Equilibrium-Binding Studies of Pig Laryngeal Cartilage Proteoglycans with Hyaluronate Oligosaccharide Fractions

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The binding of hyaluronate oligosaccharide fractions to proteoglycans from pig laryngeal cartilage has been studied by equilibrium dialysis in dilute solution. It has been shown that: (1) each proteoglycan monomer binds only one hyaluronate oligosaccharide molecule [containing about eighteen saccharide residues ($\text{HA}_{\sim 18}$) and of number-average molecule weight ($\bar{M}_n$) 3750]; (2) the dissociation constant, K_d , for interaction between proteoglycan monomer and oligosaccharide $\text{HA}_{\sim 18}$ is $3 \times 10^{-8} \text{ M}$ at 6°C at I 0.15–0.5, pH 7.4; (3) the dissociation constant has little dependence on temperature, so that K_d at 54°C is $3 \times 10^{-7} \text{ M}$ under the same conditions; (4) the aggregatability is high at 6°C , falls significantly at 54°C , but much of it can be recovered on cooling to 6°C again, demonstrating reversible denaturation; (5) a method for determining the proportion of the proteoglycan molecules capable of binding to hyaluronate by equilibrium dialysis was compared with gel-chromatographic and ultracentrifugal methods; (6) a hyaluronate oligosaccharide, $\text{HA}_{\sim 56}$ ($\bar{M}_n$ 11000), could bind more than one proteoglycan molecule; (7) consideration of ultracentrifugal data shows that when proteoglycans bind to a hyaluronate of larger size (mol.wt. 670000), an average K_d of $2 \times 10^7 \text{ M}$ fits the data in 0.5 M-guanidine hydrochloride at 20°C .

Cartilage proteoglycans display a specific interaction with hyaluronate (Hardingham & Muir, 1972). This interaction is mediated via a hyaluronate-binding region of mol.wt. 60000 (Heinegård, 1977) that occurs at one end of the proteoglycan core protein and that displays a high affinity for a deca-saccharide unit of hyaluronate (Hardingham & Muir, 1973; Hascall & Heinegård, 1974*b*). Most proteoglycans of cartilage are believed to occur in the matrix as large multimolecular aggregates (Hascall & Sajdera, 1969) involving, perhaps, 100 proteoglycan molecules bound through specific, but non-covalent, interactions with hyaluronate (Hardingham & Muir, 1972, 1973, 1974; Hascall & Heinegård, 1974*a,b*). No detailed studies of the binding behaviour of hyaluronate to proteoglycan have been reported, although the oligosaccharide-competition studies of Hardingham & Muir (1973) would suggest a dissociation constant that is approximately micromolar, and Christner *et al.* (1978) have investigated the interactions of proteoglycan core proteins with matrix-bound hyaluronate and noted an association constant of $1.8 \times 10^7 \text{ M}^{-1}$.

The present study addresses itself to the equilibrium-binding behaviour of hyaluronate towards pig laryngeal cartilage proteoglycans. The experiments have been carried out by using two oligosaccharide fractions of hyaluronate capable of

binding on average, one and two proteoglycan molecules respectively.

Materials and Methods

Proteoglycans

Proteoglycans from pig laryngeal cartilage were extracted in 4 M-guanidine hydrochloride and further purified by CsCl-density-gradient sedimentation as described in detail by Hardingham *et al.* (1976). The characteristics of each sample are shown in Table 1, and preparation A is further described in Sheehan *et al.* (1978). In addition to these A1D1D1/2 preparations (termed A, B and C), which were used for the equilibrium-dialysis experimentation, an A1–D1 preparation with a number-average molecular weight ($\bar{M}_n$) of 1630000 was used for the ultracentrifugal study that was previously reported by Hardingham (1979).

Hyaluronate fractions

Several hyaluronate fractions were used in the present study. An oligosaccharide, HA_{12} (i.e. containing twelve saccharide residues), was received as a gift from Dr. V. C. Hascall, National Institutes of Health, Bethesda, MD, U.S.A. This oligosaccharide

Table 1. *Physical characteristics of proteoglycan preparations*

Samples A and C are from the same preparation. $\bar{M}_w$ values were determined by light-scattering; $\bar{M}_n$ values were assumed (see the text). Aggregatabilities were determined by equilibrium-dialysis (see the text), ultracentrifugal and gel-filtration studies at room temperature.

Sample	Density (g/ml)	$10^{-6} \times \bar{M}_w$	$10^{-6} \times \bar{M}_n$	Aggregatability (%)
A A1D1D1	1.914	2.5	2.0	80
B A1D1D1	1.885	2.5	2.0	55
C A1D1D2	1.821–1.914	2.4	2.0	90

had been prepared by partial testicular-hyaluronidase digestion of a [^3H]glucosamine-labelled hyaluronate. Two further oligosaccharide samples were prepared for equilibrium-dialysis studies. Cock's-comb hyaluronate (a gift from Pharmacia) was degraded by ascorbate. Solid ascorbic acid was added to a sodium hyaluronate solution (1 mg/ml) to a final concentration of 5 mM and the pH was adjusted to 5.5 by addition of 3 M-NaOH. A trace of CuSO_4 ($<1 \mu\text{M}$ in the final solution) was added and the solution was left stirring for 24 h at 30°C . The solution was loaded on to a column (1000 mm $\times$ 12 mm; $V_0 = 52.8 \text{ ml}$, $V_t = 143 \text{ ml}$) of Sephadex G-100 equilibrated with 0.2 M-NaCl and eluted at 22 ml/h. The eluate was collected into seven fractions, and two of these were re-run under identical conditions and fractions eluted with K_{av} between 0.29 and 0.46 and between 0.58 and 0.75 were pooled separately. The fractions were then radiolabelled with NaB^3H_4 . To a 5 mg/ml solution (pH 9.0, unbuffered) of each fraction, solid NaB^3H_4 (8 mCi; 749 mCi/nmol) was added and the solutions were left for 6 h. Extensive dialysis four times, followed on each occasion by freeze-drying ensured that no contaminating free label remained. The $\bar{M}_n$ values for these fractions were determined by separate experiments on columns of Sephadex G-50 and G-100, which were calibrated with hyaluronate oligosaccharides prepared as described below. Sodium hyaluronate (100 mg) from cock's comb was dissolved in 20 ml of 0.15 M-NaCl/0.2 M-sodium acetate, pH 5.5, and 10 mg of testicular hyaluronidase (Miles-Seravac) were added and the mixtures stirred for 3 h at 34°C . The solution pH was adjusted to 9.0 with 3 M-NaOH and boiled to precipitate the enzyme, which was removed by centrifugation. Solid NaB^3H_4 (8 mCi; 749 mCi/nmol) was added to the supernatant. After 2 h the solution was loaded on to a preparative column (1550 mm $\times$ 30 mm; $V_0 = 342 \text{ ml}$, $V_t = 900 \text{ ml}$) of Sephadex G-50 (superfine) and the included volume was eluted into 100 fractions.

As described by Hascall & Heinegård (1974b), the positions of the individual oligosaccharides could be clearly identified as far as oligosaccharide HA_{20} , with tentative assignments to oligosaccharide HA_{26} assuming the smallest abundant peak to contain oligosaccharide HA_4 . A plot of K_{av} against $\log(\text{molecular weight})$ for the oligosaccharide peaks is linear from

oligosaccharide HA_4 to HA_{20} , and the individual oligosaccharides of known molecular weight were used to yield a linear calibration of Sephadex G-50 and G-100 columns. The $\bar{M}_n$ values for the two fractions to be used for equilibrium dialysis could be determined on these calibrated columns from the distribution of radioactivity, since the hyaluronate chains were B^3H_4^+ end-reduced. The pooled oligosaccharide fraction that eluted between K_{av} values of 0.58 and 0.75 on Sephadex G-100 was shown to have a fairly broad distribution on Sephadex G-50, with $>90\%$ of the sample between oligosaccharides HA_{12} and HA_{36} and $\bar{M}_n$ 3750, corresponding approximately to oligosaccharide HA_{18} . The other fraction, which had eluted between K_{av} values of 0.29 and 0.46 on Sephadex G-100 was analysed on the calibrated analytical Sephadex G-100 column and shown to have $>90\%$ of the sample between oligosaccharides HA_{36} and HA_{76} with $\bar{M}_n$ 11 000, corresponding approximately to oligosaccharide HA_{56} . These fractions will be referred to as ' $\text{HA}_{\sim 18}$ ' and ' $\text{HA}_{\sim 56}$ '.

The specific radioactivities of the hyaluronate oligosaccharide fractions were determined to be 300 c.p.m./ μg for oligosaccharide HA_{12} , 9.91 c.p.m./ng for fraction $\text{HA}_{\sim 18}$ and 5.89 c.p.m./ng for fraction $\text{HA}_{\sim 56}$. Samples, dried over CaCl_2 at 80°C in a vacuum oven, were weighed out on a Cahn model C (Cahn Instruments Co., Paramount, CA, U.S.A.) microbalance, and standard stock solutions were made up. Subdilution of these showed a linear response with concentration from 0 to 100 000 c.p.m., which was a larger range than that used in the experimentation. Counting efficiency was shown to be constant at 60% and no quench corrections were required. Consequently hyaluronate concentrations were determined directly from measurements of ^3H radioactivity.

Two other hyaluronate preparations were used in the present study. A hyaluronate (Miles-Seravac) from human umbilical cord was purified from chondroitin sulphate contaminants by cetylpyridinium chloride precipitation (Scott, 1960), and its weight-average molecular weight ($\bar{M}_w$) was shown by light-scattering to be 300 000. This sample was used for the column-chromatographic estimation of proteoglycan aggregatability. Another sample of

hyaluronate from cock's comb of mol.wt. 670000 was described by Hardingham (1979).

Equilibrium dialysis

Proteoglycan solutions (1.2 ml) at a fixed concentration (0.5–2.0 mg/ml) were loaded into one side of several MSE Dianorm Macro D1000 cells fitted with a solvent-imbibed Millipore membrane. The other side was filled with solutions of the radioactive hyaluronate oligosaccharides at various relative molar concentrations between 0.1 and 2.0.

The cells were sealed and then tumbled with a slow rotary motion until full equilibration had been achieved. Millipore membranes of pore size 0.1 μm (type VC) and 0.22 μm (type GS) were used for fractions HA_{~18} and HA_{~56} respectively. In each case independent experiments using only the hyaluronate oligosaccharide fractions showed that full equilibration across the membrane was achieved within 48 h, thus all experiments proceeded for at least 50 h. The leakage of proteoglycan, monitored by detection of uronic acid with orcinol, was always less than 2%, and this material was assumed to be inactive. Finally, portions (50 μl –1 ml) were removed, made up to 2 ml with water and added to 3 ml of scintillant. The scintillant was prepared by adding 1 vol. of the detergent Synperonic NXP (Cargo Fleet Chemical Co., Eaglescliffe, Stockton-on-Tees, Cleveland, U.K.) to 2 vol. of scintillation-grade toluene. To this mixture 4 g of 2,5-diphenyloxazole/litre and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/litre were stirred in. The fully mixed samples were counted for radioactivity in a Packard Tri-Carb model 3375 liquid-scintillation counter.

Other techniques

Gel chromatography on a column (340 mm $\times$ 9.5 mm) of Sepharose CL-2B was performed at 1.5 ml/h in 0.3 M-NaCl/1 mM-phosphate/1 mM-EGTA/0.02% azide, pH 7.4, at 20°C and the fractions were analysed for uronic acid with orcinol (Brown, 1946). The proportion of proteoglycan able to aggregate was estimated with a du Pont 310 (E. I. du Pont de Nemours and Co., Wilmington, DE, U.S.A.) curve analyser by using Gaussian component profiles as follows. Both the hyaluronate-promoted aggregate elution profile and the unperturbed proteoglycan elution profile were simulated and subsequently the latter profile was scaled in relative intensity to yield a best fit with the tail of the aggregate profile. By subtraction of the relative area of this scaled 'non-aggregatable' component from the total area within the aggregate envelope the proportion of aggregatable material was estimated. This method is likely to yield a minimum value for reasons discussed below.

Light-scattering experiments were performed with a Sofica model 42000 photo-gonio-diffusometer (Société Française d'Instruments de Contrôle et d'Analyses, 78 Le Mesnil-Saint-Denis, France) as previously described by Sheehan *et al.* (1978).

The sedimentation-velocity experiments on an MSE Centriscan centrifuge were as previously described by Hardingham (1979).

Results

Equilibrium dialysis was carried out with macromolecular proteoglycan and radioactive diffusible oligosaccharide fractions of hyaluronate. In order to analyse the results it was necessary to know the $\bar{M}_n$ values for the proteoglycan and oligosaccharide, and also to know the stoichiometry of the interaction. The HA_{~18} fraction was chosen for study as it contained only molecules larger than the minimum size required for binding to proteoglycan (oligosaccharides HA₉ or HA₁₀) (Hardingham & Muir, 1973; Hascall & Heinegård, 1974b). It was also too small to bind more than one proteoglycan molecule, and this was shown in two ways. Firstly, equilibrium-dialysis experiments were performed in 0.3 M-NaCl, phosphate-buffered to pH 7.4, by using 15 dialysis cells containing molar ratios of fraction HA_{~18}/proteoglycan between 20:1 and 1:25, and the resulting Scatchard plot yielded a single straight line, showing that no hyaluronate molecules capable of interacting with two proteoglycans were present. This experiment also demonstrates no evidence for weaker sites in addition to the one studied. Secondly, addition of increasing amounts of fraction HA_{~18} to proteoglycan solutions (0.4 mg/ml) on 0.3 M-NaCl phosphate-buffered to pH 7.4, showed no increases in the intensity of the light scattered at an angle of 30°. Light-scattering is highly sensitive to large particles and therefore shows that the HA_{~18} fraction contains no significant proportion of molecules capable of binding to two proteoglycans. The HA_{~18} preparation was thus univalent in its binding to proteoglycans and its $\bar{M}_n$ was determined to better than 10% accuracy by the methods described above.

The $\bar{M}_n$ of the proteoglycan was estimated from light-scattering determinations of $\bar{M}_w$. The quantities of proteoglycan available precluded the preparation of samples with sharper size distribution than those found in the A1D1D1-fractionation procedure. This involves two dissociative density-gradient runs and was expected to yield a preparation less polydisperse than an A1D1 preparation, as a proportion of small proteoglycans of higher protein content is removed from it in the second gradient (Hardingham *et al.*, 1976). Previous molecular-weight estimates for the A1D1 preparation were $\bar{M}_w$ 2400000 and $\bar{M}_n$ 1630000 (Hardingham *et al.*, 1976), so an $\bar{M}_n$ of 2000000 was estimated for the

A1D1D1 preparations used in the present study. This is probably an upper limit, as $\bar{M}_n$ must be less than $\bar{M}_w$ for a polydisperse preparation.

For the initial analysis the proteoglycan was assumed to be monovalent, as predicted by the model of proteoglycan aggregation (Muir & Hardingham, 1975). The binding of the hyaluronate oligosaccharide fraction $HA_{\sim 18}$ (HA) could thus be treated as a single-site interaction with proteoglycan (PG) of the form:

and the dissociation constant for this reaction:

$$K_d = \frac{[HA][PG]}{[HA-PG]}$$

In the present studies, hyaluronate is the radioactive diffusible species. Therefore let

$$[HA]_{\text{bound}} = [HA-PG] = B$$

and

$$[HA]_{\text{free}} = F$$

In terms of the measured parameters B and F

$$K_d = \frac{F}{B} (n - B)$$

where n is the concentration of hyaluronate-binding sites therefore

$$B = -K_d \frac{B}{F} + n \quad (1)$$

and the results were thus plotted as B against B/F, and K_d was determined from the slope of the plot and the intercept was a measure of the total concentration of hyaluronate-binding sites.

By using this graphical analysis the dissociation constant K_d for the hyaluronate-proteoglycan complex at a temperature of 6°C was found to be $3(\pm 2) \times 10^{-8} M$ in 0.15M-NaCl, or 0.3M-NaCl and 0.5M-NaCl, all at pH 7.4 (see Fig. 1 and Table 2). There was no significant variation in K_d with preparation or with proteoglycan concentration and little evidence for dependence on ionic strength within the range 1.0–0.50. Furthermore a similar K_d was obtained with the monodisperse HA_{12} preparation.

So far it has been assumed that the proteoglycan binds only one $HA_{\sim 18}$ oligosaccharide, but this assumption could also be tested in these experiments. The amount (mol) of hyaluronate-binding sites/mol of active proteoglycan, N, can be calculated from the measurement of the total concentration of binding sites for hyaluronate (n) and the proteoglycan concentration as:

$$N = \frac{n}{[PG]}$$

Fig. 1. Typical Scatchard-type plot showing the binding of proteoglycan (preparation A, 2mg/ml) to several concentrations of fraction $HA_{\sim 18}$ (a broad distribution from oligosaccharide HA_{12} to HA_{36} with $\bar{M}_n$ corresponding to polysaccharide HA_{18} , of oligomers that can only bind to one proteoglycan molecule) in 0.3M-NaCl/1M-phosphate/1mM-EGTA/0.02% azide, pH 7.4 at 6°C

B is the concentration of bound hyaluronate. B/F is the ratio of bound to free hyaluronate.

However, the proteoglycan preparations always contain some molecules that are unable to bind to hyaluronate. The active concentration of proteoglycan is therefore somewhat less than the total concentration. The proportion of proteoglycans active in binding must thus be allowed for in the calculation:

$$N = \frac{n}{[PG]_{\text{active}}} = \frac{n}{[PG]_{\text{total}}} \times \frac{[PG]_{\text{total}}}{[PG]_{\text{active}}}$$

The first term $n/[PG]_{\text{total}}$ is determined from equilibrium dialysis, and N can thus be calculated if there is an independent means of determining the proportion of proteoglycan that is active in binding. The results of gel chromatography of proteoglycans with and without added hyaluronate provides a measure of this (Hardingham & Muir, 1974), and by this means proteoglycan preparation C was found to be > 80% active (Fig. 2). Equilibrium dialysis with this preparation at 6°C showed a maximum value for the fraction bound, $n/[PG]_{\text{total}}$, of 0.9 (Table 2). Therefore

$$N = 0.9 \times \frac{1}{0.8} = 1.1$$

The amount (mol) of hyaluronate bound/mol of active proteoglycan is most likely to be integral and is therefore one.

This being so, the value of n from the Scatchard-type plots is a measure of the concentration of active proteoglycans and enables the proportion of active proteoglycan in the preparations under various

Table 2. *Proteoglycan-hyaluronate equilibrium-binding data*

Experiments linked by braces are part of a continuous individual experiment and are presented in chronological order. Aggregatabilities (expressed as percentages) were determined from the binding data.

Proteoglycan sample	[Proteoglycan] (mg/ml)	Solvent	Hyaluronate	Temperature (°C)	<i>K_d</i> (M)	Aggregatability (%)
A	2.0	0.3M-NaCl	HA ₁₈	45	2.1×10 ⁻⁷	78
	2.0	0.3M-NaCl	HA ₁₈	18	2.4×10 ⁻⁸	92
	2.0	0.15M-NaCl	HA ₁₈	18	2.0×10 ⁻⁸	79
	2.0	0.3M-NaCl	HA ₁₈	6	4.8×10 ⁻⁸	92
	2.0	0.3M-NaCl	HA ₁₈	18	1.7×10 ⁻⁸	78
	2.0	0.3M-NaCl	HA ₁₈	24.5	4.2×10 ⁻⁸	91
	2.0	0.3M-NaCl	HA ₁₈	6	5.0×10 ⁻⁸	91
	0.5	0.3M-NaCl	HA ₁₈	18	1.5×10 ⁻⁸	69
B	1.0	0.3M-KCl	HA ₁₈	21	5.3×10 ⁻⁸	53
	1.0	0.3M-NaCl	HA ₁₂	6	2.9×10 ⁻⁸	43
	2.0	0.3M-NaCl	HA ₁₈	41	2.6×10 ⁻⁷	60
	2.0	0.3M-NaCl	HA ₁₈	54	3.6×10 ⁻⁷	30
	0.93	0.3M-NaCl	HA ₁₈	54	1.1×10 ⁻⁷	21
	0.93	0.3M-NaCl	HA ₁₈	40	7.0×10 ⁻⁸	29
	0.93	0.3M-NaCl	HA ₁₈	21	6.2×10 ⁻⁸	39
	0.93	0.3M-NaCl	HA ₁₈	6	1.2×10 ⁻⁷	49
C	1.16	0.15M-NaCl	HA ₁₈	6	3.5×10 ⁻⁸	109
	1.16	0.15M-NaCl	HA ₁₈	54	4.7×10 ⁻⁷	29
	1.16	0.15M-NaCl	HA ₁₈	6	4.2×10 ⁻⁸	72
	1.16	0.5M-NaCl	HA ₁₈	6	1.7×10 ⁻⁸	88
	1.16	0.5M-NaCl	HA ₁₈	54	4.3×10 ⁻⁸	28
	1.16	0.5M-NaCl	HA ₁₈	6	1.7×10 ⁻⁸	69

conditions to be calculated (Table 2). The aggregatability is defined as the ratio $[PG]_{active}/[PG]_{total}$ and, as $N = 1$, is equal to the intercept on the $B/[PG]_{total}$ axis (see Fig. 1). There was considerable variation in the proportion that was active from one preparation to another (Table 2), but when it was followed with changing temperature in a single preparation (see Fig. 3), it was found that although the affinity of proteoglycan decreases from $3 \times 10^{-8} M$ to $3 \times 10^{-7} M$ in going from 6 to 54°C, the proportion active in binding decreased by up to 70%. Much of the loss of binding on heating thus appeared to result from a reversible denaturation involving a loss of activity of the hyaluronate-binding site on the proteoglycan.

The binding of the larger hyaluronate oligosaccharide fraction to proteoglycan was investigated as this was expected to contain a high proportion of molecules able to bind two proteoglycans. The two-stage addition of proteoglycans to an oligosaccharide can be described by two dissociation constants:

$$K_{1d} = \frac{[HA][PG]}{[HA-PG]} \quad K_{2d} = \frac{[HA-PG][PG]}{[HA-PG_2]}$$

in which HA-PG₂ represents a hyaluronate oligosaccharide simultaneously binding two proteoglycan molecules, but as only total bound (B) and free hyaluronate (F) concentrations are measured, a

Fig. 2. *Elution profiles of proteoglycan (preparation C, 1 mg) (—) and the proteoglycan (preparation C, 1 mg) plus 50 µg of a hyaluronate (mol.wt. 3 000 000) (---) monitored at 670nm for uronic acid with orcinol, and run in 0.3M-NaCl/1 mM-phosphate/1 mM-EGTA/0.02% azide, pH 7.4, at 20°C on a column (340 mm × 9.5 mm) of Sepharose CL 2B*

Fig. 3. Scatchard-type plots representing the binding of proteoglycan (preparation C, 1.16 mg/ml) to several concentrations of fraction $HA_{\sim 18}$.

Portions were taken from the same dialysis cells run (i) at 6°C, (ii) temperatures raised to 54°C and (iii) decreased to 6°C again and run respectively in: (a) 0.15 M-NaCl/1 mM-phosphate/1 mM-EGTA/0.02% azide, pH 7.4; (b) 0.50 M-NaCl/1 mM-phosphate/1 mM-EGTA/0.02% azide, pH 7.4. B is the concentration of bound hyaluronate. B/F is the ratio of bound to free hyaluronate.

single equation in B and F similar to eqn. (1) must be formulated for the analysis. It can be shown that:

$$B = \frac{-K_{1d}K_{2d}}{K_{2d} + (\alpha n - B)F} + \alpha n \quad (2)$$

where α represents the apparent change in stoichiometry of proteoglycan for hyaluronate as it changes from 1:1 ($\alpha = 1$) to 2:1 ($\alpha = 0.5$).

Plotting B against B/F yields a curve. Inspection of eqn. (2) shows two limiting conditions that are of value for the analysis:

(a) When $HA \gg PG$, $[HA-PG_2] \rightarrow 0$; $\alpha \rightarrow 1$, eqn. (2) reduces to eqn. (1):

$$B = -K_{1d} \frac{B}{F} + n$$

(b) When $PG \gg HA$, $B \ll n$; $B \rightarrow [HA-PG_2]$; $\alpha \rightarrow \frac{1}{2}$, eqn. (2) reduces to:

$$B = -\frac{2K_{1d}K_{2d}}{2K_{2d} + n} \frac{B}{F} + \frac{n}{2}$$

Therefore, in case (a), the limiting slope and the intercept on the ordinate yield the same information as would be obtained with data from a hyaluronate oligosaccharide capable of binding one proteoglycan molecule. In case (b) the limiting slope as B/F goes to infinity is

$$-\frac{2K_{1d}K_{2d}}{2K_{2d} + n}$$

from which a value of K_{2d} can be obtained. The intercept yields the value of α .

The results of experiments with fraction $HA_{\sim 56}$ are plotted as B/F against B in Fig. 4. It is clear that it cannot be represented by a single straight line, and its shape confirms that these oligosaccharides can bind more than one proteoglycan molecule. The limiting slope in Fig. 4 as $B/F \rightarrow 0$ yields $K_{1d} \approx 4.2 \times$

Fig. 4. Scatchard-type plot corresponding to the binding of proteoglycan (preparation B, 1 mg/ml) to fraction $HA_{\sim 56}$ ($\bar{M}_n$ 11 000), a substantial proportion of which could bind to two proteoglycan molecules in 0.3 M-NaCl/1 mM-phosphate/1 mM-EGTA/0.02% azide, pH 7.4 at 25°C.

The steeper line yields a $K_1 \approx 4.2 \times 10^{-8}$ M, a value similar to the K_d values obtained with oligosaccharides HA_{12} and $HA_{\sim 18}$. The slope of the other line would, in principle, enable K_{2d} to be evaluated if the $HA_{\sim 56}$ preparation contained no molecules capable of binding to more than two proteoglycan molecules. The value of the intercept on the ordinate, however, indicates that a proportion of the $HA_{\sim 56}$ preparation does not satisfy this condition.

10^{-8} M, which, within experimental error, is identical with the dissociation constants obtained with oligosaccharide HA₁₂ and fraction HA_{~18}. However, as B/F becomes large, the limiting line obtained yields an intercept on the ordinate that is less than half of the other intercept, that is, α is $< \frac{1}{2}$. This suggests that a proportion of the HA_{~56} fraction can bind to more than two proteoglycan molecules, and consequently K_{2d} cannot be reliably estimated.

We decided to determine whether the dissociation constants obtained with hyaluronate oligosaccharide fractions were representative of the values obtainable with larger hyaluronate molecules such as might be found in cartilage. To study this, controlled quantities of a fractionated hyaluronate (mol.wt. 670000) were added to an A1-D1 preparation of proteoglycan and the proportion of aggregate formed was determined by analytical ultracentrifugation (Hardingham, 1979) under the conditions described in the legend to Fig. 5. The calculated curves in Fig. 5 assume an equivalent weight of 10000 per site for hyaluronate and that all sites are identical and independent, so that an average K_d value may be used. Two calculated curves are shown, one for $K_d = 6 \times 10^{-8}$ M and another for $K_d = 2 \times 10^{-7}$ M, and the latter most closely fits the ultracentrifugal data. This dissociation constant is within an order of magnitude of that obtained in dilute NaCl solutions by equilibrium dialysis.

Discussion and Conclusion

Dilute-solution equilibrium-binding studies confirm a strong interaction between hyaluronate and cartilage proteoglycans so that the dissociation constant, K_d , at physiological ionic strength and temperature would be expected to be 10^{-7} M. It is noteworthy that the K_d of 10^{-8} – 10^{-7} M found here for the hyaluronate-intact-proteoglycan interaction is very similar to the association constant of 1.8×10^7 M⁻¹ found by Christner *et al.* (1978) for the interaction of hyaluronate with proteoglycan core protein. Since the present paper was originally submitted, Cleland (1979) has shown that proteoglycans from bovine nasal septum form 1:1 complexes with hyaluronate oligosaccharides giving association constants of $1 \mu\text{M}^{-1}$ for oligosaccharides HA₄–HA₈ and of $50 \mu\text{M}^{-1}$ for oligosaccharides HA₁₀–HA₁₄.

A method for the determination of proteoglycan aggregatability by equilibrium binding has been described that yields an independent measure of the K_d and the aggregatability. The ultracentrifugal and gel-chromatographic techniques for determining aggregatability depend on a physical separation of the hyaluronate-proteoglycan aggregate from the non-aggregating proteoglycan. However, the proportion of aggregate found is also a function of the dissociation constants, which therefore set definite

Fig. 5. Experimental data (●) obtained from analytical ultracentrifugation of link-free aggregates by the addition of controlled amounts of a hyaluronate sample (mol.wt. 670000) to an A1-D1 preparation of proteoglycans (Hardingham, 1979)

The samples were run in 0.5 M-guanidine hydrochloride/0.05 M-sodium acetate/1 mM-EDTA, pH 5.8, at 400 μ g of uronic acid/ml at 20000 rev./min and 20°C. The calculated points were obtained by using the following assumptions: proteoglycan $\bar{M}_n$ 1 630 000 (Hardingham *et al.*, 1976); hyaluronate mol.wt. 670000; proteoglycan aggregatability 58% (i.e. 58% of the proteoglycan molecules were capable of interacting with hyaluronate); total proteoglycan concentration, 1.6 mg/ml; hyaluronate concentrations were derived from the uronic acid concentrations. The two most important assumptions were that all sites on hyaluronate were identical and independent and correspond to an equivalent weight of 10000, so that calculated curves (□, $K_d = 2 \times 10^{-7}$ M; ○, $K_d = 6 \times 10^{-8}$ M) depended entirely on the average K_d assumed.

limits on the concentrations of reactants to be used in such experiments. In the gel-chromatographic method the physical separation of the aggregate from the free proteoglycan with which it is in equilibrium is an additional factor that causes underestimation of aggregatability.

In the present study, equilibrium dialysis has been used to measure aggregatabilities for given proteoglycan preparations under different conditions. However, for a fully aggregatable proteoglycan preparation and a well-defined hyaluronate oligosaccharide the same experimental system would yield a measure of the $\bar{M}_n$ for the proteoglycan distribution.

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