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Experimental Determination of the Kinetics of Calcium-Binding with Chondroitin Sulphate and the Effects of Uric Acid on this Process

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Summary. The calcium-binding kinetics of chondroitin sulphate C (*CS*) have been determined using equilibrium analysis including ^{45}Ca. There is a linear relationship between the extent of the Ca binding and the concentration of *CS* present. 1 μmol *CS* disaccharide unit binds 0.757 μmol Ca. Scatchard plots of the data have revealed a *single* constant of dissociation ($K_D = 0.1429$). In the presence of urate ions, and dependent on the pH value, the ability of *CS* to bind Ca may be impaired by as much as 31%. These measurements have supported the theory that urate ions interact with the GAGs in urine.

Key words: GAGs, Ca-binding, Interaction with uric acid.

Introduction

Numerous experiments have proved that certain disaccrides of the glycosaminoglycans (GAGs) inhibit the crystallization of calcium oxalate [3, 9, 11, 22, 24]. This inhibitory effect has been associated with the fact that GAGs bind calcium.

A number of other authors [2, 4, 17, 26] has been unable to confirm the assertion that GAG excretion is less marked in patients suffering from calcium oxalate calculi than is the case with healthy persons [1, 23].

One important point here is that high concentrations of uric acid and urate have been reported to impair the inhibitory effect of GAGs [12, 19], although these findings, too, are not undisputed.

In the series of experiments described in this article we investigated the manner in which chondroitin sulphate C binds calcium and determined the degree to which this process was affected by the action of uric acid.

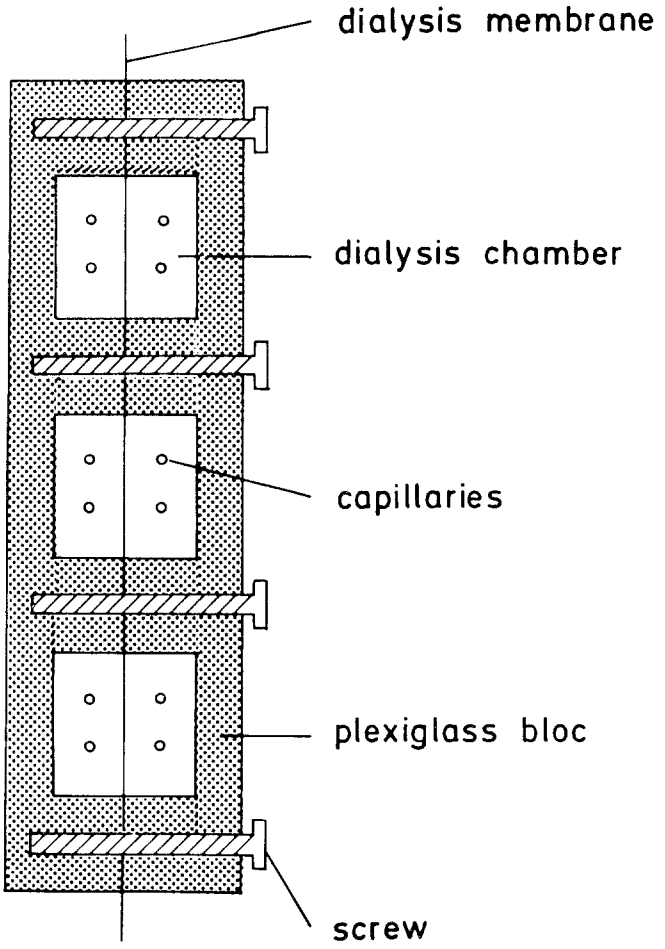

Fig. 1. System for equilibrium dialysis

Material and Methods

Measurement of the Rate of Calcium Binding to CS

Our investigations into calcium binding were based on the principle of equilibrium dialysis [5, 7, 8]. A blue-print copy of the dialysis chamber employed in our experiment is illustrated in Fig. 1. In each

case two diametrically opposed chambers (sample chamber and Ca-chamber) were separated by a dialysis membrane (cellulose acetate) with a nominal molecular size exclusion limit of 1,000 Daltons. Prior to use, the dialysis membrane was washed with demineralised water for at least 4 h. The chambers were filled using inserted capillary tubes and 1 ml disposable syringes. Each chamber had a volume of 1.3 ml. The sample solutions of calcium were prepared from an aqueous $CaCl_2$ stock and were individually labelled with ^{45}Ca (0.8 Ci). They were then added to each charge. After taking the radioactivity readings, 1 ml of each charge was introduced into the calcium chamber. 1 ml of the chondroitin sulphate C solution was then injected into the sample chamber lying opposite the calcium chamber.

Once both chambers had been filled they were vibrated at room temperature for a period of 18–20 h, after which a state of equilibrium was achieved and samples were extracted from each chamber in order to measure their radioactivity levels. The difference in radioactivity level between the sample chamber and the calcium chamber indicated the amount of calcium bound (*B*). The radioactivity in the calcium chamber corresponded to the amount of free calcium remaining (*F*) in the two chambers.

Since the level of radioactivity of the injected calcium (*E*) was known, the precise distribution of the calcium could be determined. The concentrations of bound (*B*) and free (*F*) calcium were recorded for each calcium concentration used in order to calculate the quotient *B/F*. The parameters *B* and *B/F* were then plotted – the "Scatchard plot" [25]. The shape of the curve in this Scatchard plot permits conclusions to be drawn regarding the number and affinity of the binding sites and enables their dissociation constants K_D to be determined [5, 7, 14, 25].

Migration of the calcium during the course of dialysis produces an equilibrium

$$P + L \rightleftharpoons P - L \quad P\text{: polymer } (CS),\ L\text{: ligand } (Ca^{2+})$$

such that the constant of dissociation (K_D) can be determined according to the Law of Mass Action:

$$K_D = \frac{(Ca^{2+})_n\ (CS^{2-})_n}{(CS - Ca)_n}$$

The quantity of free (*F*) and bonded (*B*) calcium is determined from the results of the equilibrium dialysis. Thus we have:

$$K_D = \frac{F \times (n - B)}{B} \qquad n = \text{number of binding points}$$

Solving the equation for *B/F* produces a first-degree equation of gradient $1/K_D$ and ordinate value n/K_D

$$\frac{B}{F} = \frac{(n - B)}{K_D} = \frac{1}{K_D} \times B + \frac{n}{K_D}$$

If *B/F* is plotted as a function of *B*, a plane curve will indicate the presence of one binding point. If many and varied binding points are present a concave curve will result.

Inhibition of the Calcium-Binding Capacity of the Chondroitin Sulphate Using Uric Acid/Urate

The difference in the calcium-binding capacity of chondroitin sulphate with and without the addition of uric/urate was determined by adding sodium urate and uric acid solutions to both chambers of the equilibrium dialysis systems.

The following Na-urate and uric acid stock solutions were employed:

I. 4.6 mmol/l sodium urate, pH 6.8
II. 4.07 mmol/l uric acid, pH 6.0
III. 4.6 mmol/l uric acid, pH 8.0

Table 1. The extent of Ca binding at different concentrations of chondroitin sulphate expressed in percent ($n = 10$, $^{+}n = 20$, $E = 0.912$ µmol/1050 µl)

Chondroitin sulphate concentration (µmol/1050 µl)	Calcium binding (%)		*CV* (%)
	$\bar{x}$	SD	
0.0099	0.54	0.27	50.0
0.0198^{+}	1.54	0.44	28.2
0.0298^{+}	2.03	0.62	30.5
0.0397^{+}	2.69	0.69	25.6
0.0497	3.38	0.18	5.3
0.0596	3.89	0.54	13.8
0.0696	4.71	0.42	8.9
0.0795	5.41	0.57	10.5
0.0894	6.17	0.32	5.2
0.0994^{+}	6.67	0.41	6.1
0.1093	7.44	0.50	6.7
0.1193	8.05	0.42	5.2
0.1491	10.31	0.56	5.4
0.1988	13.15	0.55	4.2
0.2485	16.78	0.64	3.8
0.2982	19.66	0.61	3.1
0.3976	25.79	0.57	2.2
0.4970	31.28	0.90	2.8
0.5964	37.00	0.54	1.4
0.6958	42.62	0.87	2.0

Reagents

$CACl_2$ (Merck)

$^{45}CaCl_2$ (Amersham Buchler); ^{45}Ca in aqueous solution with a specific activity of 10–40 mCi/mg Ca. Dilution: 0.8 µCi/100 µl.

Scintillation-Cocktail 299^{TM} (Packard)

Chondroitin sulphate C (Di-Na-salt) (Sigma): theoretical molecular weight of the disaccharide unit = 503

Sodium hydrogen urate (Sigma)

Uric acid (Merck)

Abbreviations

E = employed calcium (µmol); *B* = bound calcium (µmol); *F* = free calcium (µmol); K_D = constant of dissociation; *CS* = chondroitin sulphate.

Results

The Percentage of Calcium-Binding Capacity of the CS with Increasing CS Concentration

Using a constant quantity of calcium ($E = 0.912$ µmol Ca/1050 µl measured volume) the rate of calcium binding increased in direct proportion to the increase in *CS* concentration.

As may be seen from the measurements obtained (Table 1), the degree of precision achieved with this method of measurement (*CV* = coefficient of variation) is very poor

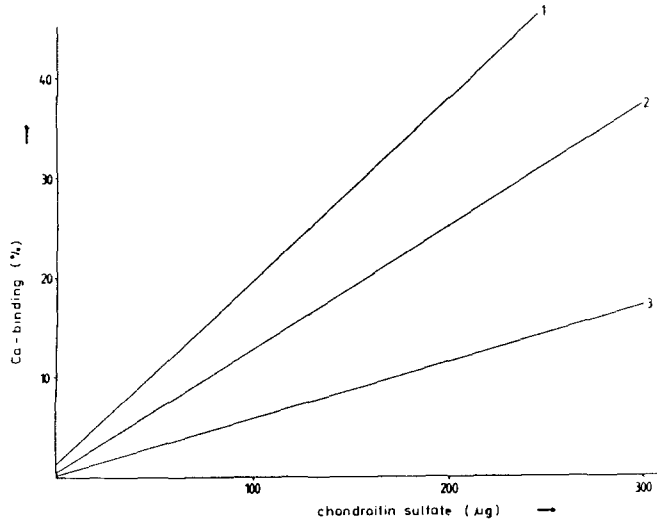

Fig. 2. Linear regression between the Ca-binding capacity of *CS* (y) and the *CS* concentration (x) at three different Ca levels

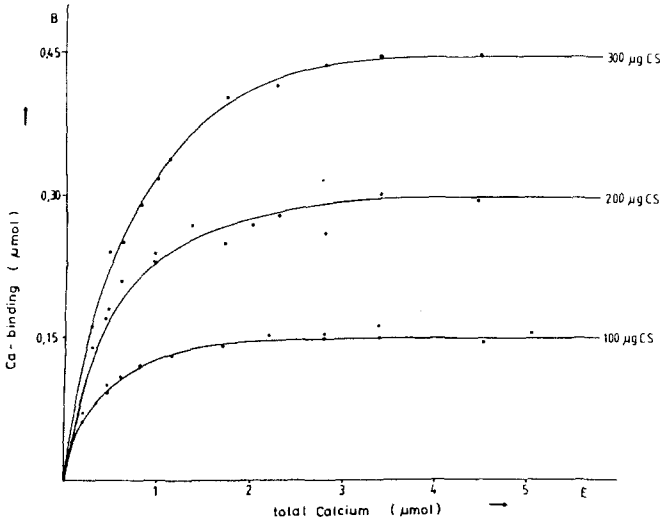

Fig. 3. The saturation of the Ca-binding capacity of *CS* (B) at three different *CS* concentrations in relation to the calcium level (E)

for concentrations in the region up to 0.020 mg CS/1050 µl. At higher *CS* concentrations *CV*s of under 5% were achieved. Figure 2 shows the linear increase in the Ca-binding capacity with increasing Ca concentration expressed as a percentage.

Plane regression curve 1: E = 0.455 µmol/1050 µl
y = 0.1848 x + 1.200
r = 0.998
n = 13

Plane regression curve 2: E = 0.912 µmol/1050 µl
y = 0.123 x + 0.532
r = 0.998
n = 44

Plane regression curve 3: E = 2.283 µmol/1050 µl
y = 0.055 x + 0.253
r = 0.998
n = 28

Saturation Functions

If the *CS* concentration is held constant, increasing calcium dosage (E) will produce Ca-binding saturation from a certain Ca-concentration onwards.

The curves in Fig. 3 indicate calcium doses exceeding 2.5 µmol (per experimental dose), corresponding to a calcium concentration of 1.25 µmol/ml, and the saturation point for Ca-binding by *CS*. 100 µg (0.198 µmol) *CS* bind a maximum of 0.15 µmol of calcium. If the *CS* dose is increased to 200 or 300 µg its Ca-binding capacity doubles or trebles respectively. The fact that 0.198 µmol *CS* bind 0.15 µmol Ca implies a ratio of 0.757 µmol Ca per 1 µmol *CS* (disaccharide unit).

If the results are plotted according to Scatchard [25] the regression illustrated in Fig. 3 is produced.

The resulting function

$$\frac{B}{F} = -6.9958\,B + 1.068$$

corresponds to the ratio

$$\frac{B}{F} = \frac{1}{K_D}\,B + \frac{n}{K_D}$$

This produced a K_D = 0.1429 and a figure for the binding points of n = 0.1526 per 0.198 µmol *CS*. These values derived from the Scatchard plot are almost identical to those for the saturation functions (Fig. 4).

The Scatchard plot produces a maximum Ca-binding limit of 0.77 µmol *CS* disaccharide unit.

Inhibition of the Calcium-Binding Capacity of CS Using Uric Acid

The influence of uric acid on Ca-binding capacity was measured in the dialysis chambers using identical initial concentrations of *CS* (100 µg = 0.198 µmol) and Ca doses (1.141 µmol) for three different pH values.

In the current experiment in each case 10 measurements of the calcium-binding capacity without the addition of uric acid were compared with 10 measurements where uric acid had been added (Table 2). Uric acid or urate inhibited the Ca-binding capacity of the *CS:* at pH 6.0 19% less calcium was bound, at pH 6.8 22.5% and at pH 8.0 the figure reached 30.9%. In each case, the inhibition of the capacity of the *CS* to bind calcium was shown by the U-test to be significant.

Table 2. The capacity of chondroitin sulphate to bind calcium, related to pH value and the presence of uric acid (urate). Dosage in the dialysis chambers: 0.198 μmol *CS*, 0.141 μmol Ca and 1.85 μmol/ml uric acid (pH 6.0), 2.13 μmol/ml mono-Na urate (pH 6.8), 2.13 μmol/ml uric acid (pH 8.0)

Percentage Ca-Binding (%), $\bar{x}$ (± S)					
	pH 6.0		pH 6.8		pH 8.0
without ($n = 10$)	with uric acid ($n = 10$)	without ($n = 10$)	with Na urate ($n = 10$)	without ($n = 10$)	with uric acid ($n = 10$)
9.67 (1.04)	7.83 (1.05) $p < 0.001$	10.16 (0.84)	7.88 (1.08) $p < 0.0003$	10.48 (0.64)	7.24 (0.75) $p < 0.0001$

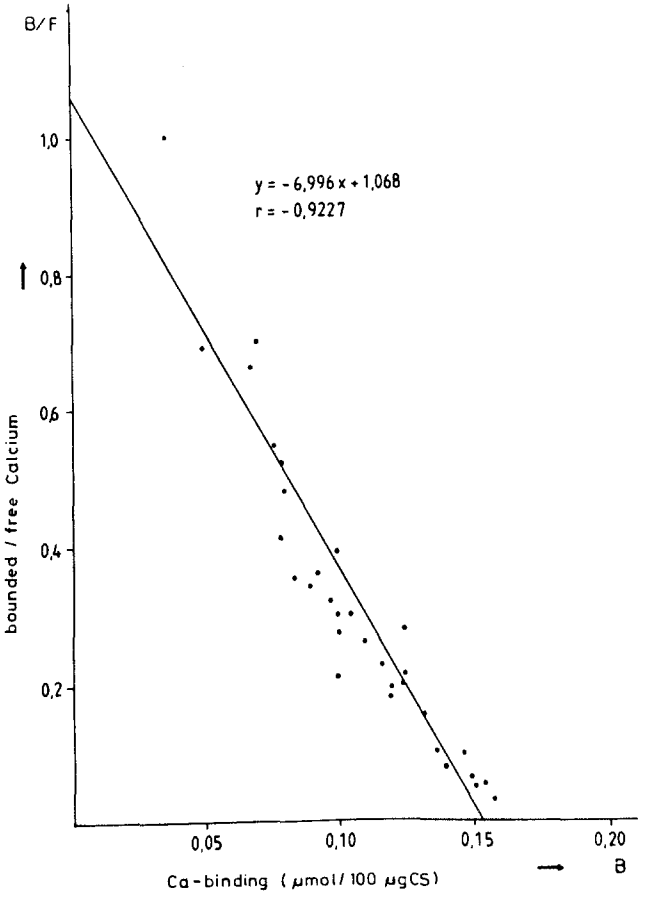

Fig. 4. Scatchard plot for the Ca-binding capacity of *CS*

Discussion

The affinity for calcium and the calcium-binding ability of the polyanionic GAGs is an important presupposition for the validity of the theory that GAGs inhibits the genesis of calcium oxalate calculi.

In the course of these experiments, which were based on equilibrium dialysis and ^{45}Ca, we were able to confirm the fact that *CS* binds Ca. This binding capacity increases linearly to the increase in the *CS* concentration. When calculating the Ca-binding capacity due consideration was given to the binding ability of the dialysis membrane, as had also been demonstrated in Reed's [21] experiments. For our binding studies, however, concentrations of *CS* had to be selected which lay above the normal GAG content of urine. Adequate analytical accuracy (*CV* 5%) was only achieved at concentrations above 100 μmol/l (see Table 1). According to our method of analysis [13] the GAG concentrations in urine lie between 10–25 μmol/l. Using saturation functions we were able to estimate the binding capacity of *CS* at 0.77 μmol Ca/l disaccharide unit. Irrespective of the *CS* concentration, saturation is achieved at a Ca concentration of 1.25 mmol/l. This corresponds approximately to the concentration of ionised calcium present in normal urine. *CS* is also able, however, to bind other di-, mono- and trivalent ions [6, 10, 18].

Carboxyl and sulphate groups must also be taken into account as binding sites for calcium. Analysis of the calcium-binding data according to Scatchard's method [25] revealed that there is only *one* constant of dissociation, namely $K_D = 0.1429$. This means that only one of the binding sites mentioned above is occupied by calcium.

De Jong et al. [8] have demonstrated with water-soluble, acidic polysaccharides that carboxyl groups are actively involved in calcium binding. In contrast to this, other authors [16, 27] have presented a hypothesis based on the formation of a Ca-chelate complex with two sulphate groups from two *CS* molecules. Ca binding activity is frequently attributed to both these groups (carboxyl and sulphate) [10, 15, 18]. Our measurements indicated that there is most likely only one binding site and paying due regard to the references quoted, it would appear that in all probability the primary role is played by the sulphate groups.

In the course of our experiments we used urate ions to inhibit the Ca-binding properties of *CS*, depending on the pH value. Within the pH range from 6.0–6.8 the degree of inhibition was 10–22.5%. In the alkaline range (pH 8.0) an inhibition limit of 31% was recorded for the same urate concentration. Inhibition of the Ca-binding capacity of *CS* through the presence of urate ions was independent of the *CS* concentration. The percentage inhibition remained constant as the *CS* concentration increased.

At the present time we can only hypothesize as to the manner in which urate ions inhibit calcium binding:

- urate reduces the local concentration of ionised Ca, thus preventing the macromulecules from binding Ca,

- urate influences the polyanionic macromolecules and alters their ability to bind Ca.

The ability of urate to absorb GAGs has already been described [12, 19] and it is assumed that GAGs stabilise urate in colloidal form [20]. If it is also assumed that GAGs play an important role in the inhibition of calcium oxalate crystallization in urine, then the effect produced on them by uric acid (urate ions) must impair their inhibitory impact.

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