Nature of the Interaction of Chondroitin 4-Sulphate and Chondroitin Sulphate-Proteoglycan with Collagen

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The electrostatic interaction of chondroitin sulphate and the chondroitin sulphate-proteoglycan with collagen was studied by chromatography of the glycosaminoglycan and the proteoglycan on a collagen gel. The observed binding between the macromolecules increased with decreasing pH and ionic strength, and was significant under physiological conditions. A study of the interaction between chondroitin sulphate and a preparation of soluble collagen, with a partition-equilibrium technique, afforded similar results.

Connective tissue is characterized by a large extracellular space, occupied by glycosaminoglycan-protein complexes and collagen fibres. Connective tissues, differing with respect to origin, age and function, are distinguished by the types, concentrations and mutual proportions of the glycosaminoglycans present (Jackson & Bentley, 1968). Likewise variations in the glycosaminoglycan pattern are paralleled by changes in the dimensions and spatial orientation of the fibres in the collagen network (Gross, 1961). These observations have led to the theory that the glycosaminoglycans interact with collagen and influence fibrillogenesis and the organization of connective tissue (Jackson & Bentley, 1968; Mathews, 1965).

Evidence that an interaction of some kind between collagen and glycosaminoglycans may determine the physical properties of connective tissue has been presented (Jackson & Bentley, 1968). Furthermore it has been demonstrated that the presence of glycosaminoglycans and proteoglycans may under certain conditions influence the formation of fibres of tropocollagen *in vitro* (Wood, 1960; Toole & Lowther, 1968; Mathews & Decker, 1968). There are, however, at present very few direct studies on the interaction between collagen and glycosaminoglycans. Mathews (1965) studied the interactions of a solubilized collagen with chondroitin sulphate, hyaluronate or heparin by free electrophoresis, and observed the reversible formation of a complex of electrostatic nature between collagen and these polysaccharides. The extent of complex-formation depended on the type of glycosaminoglycan, its molecular weight and the ionic strength and pH of the environment. Steven, Jackson & Broady (1968) and Steven, Knott, Jackson & Podrazky (1969) showed that collagen

forms a complex of ionic character with proteoglycan isolated from human intervertebral discs, and claimed that this complex is present *in vivo*.

The present investigation was undertaken to provide further evidence for an electrostatic interaction between collagen and chondroitin 4-sulphate, with special regard to the effects of pH and ionic strength. Preliminary results have been reported (Wasteson & Öbrink, 1968; Öbrink, 1969).

MATERIALS

Collagen was isolated from human umbilical cords by digestion of the tissue with pepsin according to the method of Balazs & Sweeny (1968), and was stored in the freeze-dried state until used. As shown in the analyses in Table 1 the material was free from other proteins (cf. Eastoe, 1967) and contained no hexosamine. Galactose and glucose were the only neutral sugars found, further confirming the purity of the collagen preparation (Table 1).

Chondroitin sulphate was prepared from bovine nasal septa by precipitation with cetylpyridinium chloride from 0.3M-NaCl after digestion of the tissue with papain (Scott, 1960). The polysaccharide was fractionated by gel chromatography on Sephadex G-200 (Wasteson, 1971); physical data for the subfractions used in the present investigation are given in Table 2.

Chondroitin sulphate-proteoglycan was prepared from bovine nasal septa by the technique of Gerber, Franklin & Schubert (1960). The preparation contained 18% (w/w) of protein. The PPL* was separated on Sepharose 2B (Pharmacia AB, Uppsala, Sweden) into two fractions, A and B. The weight-average molecular weights of the fractions, determined by light-scattering, were 6.7×10^6 and 2.4×10^6 respectively.

A dextran fraction, D-4-B, with a K_{av} value of 0.39 on Sephadex G-200, was available in our laboratory (Laurent

* Abbreviation: PPL, chondroitin sulphate-proteoglycan.

& Granath, 1967). Agarose was supplied by Behringwerke AG, Marburg-Lahn, Germany.

Buffers. Buffers were 0.01M-phosphate, pH7.0 or 7.4, and 0.02M-veronal, pH8.6. The ionic strength was adjusted with NaCl.

METHODS

Analytical methods. The amino acid and hexosamine analyses were performed with a Bio-Cal model BC-200 amino acid analyser after hydrolysis of the sample *in vacuo* in 6M-HCl at 110°C for 24h.

Neutral sugars were determined as alditol acetates by gas-liquid chromatography according to the method of Albersheim, Nevins, English & Karr (1967), as modified by Lindahl (1970). Hydrolysis of the protein was performed in 2M-trifluoroacetic acid at 100°C for 4h.

Measurements of light-scattering at 436nm were performed in a Sofica PGD 42000M photometer at

pH7.1, 10.4 and 25°C, and the data were treated according to Zimm (1948), a refractive index increment being used for the proteoglycan of 0.172 (Mathews & Lozaityte, 1958).

Collagen concentrations were determined by semi-micro nitrogen analyses by the Kjeldahl method (Ma & Zuazaga, 1942), with a value of 17% (w/w) of nitrogen being assumed. Chondroitin sulphate, PPL and dextran were determined by the carbazole method, modified for autoanalysis (Balazs, Berntsen, Karossa & Swann, 1965). (Although the carbazole method is specific for hexuronic acid, it worked satisfactorily for dextran at the high concentrations used.)

Preparation of collagen solutions. Collagen was dissolved in 0.5% acetic acid and dialysed in the cold against the buffer to be used in the actual experiment.

Preparation of collagen gel. Collagen was dissolved in 0.5% acetic acid and the solution was dialysed in the cold against 5mM-phosphate buffer, pH7.3, containing 0.14M-NaCl. The collagen concentration of the dialysed solution was 2% (w/v). This solution was then mixed with aq. 25% glutaraldehyde in the ratio 9:1, stirred effectively and then left at 4°C for 24h. Under these conditions a rigid gel was formed. The gel was granulated by passage through a wire-mesh screen (Hjertén, 1962).

Chromatography of chondroitin sulphate and PPL on collagen gels. Collagen gel was packed into chromatographic columns. Chondroitin sulphate was chromatographed on a column (0.6cm × 100cm), maintained at 9°C by means of a cooling jacket, and was eluted by hydrostatic pressure at a flow rate of 10mlh⁻¹cm⁻². Samples of 400μg of chondroitin sulphate in 0.2ml of buffer were applied to the column and effluent fractions of 0.7–1.0ml were collected.

PPL was chromatographed on a 1.5cm × 35cm column at 4°C. The column was eluted at a flow rate of 6mlh⁻¹cm⁻² by means of a peristaltic pump. Samples of 600μg of PPL in 0.3ml of buffer were applied to the column, and effluent fractions of 2.4–2.6ml were collected.

Buffers of various ionic strengths and pH values were used as eluents. Effluent volumes were determined by gravimetry. Before chromatography at least 5 bed vol. of buffer was passed through the columns.

The void volume (V_0) and the total volume (V_t) of the columns were determined with dextran (mol.wt. 12×10^6) and tritiated water respectively as described elsewhere (Öbrink, Laurent & Rigler, 1967; Marsden, 1965). V_0 and

Table 1. *Amino acid and neutral sugar composition of the collagen*

Data for the amino acids and sugars are expressed as residues per 1000 amino acid residues.

Asp	45.1
Hyp	109.1
Thr	16.4
Ser	36.9
Glu	73.7
Pro	108.2
Gly	337.7
Ala	103.9
Val	21.3
Met	6.5
Ile	11.4
Leu	22.9
Tyr	1.6
Phe	11.5
Hyl	14.1
Lys	22.6
His	5.3
Arg	51.8
Glucose	2.9
Galactose	2.3

Table 2. *Physical data for dextran and chondroitin sulphate fractions*

K_{av} denotes the available volume fraction for the preparation on Sephadex G-200, calculated from the elution volume according to Laurent & Killander (1964); r_s denotes Stokes radius and $\bar{M}_w$ the weight-average molecular weight.

Preparation	K_{av}	r_s (Å)	$\bar{M}_w$
Chondroitin sulphate fraction 1	0.11	58.5	36000
Chondroitin sulphate fraction 2	0.20	49.5	26000
Chondroitin sulphate fraction 3	0.28	43.5	19000
Chondroitin sulphate fraction 4-B	0.38*	—	18000
Dextran fraction D-4-B	0.39*	—	—

* The K_{av} value was obtained with a batch of Sephadex G-200 other than that used for fractions 1, 2 and 3.

V_t were checked after each change of buffer and were found to remain constant during all the experiments.

The coefficient for the distribution of chondroitin sulphate between the gel and the actual buffer was calculated from $K = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the polysaccharide. The position of the maximum of the peak was taken as the elution volume even in those cases where the peak was asymmetrical.

Potentiometric titration. Granulated collagen gel was carefully washed with water and homogenized in a glass homogenizer. Soluble collagen was extensively dialysed against distilled water. After freeze-drying, the water and nitrogen contents of both types of collagen were determined by drying to constant weight and by the Kjeldahl procedure (Ma & Zuazaga, 1942) respectively.

The titrations (Kenchington, 1960) were performed at 4°C in glass tubes, in which 25 mg of freeze-dried material was suspended in 2 ml of acid or base (HCl or KOH) containing 0.2 M-KCl. The tubes were flushed with N_2 , stoppered and put in a cold-room (4°C). After 1 week, with mixing several times a day, the pH values of the solutions or suspensions were determined at 4°C with a glass electrode. Back-titrations were performed by stepwise addition of HCl or KOH. The pH values were determined after an equilibration time of several days. The back-titration procedure was carried out on samples covering the entire pH range.

Partition equilibrium experiments. Agarose gels were prepared by cooling 3 ml of a hot 4% (w/v) agarose solution in 25 ml Erlenmeyer flasks. After layering 3 ml of the chondroitin sulphate solutions (fraction 4-B; 0.2 mg/ml) on top of the gel discs, the polysaccharide was allowed to equilibrate at 4°C between the gel and the buffer phase. The equilibration was performed at different ionic strengths and pH values (Table 4) with or without soluble collagen (5 mg/ml) in the solution. Dextran D-4-B (1.0 mg/ml) was similarly equilibrated under varying ionic conditions between the agarose gels and buffer solutions containing soluble collagen (5 mg/ml). The concentrations of polysaccharide in the buffer solutions were determined before and after equilibrium was attained.

RESULTS

Chromatography on collagen gels. The elution patterns obtained after chromatography of the chondroitin sulphate fractions on the collagen gel were markedly affected by changes in pH and ionic strength of the eluent (Fig. 1). When the ionic strength and pH of the buffer decreased all the fractions were displaced towards larger elution volumes, as compared with the chromatograms obtained at pH 8.6 and I 0.4. At ionic strengths greater than 0.4 (pH 8.6) no further displacement of the peaks was observed. The position and shape of the chondroitin sulphate peak was more profoundly affected by the changes in ionic strength than the changes in pH. Further, the effects of ionic strength and pH increased with the molecular weight of the chondroitin sulphate, as seen from the distribution coefficients of fractions 1, 2 and 3 in Table 3.

Under certain conditions, chromatography of

Fig. 1. Chromatography of chondroitin sulphate (fraction 1) on collagen gel, under various conditions of pH and ionic strength: (a) pH 7.0; (b) pH 8.6. (I)–(IV) refer to elution at the ionic strengths 0.40, 0.15, 0.12 and 0.10 respectively. The chromatograms have been normalized to equal areas.

PPL on the collagen gel apparently resulted in a complex-formation similar to that observed with chondroitin sulphate. No differences were observed when the two PPL fractions A and B were compared. Generally, three different modes of chromatographic behaviour were distinguished.

(a) In the first group of experimental conditions (pH 7.0, I 0.15; pH 7.0, I 0.4; pH 7.4, I 0.15; pH 8.6, I 0.4) all of the proteoglycan was recovered in the void volume, indicating that no adsorption to the gel had occurred.

(b) In the second group (pH 7.0, I 0.12; pH 8.6, I 0.1) more than 50% of the material applied to the column was eluted with the void volume. The remainder could not be recovered even by prolonged elution under the initial conditions, but was released when the pH and ionic strength were raised to 8.6 and 0.4 respectively.

(c) In the third group (pH 7.0, I 0.10; pH 7.4, I 0.10) no proteoglycan appeared in the eluate, even after prolonged elution, indicating complete adsorption to the gel. Attempts to recover the protein-polysaccharide were carried out by stepwise elution with buffers of increasing pH and ionic strength. Merely raising the ionic strength to 0.4 while maintaining pH at 7.0 was not sufficient for this purpose.

Table 3. *Distribution coefficients (K) for chondroitin sulphate fractions subjected to chromatography on the collagen gel*

The minimum value of K (pH 8.6 and $I 0.4$) has been denoted $K_{\min.}$. Values in parentheses were obtained when the collagen gel had been used for a long time. As the gel had lost some of its binding ability these values are too low.

I	pH	Fraction 1 (M_w 36 000)		Fraction 2 (M_w 26 000)		Fraction 3 (M_w 19 000)	
		K	$K/K_{\min.}$	K	$K/K_{\min.}$	K	$K/K_{\min.}$
0.10	7.0	2.7	8.4	2.3	4.7	1.7	2.8
0.10	8.6	1.7	5.3	1.4	2.9	1.4	2.3
0.12	7.0	1.1	3.4	—	—	—	—
0.12	8.6	0.89	2.8	—	—	—	—
0.15	7.0	0.73	2.3	—	—	—	—
0.15	7.4	0.61	1.9	(0.63)	(1.3)	0.80	1.3
		(0.51)	(1.6)	—	—	(0.72)	(1.2)
0.15	8.6	0.53	1.7	—	—	—	—
0.4	7.0	0.35	1.1	0.52	1.1	0.80	1.3
0.4	8.6	0.32	1.0	0.49	1.0	0.60	1.0

Some of the material was released at pH 8.6 and $I 0.4$ and most of the remaining PPL dissociated from the gel at pH 8.6 and $I 1.0$.

Potentiometric titration. The results of the potentiometric titration of the collagen gel and of the soluble collagen were treated according to Kenchington (1960) and are shown in Fig. 2. The two titration curves are almost identical in the acidic region, but deviate from each other in the alkaline region. This finding is in accordance with the titration properties of collagen and other proteins after cross-linking with glutaraldehyde (Bishop & Richards, 1968; Bowes & Cater, 1968a). It has been claimed that glutaraldehyde reacts preferentially with lysine residues, giving rise to a hitherto unidentified product (Bowes & Cater, 1968a). The back-titrations indicated that the titration process was reversible between pH 2 and 12.

Partition-equilibrium experiments. The results of the partition-equilibrium experiments are recorded in Table 4. Since the collagen molecules are larger and more asymmetric than the polysaccharide molecules, a larger fraction of the gel volume was available to the polysaccharide than to the protein. As a result the polysaccharide molecules should be distributed between the gel and the buffer phases, whereas the collagen molecules should largely remain in the latter phase. The formation of a complex between chondroitin sulphate and collagen must necessarily influence the partition of the polysaccharide. Consequently, the observation that the concentration of chondroitin sulphate in the collagen solution was higher at low than at high ionic strength (Table 4) indicated an electrostatic binding between the polysaccharide and the collagen, in full agreement with the results of the

Fig. 2. Potentiometric titration of soluble collagen (○) and of collagen gel (●).

chromatography experiments. The distribution of chondroitin sulphate under unfavourable conditions for ionic binding ($I 0.4$) probably reflects the exclusion properties of the collagen in the buffer phase. Apparently dextran did not interact ionically with collagen, since the concentration of dextran in the collagen solution was not affected by the composition of the buffer.

Table 4. *Partition of chondroitin sulphate and dextran between an agarose gel and a collagen solution*

The initial polysaccharide concentration in the collagen solution applied to the gel is denoted C_0 ; C_e is the concentration after equilibrium had been attained.

<i>I</i>	pH	C_e/C_0		
		Chondroitin sulphate (fraction 4-B) in the absence of collagen	Chondroitin sulphate (fraction 4-B) in the presence of collagen	Dextran (D-4-B) in the presence of collagen
0.1	7.0	0.71	0.66	0.57
0.1	8.6	0.73	0.64	0.56
0.4	7.0	0.71	0.54	0.57
0.4	8.6	0.76	0.52	0.56

DISCUSSION

The interactions between collagen and glycosaminoglycans *in vivo* have been a subject of great interest in connective-tissue research, and many authors have presented more-or-less direct evidence that such interactions occur (see review by Jackson & Bentley, 1968). It is now generally accepted that these macromolecules do indeed interact *in vivo*; however, it is still doubtful whether steric effects and molecular entanglement are operating alone or in conjunction with attraction forces of some kind. Experiments suggesting that collagen binds with glycosaminoglycans under conditions close to those existing *in vivo* were first performed by Mathews (1965). He demonstrated that free electrophoresis of mixtures of soluble collagen with chondroitin sulphate, hyaluronate or heparin gave rise to an extra peak, which was ascribed to a reversible electrostatic complex between collagen and the polysaccharide. However, as pointed out by Davies, Nichol & Ogston (1963) the interactions between components in a dynamic system may not be the same as those present under other conditions. It thus seemed desirable to confirm Mathews' results for collagen-glycosaminoglycan by an alternative method. In the present work chromatographic and partition-equilibrium techniques have been used.

Chromatography of chondroitin sulphate on a collagen gel showed that the elution behaviour of the polysaccharide strongly depended on the ionic strength and pH of the medium. The increase in the elution volume observed on lowering the pH and ionic strength of the eluent indicates the formation of weak electrostatic bonds between the polysaccharide and the gel. At pH 8.6 and *I* 0.4, where the interaction was negligible for all the fractions, the chromatographic behaviour was mainly a function of steric exclusion, and the chondroitin sulphate was thus eluted according to size. From the distribution coefficients given in Table 3, all the chondroitin sulphate fractions formed complexes under physiological conditions (pH 7.4 and *I* 0.15).

Further, it is clear that the gel-binding depends on the molecular weight of the chondroitin sulphate, as the ratio $K/K_{\min}$ (see Table 3) increased with increasing molecular weight. This size-dependence, particularly evident at low pH and ionic strength, was also significant under physiological conditions.

Another phenomenon that might have influenced the elution of the polysaccharide is the effect of the ionic strength on the degree of coiling of the negatively charged chondroitin sulphate chains. Any change in size should, for steric reasons, result in a corresponding change in the elution volume. However, Wasteson (1971) has shown by chromatography of the polysaccharide on Sephadex G-200 that the values of Stokes radius for fraction 3 at *I* 0.4 and 0.1 were 42.5 and 45.5 Å respectively. Thus the difference in size at the two ionic strengths would be too small to influence the elution behaviour to any significant extent.

Chondroitin sulphate *in vivo* is covalently bound to protein (see e.g. Rodén, 1968) and it should therefore be of major interest to study the interactions between the proteoglycan and collagen. By use of model systems involving chondroitin sulphate proteoglycans and various kinds of collagen, a number of authors were able to demonstrate the formation of complexes of an ionic nature (Mathews, 1965; Mathews & Decker, 1968; Steven *et al.* 1969; Toole & Lowther, 1968).

The chromatographic experiments performed in the present study clearly indicated that PPL, like chondroitin sulphate under certain circumstances, forms an ionic complex with collagen gel. However, there are some differences in the behaviour of chondroitin sulphate and PPL which require further comment. Under conditions that least favoured binding of the proteoglycan to the gel all the material was eluted with the void volume, whereas under conditions favouring interaction some or all of the material applied to the column remained adsorbed to the gel. Thus, unlike chondroitin sulphate, the adsorbed material could not be eluted as long as pH and ionic strength were

constant. Complexes formed at low pH and ionic strength (pH 7.0 or 7.4 and $I 0.1$) did not dissociate even when the pH and ionic strength were raised to values where the complex could not be formed. This observation indicates that at low pH and ionic strength a very pronounced ionic association takes place between negatively charged groups on the glycosaminoglycan and positively charged groups on the collagen. A similar mechanism could account for the strong binding of PPL to collagen observed in the experiments of Steven *et al.* (1968, 1969). The fact that chondroitin sulphate, but not PPL, formed a complex with the gel at pH 7.4, $I 0.15$, might be due to the larger size of the PPL molecules than those of chondroitin sulphate. As the PPL fractions were apparently completely excluded from the gel it is probable that only a limited number of the charged groups could interact with the collagen side chains. The similarity in the elution properties of the two PPL fractions might be explained likewise.

The main objection to the use of the collagen gel is that the cross-linking of the collagen could have changed the charge distribution of the protein in such a way as to favour the observed electrostatic interaction with the negatively charged polysaccharide chains. However, this possibility seems highly unlikely since glutaraldehyde has been shown to react with the ϵ -amino groups of the lysine residues (Bowes & Cater, 1968a,b; Richards & Knowles, 1968), producing a compound that is slightly more acidic than the unchanged collagen (Bishop & Richards, 1968; Richards & Knowles, 1968). This was substantiated by the titration experiments with the collagen gel and the unmodified soluble collagen. The two titration curves were essentially identical between pH 7.0 and 8.6, and the collagen gel should therefore have the same net charge as the soluble collagen in this range. Furthermore, in the cross-linked collagen some of the ϵ -nitrogen atoms of the lysine side chains involved in the cross-links might for steric reasons be inaccessible to the polysaccharide chains. It thus seems reasonable to conclude that the electrostatic interaction observed between chondroitin sulphate and the collagen gel is paralleled by a similar interaction with unmodified collagen.

It should be pointed out that the preparation of the collagen included pepsin digestion, which may lead to the loss of some of the *N*-terminal amino acids (Piez, 1967). However, this modification should not alter the properties of the tropocollagen triple helix.

The partition equilibrium experiments (Table 4), which were designed to demonstrate that chondroitin sulphate and soluble collagen form an electrostatic complex, corroborate the results obtained by chromatography on the gel. However,

the partition of chondroitin sulphate in the system will depend on the degree of aggregation of the tropocollagen molecules, as such variations would influence the steric exclusion of the polysaccharide from the domain of the collagen (Laurent, 1968). Similarly those molecules that do not form complexes with collagen would also be affected. As the dextran fraction D-4-B was equally distributed at different ionic strengths and pH values, it is concluded that the observed variations in the concentration of chondroitin sulphate were a function of attractive forces between this polysaccharide and the collagen.

An attempt was made to determine the fibre parameters of the collagen gel by chromatography of chondroitin sulphate fractions, differing in size, under conditions precluding ionic interaction. According to Laurent & Killander (1964) and Siegel & Monty (1966) a plot of $(-\ln K_{av})^{1/2}$ against r_s should intercept the r_s axis at $-r_s = r_f$ (K_{av} is the partition coefficient for a molecule equilibrated between a gel and a buffer; r_s is Stokes radius of this molecule and r_f the radius of the gel fibres). However, when such plots were made, by using the values of the partition coefficients obtained at pH 8.6 and $I 0.4$ (Table 3), the curves invariably passed through the origin or to a value corresponding to a negative fibre radius. The same phenomenon was observed when various molecular-weight fractions of Ficoll were chromatographed on the collagen gel (B. Öbrink, unpublished work). This observation suggests that, although the electrostatic interaction was abolished, some kind of weak interaction between the polysaccharides and the collagen gel, other than the steric exclusion, must have been operative. The nature of this interaction is unknown.

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