobilities relative to solvent were close to those observed in the absence of collagen gel (Mathews, 1959, and unpublished work), in accord with the theory of electro-osmosis (Alexander & Johnson, 1949).

Electro-osmosis was not observed with CS chains of molecular weight 50000 or lower. This result indicated that these macromolecules passed relatively unretarded through the collagen gel. However, when the molecular weight for either CS or hyaluronate reached 100000, an electro-osmotic effect, which increased with increased molecular size of hyaluronate, was noted. The high-molecular-weight CS-proteins produced a high degree of electro-osmotic flow and were also trapped within the gel pellet. These results suggested a possible contribution of molecular entanglement to the observed phenomena. Though of low molecular weight, heparin produced a small electro-osmotic flow and was appreciably trapped. With this very highly charged polyanion it appeared that electrostatic interaction produced strong binding to collagen.

DISCUSSION

Investigations of fibril formation encounter complexities deriving from the capacity of collagen in solution to bind a wide variety of inorganic cations and anions that have profound influences on the isoelectric point (Veis, Anesey & Cohen, 1958) and the degree and rate of fibril formation (Bensusan & Hoyt, 1958; Davison & Drake, 1966; Weinstock, King & Wuthier, 1967; Gross & Kirk, 1958). Fibril morphology is also highly dependent on many variables including the temperature, ionic environment and the history of previous manipulations (Kahn, Carrol & Witnauer, 1962; Wood & Keech, 1960; Keech, 1961). Added difficulties arise because the kinetics of fibril formation cannot be expressed in molecular forms that are directly related to the measured quantities. Bensusan & Hoyt (1958) and Wood & Keech (1960) noted that the precipitation of fibrils from collagen solutions occurred in two steps, the first occurring during the lag period or nucleation phase, the second occurring as a growth phase. Wood (1960a) showed that the final distribution of fibril width was determined during the lag period and that the measured turbidity was a linear function of the degree of precipitation of collagen and hence related to the extent of fibril growth.

Wood (1960b) found that prior incubation of the

collagen in phosphate-buffered sodium chloride solution at 0° effectively nucleated the solution so that subsequent addition of acid mucopolysaccharides and raising the temperature of the mixture resulted in observation of the effect of the agent on the growth phase only. Since our collagen solutions had been dialysed against phosphate buffer before use, it is thus likely that our observations were limited mainly to the growth phase of fibril formation. As a consequence, we did not observe the various nucleating effects of acid mucopolysaccharides reported by Wood (1960b). The results obtained are associated with particular ionic conditions, the nature of the collagen preparation and the properties of the various agents used. Soluble collagen from normal tissues of the same species may differ slightly in primary composition. However, it may be more significant for studies of fibrillogenesis that such preparations may differ widely in tertiary and higher structures (Seifter & Gallop, 1966; Harding, 1965). This is evident from analysis of denatured collagens for components arising from cross-linking of the primary polypeptide chains. In addition, most soluble preparations, including our own, contain polymers as well as tropocollagen monomer (Veis, Anesey & Mussel, 1967; Davison & Drake, 1966). Because of these evident complexities, the present study was confined to a single preparation of collagen.

All the major types of non-covalent bonding have been suggested to account for fibril formation from collagen solutions [hydrogen bonds (Gross & Kirk, 1958); electrostatic forces (Wood & Keech, 1960; Gross & Kirk, 1958); hydrophobic bonding (Cassel, 1966; Cassel & Christensen, 1967; Bianchi, Conio & Ciferri, 1966)]. Though the relative contributions of various forces in fibrillogenesis are not readily evaluated, it is probable that electrostatic interactions have a significant role. Thus an electrostatic influence of highly charged polyanions on collagen aggregation would account for the observed lowering of effectiveness of acid mucopolysaccharides with increasing ionic strength of the medium. Further, the increase of effectiveness with increase in linear charge density and with molecular chain length of acid mucopolysaccharides supports a previous suggestion that collagen-polyanion complexes are formed in a reversible manner (Mathews, 1958). The stability of the complexes would increase with increase in number of possible sites for electrostatic interaction of the components. Such collagen-macroion complexes might be essentially 'inactive' with respect to the fibril-forming process in a manner analogous to that suggested for collagen-microion complexes by Bensusan & Hoyt (1958).

General reversibility of the association of polysaccharide with collagen, as also suggested by

Wood (1960b), is indicated by the observation that precipitated collagen did not retain detectable amounts of acid mucopolysaccharide, with the exceptions of heparin, heparitin sulphate and squid CS. An exceptionally high linear density of anionic charge of the heparin molecule and of segments of the heparitin molecule (J. A. Cifonelli, personal communication) could confer high stability on complexes containing these molecules. Squid CS combines a high average linear density of charge with an unusually long molecular chain; in addition, it is exceptional for the possession of galactosamine residues bearing two sulphate groups (Kawai, Seno & Anno, 1966). The effect of increasing acid mucopolysaccharide concentration (Fig. 5) also suggests a mass-action effect on a reversible association. The high effectiveness of acid mucopolysaccharide-proteins (Table 1) is probably due to the multiple interactions available to structures consisting of many individual polysaccharide chains united by a polypeptide chain into a single macromolecule (Mathews, 1965), with possible contributions from excluded-volume effects.

The gelation process is too complex to permit explicit analysis of the light-scattering results. However, the qualitative assumptions made above are consistent with present theory (Stacey, 1956). In all stages of gel formation (Fig. 6), the decrease in transmission could be due to both elongation and increase in diameter of thread-like units. The initial dissymmetry is apparently due to a very small number of large aggregates representing only a minor fraction of the collagen present. Shortly thereafter, though the light-intensities at 45° and 135° rise quickly, the ratio of these intensities (dissymmetry, Z) falls. This suggests increase in length in preference to increase in thickness. The rise in dissymmetry in the final stages is probably due mainly to increase in thickness of established fibrils.

With respect to the process of aggregation, fibrinogen-fibrin systems are similar in many respects to collagen systems (Scheraga & Laskowski, 1957; Stacey, 1956). Of particular interest is the light-scattering study by Sheppard, Imperante & Wright (1956) of the effect of heparin on the formation of a fibrin gel from fibrinogen. As in the collagen system, heparin delayed gelation and produced dissymmetry values much lower than those of the control. This result suggested that high concentrations of heparin favoured end-to-end conformation over lateral aggregation.

The effect of heparin at $2.5 \mu\text{g./ml.}$ in the collagen system (Table 2) may be due to the initial near-equivalence in numbers of molecules or stable aggregates of both reactants and to a high extent of interaction with collagen. Electrostatic repulsions between kinetic units of collagen-heparin com-

plexes apparently interfered to a greater extent with lateral associations than with associations leading to elongation of fibrils. This effect was diluted out with heparin at $0.25 \mu\text{g./ml.}$, and lateral association was promoted in a manner similar to that of the other acid mucopolysaccharides of lower linear charge density, e.g. CS-A of molecular weight 50 000. These concentration effects of heparin paralleled those found in the rate experiments. The gelation process was influenced to a smaller extent by CS-A of molecular weight 6000 either because of the low degree of complex-formation or because the contribution of negative charges to a 1:1 complex was lower for the smaller polysaccharide. Similar interpretations may apply to the sialoproteins. The decrease in dissymmetry caused by methocel suggests involvement of hydrophobic-group associations.

The CS-proteins have a greater effect on both rate of gelation and fibril aggregation than do the corresponding polysaccharide chains alone. The effect presumably depends on the branched structure of the macromolecule and also on the linear charge density and length of the polysaccharide chain.

The stability of Pronase-treated collagen suggests that the observed effects of CS-proteins are linked to the process of aggregation of undegraded collagen, i.e. the agents modified, but did not determine, the basic features of fibril formation. This viewpoint is supported by the following evidence that the enzyme produces only minimal alterations in collagen. Though fibres are not formed readily from solutions of Pronase-treated collagen, ATP can induce formation of fibres with $640 \text{ \AA}$ spacing; inorganic-ion binding is similar to that of undegraded collagen; the isoelectric point in phosphate buffer is about pH 6.0. The peptides removed by Pronase probably have a role in orientation of collagen molecules during fibril formation but appear to have little effect on other properties of collagen (Davison & Drake, 1966). It thus appears that CS-proteins, which are incapable of precipitating Pronase-treated collagen, primarily affect kinetics of fibril formation from undegraded collagen.

It seems unlikely that the apparent correlation of the electron-microscopic appearance of gels with the light-scattering observations was due to artifacts produced during preparation of the gels for microscopy. Thus our results with heparin at two different concentrations are similar to those reported by Keech (1961), who used different techniques. However, Keech (1961) found that the presence of CS-C produced individual fibrils of smaller diameter than those from control solutions of collagen. Since in these measurements larger aggregates were not included, a direct comparison with our results is not possible. We therefore

Table 4. *Ratio of 'available volume' of agent to total volume*

The calculations were based on assumed radii for collagen-gel fibrils and tropocollagen monomer of 500 Å and 7 Å respectively.

Agent	Radius of equiv. sphere (Å)	Ratio for collagen gel		Ratio for tropocollagen monomer	
		(0.68 mg./ml.)	(0.085 mg./ml.)	(0.68 mg./ml.)	(0.085 mg./ml.)
Albumin	34	0.999+	0.999+	0.975	0.997
CS (mol.wt. 10000)	50	0.999+	0.999+	0.96	0.995
CS (mol.wt. 50000)	250	0.999+	0.999+	0.43	0.90
Squid CS	500	0.998	0.999+	0.04	0.45
Hyaluronate-peptide	1000	0.995	0.999+	< 0.001	0.21
CS-C-protein	2000	0.987	0.998	< 0.001	0.002

suggest that the fibril aggregates or bundles seen in the electron microscope were probably present in the undisturbed gel and contributed largely to the light-scattering results.

Estimates of steric factors can be obtained from a theoretical treatment by Ogston (1958) of cases of random distribution of thin straight fibres and spheres. The equations permit calculation of the fraction of the total volume that is freely available to the spheres without contact with the fibres. Such calculations, with reasonable approximations for collagen-fibre dimensions and for the radius of the equivalent sphere for each agent, are given in Table 4. Values of 'available volume' for collagen gels at 0.68 mg./ml. indicate that steric factors are very small except for CS-proteins and possibly hyaluronate-peptides. This is in approximate agreement with the observed electro-osmotic flow results in Table 3. The obvious exception is heparin, which has molecular dimensions comparable with those of CS of molecular weight 10000. It is apparent from this observation, as well as from the extent of trapping of heparin (Table 3), that binding to collagen fibrils was the factor of major importance.

With hyaluronate-peptide and the remaining three agents of Table 3 that induced an electro-osmotic flow, no trapping of agent was observed. However, Table 1 shows that appreciable trapping of squid CS, but not of hyaluronate-peptide, occurred during fibril formation. Reference to Table 4 reveals low values of 'available volume' for both agents in solutions of monomeric collagen at 0.68 mg./ml. The trapping of squid CS in these experiments may therefore be due to binding to collagen during early stages of fibril formation from monomer, when the probability of intermolecular contacts is high. Though the probability of such contacts is very much greater for hyaluronate-peptide, this molecule does not bind as strongly to collagen, probably because it possesses a much lower linear charge density.

The effects of electrostatic interactions are particularly difficult to observe under conditions

where a large degree of molecular entanglement must occur, as during the initial stages of fibril formation in the presence of CS-proteins. Intermolecular binding was considered a possibility in such circumstances by Disalvo & Schubert (1966) in view of earlier results by Gerber & Schubert (1964) that showed binding between CS-proteins and albumin at low ionic strengths. Toole & Lowther (1967) observed that sulphated acid mucopolysaccharide-proteins were precipitated with collagen at low temperatures, whereas hyaluronate-peptides were not. They interpreted this finding as indicating that the precipitation required the presence of the ionizable sulphate groups. Binding of acid mucopolysaccharide-proteins to collagen has not been shown unequivocally in the present study. However, its occurrence, supported by the observations on the acid polysaccharide segments of the same macromolecules, is highly probable.

Heterogeneous molecular interactions probably have significant influences on processes of fibril formation and growth and on higher-level organizations of mature connective-tissue matrices (Jackson, 1966). In mature connective tissues, particularly in cartilage, where very high concentrations of collagen and polysaccharides are present, electrostatic interactions may assume increased significance with respect to physical properties (Sokoloff, 1963; Fry & Robertson, 1967). It should be noted that interactions of acid mucopolysaccharide-proteins with large cross-linked collagen fibrils, as described by Smith, Peters & Serafini-Fracassini (1967), may differ sterically from those suggested for the early stages of fibril formation (Mathews, 1965). Also, the tightly knit network of collagen fibrils in mature human (Johnson & Schubert, 1960) and shark (Mashburn & Hoffman, 1967) tissues apparently traps a large proportion of acid mucopolysaccharide-proteins and other macromolecules.

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REFERENCES

- Alexander, A. E. & Johnson, P. (1949). *Colloid Science*. London: Oxford University Press.
- Andrews, A. T. de B., Herring, G. M. & Kent, P. W. (1967). *Biochem. J.* **104**, 705.
- Bensusan, H. B. & Hoyt, B. L. (1958). *J. Amer. chem. Soc.* **80**, 719.
- Bianchi, E., Conio, G. & Ciferri, A. (1966). *Biopolymers*, **4**, 957.
- Cassel, J. M. (1966). *Biopolymers*, **4**, 989.
- Cassel, J. M. & Christensen, R. G. (1967). *Biopolymers*, **5**, 431.
- Davison, P. F. & Drake, M. P. (1966). *Biochemistry*, **5**, 313.
- Disalvo, J. & Schubert, M. (1966). *Biopolymers*, **4**, 247.
- Drake, M. P., Davison, P. F., Bump, S. & Schmitt, F. O. (1966). *Biochemistry*, **5**, 301.
- Fry, H. & Robertson, W. Van B. (1967). *Nature, Lond.*, **215**, 53.
- Gerber, B. R. & Schubert, M. (1964). *Biopolymers*, **2**, 259.
- Gross, J. & Kirk, D. (1958). *J. biol. Chem.* **233**, 355.
- Harding, J. J. (1965). *Advanc. Protein Chem.* **20**, 109.
- Jackson, S. F. (1966). In *Cells and Tissues in Culture*, p. 1. Ed. by Willmer, E. N. New York: Academic Press Inc.
- Johnson, B. & Schubert, M. (1960). *J. clin. Invest.* **39**, 1752.
- Kahn, L. D., Carrol, R. J. & Witnauer, R. J. (1962). *Biochim. biophys. Acta*, **63**, 243.
- Kawai, Y., Seno, N. & Anno, K. (1966). *J. Biochem., Tokyo*, **60**, 317.
- Keech, M. K. (1961). *J. biophys. biochem. Cytol.* **9**, 193.
- Mashburn, T. A., jun. & Hoffman, P. (1967). *Biochem. biophys. Res. Commun.* **29**, 686.
- Mathews, M. B. (1958). *Nature, Lond.*, **181**, 421.
- Mathews, M. B. (1959). *Biochim. biophys. Acta*, **35**, 9.
- Mathews, M. B. (1962). *Biochim. biophys. Acta*, **58**, 92.
- Mathews, M. B. (1965). *Biochem. J.* **96**, 710.
- Mathews, M. B. (1966a). *Clin. Orthop.* **48**, 267.
- Mathews, M. B. (1966b). In *Methods in Enzymology*, vol. 8, p. 654. Ed. by Neufeld, E. F. & Ginsburg, V. New York: Academic Press Inc.
- Mathews, M. B. (1967). *Biol. Rev.* **42**, 499.
- Mathews, M. B. (1968). *Fed. Proc.* **27**, 529.
- Mathews, M. B. & Decker, L. (1967). *Abstr. 7th int. Congr. Biochem., Tokyo*, p. 711.
- Mathews, M. B. & Glagov, S. (1966). *J. clin. Invest.* **45**, 1103.
- Mathews, M. B. & Inouye, M. (1961). *Biochim. biophys. Acta*, **53**, 509.
- Mathews, M. B. & Lozaityte, I. (1958). *Arch. Biochem. Biophys.* **74**, 158.
- Ogston, A. G. (1958). *Trans. Faraday Soc.* **54**, 754.
- Rubin, A. L., Drake, M. P., Davison, P. F., Pfahl, D., Speakman, P. T. & Schmitt, F. O. (1965). *Biochemistry*, **4**, 181.
- Scheraga, H. A. & Laskowski, M. (1957). *Advanc. Protein Chem.* **12**, 1.
- Schiller, S., Mathews, M. B., Jefferson, H., Ludowieg, J. & Dorfman, A. (1954). *J. biol. Chem.* **211**, 717.
- Seifter, S. & Gallop, P. M. (1966). In *The Proteins*, p. 155. Ed. by Neurath, H. New York: Academic Press Inc.
- Sheppard, E., Imperante, J. & Wright, I. S. (1956). *J. phys. Chem.* **60**, 584.
- Smith, J. W., Peters, T. J. & Serafini-Fracassini, A. (1967). *J. Cell Sci.* **2**, 129.
- Sokoloff, L. (1963). *Science*, **141**, 1055.
- Stacey, K. A. (1956). *Light Scattering in Physical Chemistry*, pp. 146, 147. New York: Academic Press Inc.
- Tamm, I., Bugher, J. C. & Horsfall, F. L., jun. (1955). *J. biol. Chem.* **212**, 125.
- Toole, B. P. & Lowther, D. A. (1967). *Biochem. biophys. Res. Commun.* **29**, 515.
- Veis, A., Anesey, J. & Cohen, J. (1958). In *Recent Advances in Gelatin and Glue Research*, p. 155. Ed. by Stainsby, G. London: Pergamon Press Ltd.
- Veis, A., Anesey, J. & Mussell, S. (1967). *Nature, Lond.*, **215**, 931.
- Weimer, H. E. & Winzler, R. J. (1955). *Proc. Soc. exp. Biol., N.Y.*, **90**, 458.
- Weinstock, A., King, P. C. & Wuthier, R. E. (1967). *Biochem. J.* **102**, 983.
- Wood, G. C. (1960a). *Biochem. J.* **75**, 598.
- Wood, G. C. (1960b). *Biochem. J.* **75**, 605.
- Wood, G. C. & Keech, M. K. (1960). *Biochem. J.* **75**, 588.
- Zamoscanyk, H. & Veis, A. (1966). *Fed. Proc.* **25**, 409.