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Biochim. Biophys. Acta, 42 (1960) 462–469

METAL BINDING PROPERTIES OF CHONDROITIN SULPHATE

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(Received January 14th, 1960)

SUMMARY

The interactions of H^+, Ca^{2+}, Y^{3+}, La^{3+}, with chondroitin sulphate of cartilage have been studied using a micropotentiometric titration technique. These ions are bound to the carboxyl groups of chondroitin sulphate by the formation of 1:1 complexes, with intrinsic association constants in KCl solution (with the ionic strength in parentheses); H^+ log $K = 3.06 \pm 0.02$ (0.1); 2.92 ± 0.02 (1.0); Ca^{2+} log $K = 0.42 \pm 0.09$ (1.0); Y^{3+} log $K = 0.61 \pm 0.05$ (1.0); La^{3+} log $K = 0.79 \pm 0.03$ (1.0). The electrostatic contribution to the binding constant, due to the charge of the polymeric molecule, accords with the "nearest-neighbour interaction" description of the titration curve. The influence of the sulphate groups on these results is discussed and is considered small.

INTRODUCTION

Chondroitin sulphate is a poly-β-D-glucopyranosyluronic acid 2-deoxy-2-acetylamino-galactose sulphate[1], which occurs in bone, and as a major constituent of cartilage, probably in the form of chondromucoprotein, in which it is bound to a noncollagenous protein[2]. There appear to be two closely related forms, the A and C isomers, in which the ester sulphate group is in position 4 and 6 of the galactose ring respectively[3].

The fundamental importance of this polyelectrolyte in providing sites for the deposition of calcium ions during the calcification of cartilage has often been postulated[4], and it has also been suggested as binding radio-yttrium in the mammalian skeleton[5]. In spite of these hypotheses no precise measurements have hitherto been reported of the interactions of these metal ions with purified chondroitin sulphate. In this paper is reported a potentiometric titration study of these systems, and also of the interactions with hydrogen and with lanthanum ions.

EXPERIMENTAL

2-ml aliquots of solutions containing up to 10 mg of polysaccharide were titrated with magnetic stirring in small flat-bottomed glass tubes, using standard size wide-range glass electrodes, with agar bridge connections to a saturated calomel electrode. Titrant was added from a calibrated "Agla" microburette with a well-fitting glass capillary nozzle. The ionic strength of the solutions was either 0.1 *M* or 1.0 *M*, allowing the use of high concentrations of di- or trivalent metal ion in the presence of excess KCl. Owing to the difficulty of handling small volumes of solution in carbon dioxide-free conditions, the whole titration assembly was enclosed in a specially constructed glove box of low leakage rate, containing CO_2-free nitrogen at a small positive pressure. For ease of use several titration vessels were mounted on a rotating stand. A Cambridge Bench Type pH meter was used outside the glove box, connection being made with the electrodes through screened bulkhead fittings. Temperatures were 22 ± 2° throughout.

Stock calcium solutions were made from analytical grade $CaCl_2 6H_2O$ followed by determination of Ca^{2+} with oxalate; yttrium solutions from 99% pure Y_2O_3 and lanthanum solutions from "Specpure" La_2O_3 (both obtained from Messrs. Johnson, Matthey and Co. Ltd.), by dissolution in HCl and removal of excess acid. Two sources of mucopolysaccharide were used: (a) chondroitin sulphate supplied by Messrs. L. Light and Co. Ltd., prepared from alkaline extracts of bovine trachea and thus a mixture of the A and C isomers[6]; (b) chondromucoprotein obtained by aqueous extraction of bullock nasal septum, by the method of MALAWISTA AND SCHUBERT[2]. Material (a) was subsequently further purified by a cetyltrimethyl ammonium bromide precipitation procedure[7], and the final product contained N, 2.27%; S, (see ref. 8) 5.10%; glucuronic acid (by titration), 31.8%. Theoretical values for $C_{14}H_{19}NSO_{14}K_2 \cdot 4\frac{1}{2} H_2O$ are N, 2.27%; S, 5.16%; glucuronic acid 31.4%. This preparation migrated on paper electrophoresis as a single band, staining metachromatically with toluidine blue; two small u.v. fluorescent bands shown by the crude material were removed by the purification, which also lowered the N content by about 0.4%. The spectrophotometric absorption at 280 mμ, partially attributed to protein contaminants, of a 2 mg/ml solution in 1-cm cells was lowered by the purification to 0.080, which accords with that of material after repeated precipitation on kaolin[9].

RESULTS

Hydrogen ion binding

The degree of H^+ association has been derived from the titration curve of the mucopolysaccharide by comparison with the corresponding titration curve in the

absence of mucopolysaccharide. A small correction has been made at the lower pHs for volume differences between the two solutions. The results are plotted in Fig. 1. The chondroitin sulphate titration has two distinct regions: on the acid side the carboxyl groups are titrated and on the alkaline side about 2.5 % of a carboxyl equivalent is titrated with a pK of about 8.2. The possibility of titration of the ester

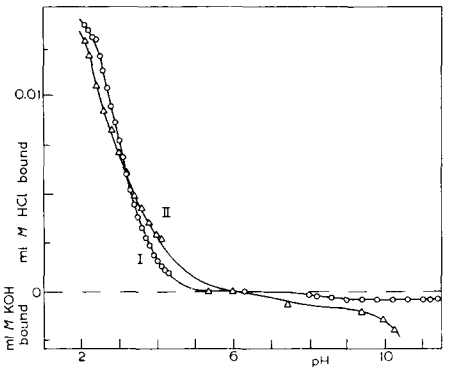

Fig. 1. H^+ binding of chondroitin sulphate (curve I), and of a chondromucoprotein preparation (curve II).

sulphate groups in the more acid solutions was investigated by a graphical method, which also gave an accurate equivalent weight.

At the ionic strengths used the comparatively wide spacing of the carboxyl groups along the chondroitin sulphate chain permits analysis of the titration curve in terms of the "nearest neighbour interaction" model of HILL[10]. The unit of the linear polymer may be regarded for this purpose as the trisaccharide in which a glucuronic acid ring is flanked by two sulphated acetylamino-galactopyranose rings, and the relevant equation becomes:

$$\log K = \mathrm{pH} + \log \frac{\alpha}{1-\alpha} - A(1-\alpha) \qquad (1)$$

where α = the degree of association of protons with the carboxyl groups, K = the intrinsic association constant, A = the electrostatic coefficient.

If y = the bound protons in moles/l, a = the concentration of chondroitin sulphate in carboxyl equivalents/l and h = the activity of hydrogen ions, incorporating the mean activity coefficient, (1) may be written

$$\log K - \mathrm{pH} = \log \frac{y}{a-y} - A(1 - y/a) \qquad (2)$$

hence

$$y = a(1 - y/Kah.\ \varepsilon^{-2 \cdot 30A(1-y/a)}) \qquad (3)$$

For y/a near unity, that is at low pH, since A is small, (3) approximates to:

$$y = a(1 - By/h) \qquad (4)$$

where B is an almost constant factor.

A plot of y against y/h (Fig. 2) should be linear with $y = a$ as its extrapolated intercept, and hence the equivalent weight may be calculated. Binding of protons to the sulphate groups at lower pHs would cause enhanced values of y and positive

deviations from linearity. The plots of Fig. 2 therefore show that such binding is negligible within the experimental range, that is down to pH 2.

Equation (2) shows that, for this model, pH + log $y/(a-y)$ should be linear with $y/a = \alpha$, with an intercept at $\alpha = 1$ of log K, and a slope of $-A$. This is demonstrated in Fig. 3, and the derived constants are given in Table I.

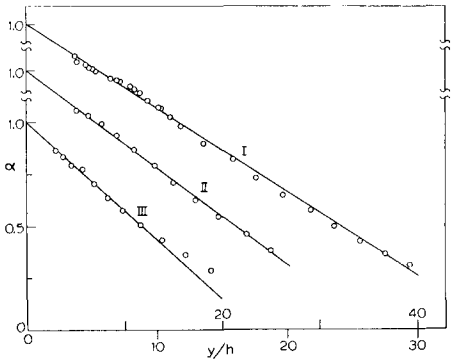

Fig. 2. Determination of equivalent weight by extrapolation (curves I and II in 1 M solution, lower scale; curve III in 0.1 M solution, upper scale).

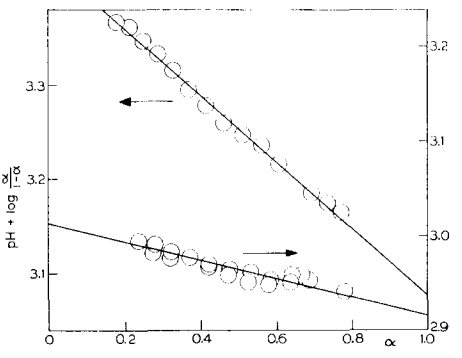

Fig. 3. The electrostatic contribution to the association constant, and the determination of the intrinsic constant.

On the acid side the titration curve of the chondromucoprotein preparation is broadly similar to that of chondroitin sulphate, as suggested by its composition[2]. The differences in the alkaline titrations are discussed below.

TABLE I

INTRINSIC ASSOCIATION CONSTANTS

Cation	*Ionic strength*	*A*	$\log_{10} K$
H^+	0.10	0.354	3.06 ± 0.02
H^+	1.00	0.098	2.92 ± 0.02
Ca^{2+}	1.00	0.098	0.42 ± 0.09
Y^{3+}	1.00	0.098	0.61 ± 0.05
La^{3+}	1.00	0.098	0.79 ± 0.03

Calcium binding

In the presence of excess calcium, lanthanum, or yttrium ions, the acid titration curve of chondroitin sulphate was displaced to more acid values, indicating association of metal ions with the carboxyl groups. The displacements were small, and measurements were restricted to the higher ionic strength to permit the use of high concentrations of added metal ion. The degree of proton binding was derived as described above, using as blank a titration curve of the same salt solution. The low hydrolysis of Ca^{2+} allowed alkaline titrations also, and here no displacement of the titration curve could be detected, indicating negligible complex formation with the corresponding groups.

In the presence of metal ion the analogue of equation (1) is:

$$\log K = \mathrm{pH} + \log \frac{\alpha'}{1 - \alpha' - \alpha_M} - A(1 - \alpha' - n\alpha_M) \qquad (5)$$

where α', α_M, are the degrees of association of H^+ and of M^{n+} respectively at the carboxyl groups. Considering pairs of titrations, identical except for the presence or absence of M^{n+}, at the same pH, (1) and (5) give:

$$\log \frac{\alpha'}{1-\alpha'-\alpha_M} - A(1-\alpha'-n\alpha_M) = \log \frac{\alpha}{1-\alpha} - A(1-\alpha) \tag{6}$$

$$\therefore \log \frac{\alpha(1-\alpha'-\alpha_M)}{\alpha'(1-\alpha)} = A(\alpha' + n\alpha_M - \alpha) \tag{7}$$

For Ca^{2+} α_M is small enough for the right hand side to tend to zero, and

$$\alpha_M = \frac{\alpha - \alpha'}{\alpha} \tag{8}$$

Better approximations were then derived from (7) where necessary by successive approximation. The analogue of (5) for the metal ion binding is:

$$\log K_M = -\log C_M + \log \frac{\alpha_M}{1-\alpha'-\alpha_M} - nA(1-\alpha'-n\alpha_M) \tag{9}$$

where C_M = the concentration of metal ion, corrected for the small proportion bound to the polysaccharide, and K_M = the intrinsic association constant, in terms of ionic concentrations at constant ionic strength. Hence using α_M derived from (8) and (7), $\log K_M$ follows from (9). These results are tabulated in Table II, and the mean

TABLE II

TITRATION RESULTS

Cation	*pH*	α'	α_M	$nA(1-\alpha'-n\alpha_M)$	$\log_{10} K_M$
La^{3+} (0.05 M)	3.64	0.130	0.207	0.07	0.74
	3.48	0.174	0.219	0.05	0.82
	3.16	0.302	0.188	0.04	0.84
	3.08	0.345	0.169	0.04	0.80
	2.94	0.423	0.142	0.04	0.78
	2.83	0.492	0.123	0.04	0.77
	2.63	0.599	0.102	0.03	0.81
	2.46	0.691	0.074	0.03	0.78
Y^{3+} (0.1 M)	3.59	0.135	0.256	0.03	0.61
	3.44	0.174	0.253	0.03	0.60
	3.32	0.216	0.242	0.02	0.64
	3.22	0.258	0.243	0.00	0.69
	3.14	0.300	0.207	0.02	0.61
	3.05	0.337	0.208	0.01	0.66
	2.92	0.415	0.174	0.02	0.61
	2.81	0.489	0.139	0.03	0.55
	2.61	0.605	0.101	0.03	0.51
Ca^{2+} (0.1 M)	3.42	0.274	0.133	0.09	0.26
	3.30	0.327	0.153	0.07	0.40
	3.21	0.368	0.124	0.07	0.31
	3.13	0.414	0.134	0.06	0.41
	2.99	0.493	0.115	0.05	0.41
	2.86	0.562	0.098	0.05	0.41
	2.64	0.665	0.084	0.03	0.49
	2.47	0.746	0.073	0.02	0.59
	2.33	0.795	0.052	0.02	0.51

log K_{Ca} in Table I. In the derivation of all the log K_M, values have been taken within a range of 0.5 pH units on either side of the mid-point of the titration curve where percentagewise errors are minimal.

Lanthanum and yttrium binding

These metals are more strongly complexed than calcium and the results have been calculated by an alternative method. At any point on the titration curve all quantities of (5) are known, using the value of K already derived, except α_M, which can be calculated by successive approximations. In the first step the electrostatic contribution is put equal to zero, and in following steps is included using the approximated α_M. A maximum of four steps was found adequate. K_M was then calculated at each point by (9). Results are given in Tables I and II.

DISCUSSION

The most accurate of previous determinations of the pK of the carboxyl groups of chondroitin sulphate are those of MATHEWS[11] who gives intrinsic association constants at 0.1 M ionic strength only: log $K = 3.07 \pm 0.08$ and $3.06 + 0.06$ for two different preparations from bovine nasal septum. The agreement with corresponding results of the present work is within the quoted experimental error. Comparison with earlier determinations[12] is uncertain, because of invalid derivations of the intrinsic constants.

The alkaline titration curve shows the presence of about 2 groups, which are titrated completely between pH 7 and 9, with log $K = 8.2$, for each 100 carboxyl groups present. No further titration is apparent, at least up to pH 11.6. This behaviour is quite unlike that of mucoprotein preparations (Fig. 1) and, together with the analyses, suggests that the groups are not those of protein contaminants. This accords with the conclusion of LOEVEN[12], and the groups in question are considered to be primary amino groups formed by slight hydrolysis of acetylamino groups during extraction. The negligible Ca^{2+} complexing with these groups supports this identification.

The metal ion binding data have been evaluated assuming a "single site" association between metal ions and carboxyl groups, which is confirmed by the lack of drift in K_M values with increasing carboxyl ionisation. In any case the single site association is weak, and steric factors hinder linkage of a metal ion to groups on more than one ring (see ref. 13). The association constants are in fact very close to those for isolated carboxyl groups; they may be compared with published values for acetate ions: Ca^{2+} in 0.2 M ionic strength, log $K = 0.53$ (see ref. 14), La^{3+} in 2.0 M ionic strength, log $K = 0.49$ (see ref. 15), although differences in ionic strength do not allow exact comparison. The comparatively small Ca^{2+} association explains why an earlier qualitative study of the system failed to demonstrate complex formation[16].

The solutions used have contained high concentrations of chloride ion and allowance for metal-chloride association should be made in applying the results to different systems. For Ca^{2+} and Y^{3+} the effects seem slight or absent[17,18], but for La^{3+} the $La^{3+}/LaCl^{2+}$ ratio at the same ionic strength[19] necessitates an increase of 0.20 in log K_{La} in chloride free systems.

A possible cause of small systematic errors in this work is ion-pair formation between metal ions and the ester sulphate groups. Firstly, this could affect the calcu-

lated concentration of free metal ion; however, the greatest error possible is about the same as the experimental error in the case of La^{3+}, and considerably less for Ca^{2+} and Y^{3+}. Secondly, this could introduce an additional electrostatic contribution to the binding constant. It is considered that binding of these metal ions to the sulphate groups in the present experimental conditions of moderate to high ionic strength is small. This is shown by the high concentration (0.33 *M*) of La^{3+} needed to reverse the mobility of adsorbed chondroitin sulphate in microelectrophoretic measurements[20]. In controlled conditions such mobilities are closely related to the charge on the adsorbed polyelectrolyte[21], and at this concentration the present results show that sufficient La^{3+} ions are bound to the carboxyl groups to neutralise the whole charge on the molecule, and it is unnecessary to postulate appreciable pairing with the sulphate groups. Similar considerations apply to Ca^{2+}, and to Y^{3+} by implication. Thus, although small degrees of association at the sulphate groups are not impossible their influence on the present results is slight.

ACKNOWLEDGEMENTS

We thank the Governors of the Royal Marsden Hospital for the award of a studentship (to N.A.M.), Professor W. V. MAYNEORD for his interest and encouragement, and Mr. D. P. SMITH and Mr. M. PHILLIPS for the construction of apparatus.

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