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INVESTIGATION OF THE HYALURONIC ACID–COPPER COMPLEX BY N.M.R. SPECTROSCOPY

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ABSTRACT

Analysis of the ^{13}C and ^{1}H relaxation data of the hyaluronic acid–copper complex indicates a binding site involving the carboxyl group and O-1 of the D-glucuronic acid moiety. The paramagnetic relaxation of Cu^{2+} is discussed within the framework of the Solomon–Bloembergen formalism and it is shown that various atoms experience, in addition to the dipolar paramagnetic relaxation, a strong scalar relaxation contribution. E.s.r. spectra have also been obtained in order to determine the binding constants, and measurements at 69 K gave the *g*-values of the complex.

INTRODUCTION

Hyaluronic acid (**1**, HA), which is an unbranched polymer $(GlcNAc–GlcA)_n$, is an important structural glucosaminoglycan of connective tissues, and is present in vitreous body, synovial fluid, umbilical cord, skin, and the capsules of several bacteria. The interaction of HA with copper ions has biological significance and depends on their oxidation state. Cu^{+} depolymerises HA in an oxidative–reductive reaction involving mainly HO· radicals[1,2]. This degradation leads to the liquefaction of the vitreous body gel in copper-induced metalloses of the eye[3]. Also, the antibacterial efficacy of Cu^{+} can be ascribed to the depolymerisation of the HA mediated by HO radicals[4].

On the other hand, Cu^{2+} forms complexes with HA without depolymerisation of the glucosaminoglycan[5,6] and produces gels[7].

In order to understand the above biological activities of copper ions, it is important to determine the specifity of the copper binding and we have now applied n.m.r. and e.s.r. spectroscopy to this problem.

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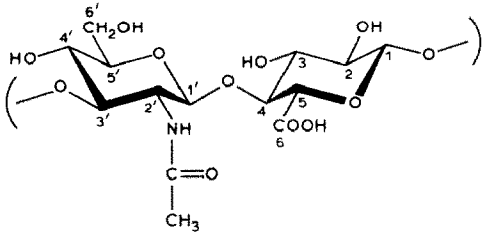

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RESULTS

Tables I–III contain the T_1 and T_2 relaxation times of the ^{13}C atoms of HA, D-glucuronic acid (GlcA), and 2-acetamido-2-deoxy-D-glucose (GlcNAc).

The largest change of the relaxation time with respect to the concentration of Cu^{2+} was seen for the carboxyl group of GlcA. The carbonyl group and the methyl group of GlcNAc showed minor effects and all the other carbon atoms were unaffected. The T_2 experiments indicated a strong influence of the Cu^{2+} on the relaxation rate of C-5 of GlcA.

In order to obtain more complete information on the structure of the complexes and to check if structural distortions of HA in the complexes occurred, the ^1H T_1 relaxation times were measured in the presence and absence of Cu^{2+}, and the results are summarised in Table IV. Structural distortions are unlikely since only those protons located in the vicinity of the binding site are influenced.

The ^1H and ^{13}C investigations performed on GlcA and GlcNAc in the presence of copper allowed a comparison to be made with the HA–Cu complex.

The e.s.r. spectrum of the frozen HA–Cu^{2+} complex (measurements at 69 K) showed a spectral behaviour similar to that of copper acetate[8]. The g-values were $g_{/\!/}$ 2.436, $g_{\perp}$ 2.070, and $\langle g \rangle$ 2.192. The hyperfine splitting was detectable only at $g_{/\!/}$ and was 417.9 MHz. No evidence for Cu dimerisation was seen[9,10]. At room temperature, only those e.s.r. lines can be measured which belong to the free copper ion and this fact was used to perform a so-called[11] "M titration". The dissociation constant thus determined was 0.08 mol/L, the number of equivalent binding-sites within a molecule being 8.

DISCUSSION

Copper-induced changes in the paramagnetic relaxation of ^{13}C and ^1H can be understood, using the well-known equations (*1–5*) of Solomon–Bloembergen and Luz–Meiboom, respectively[12–15], where w_s and w_I are the electron and nuclear Larmor precession frequencies, γ_I is the magnetogyric ratio of the nucleus, β is the Bohr magneton, r is the distance between the nucleus and the paramagnetic ion, τ_m is the life time of the paramagnetic nucleus in the bound site, τ_r is the rotational

TABLE I

EFFECT OF Cu^{2+} ON THE ^{13}C RELAXATION TIMES (s) OF HYALURONIC ACID (1)[a]

mM Cu^{2+}	C-1	C-1′	C-3′	C-4	C-5′	C-3	C-5	C-2	C-4′	C-6	C-2′	CO	COO	CH_3	
—	0.14	0.14	0.14	0.14	0.14	0.13	0.13	0.14	0.15	0.07	0.14	1.90	2.80	0.70	T_1
—	0.10	0.08	0.08	0.10	0.08	0.10	0.10	0.10	0.08	0.06	0.08	0.66	0.75	0.35	T_2
0.14	0.13	0.15	0.14	0.14	0.14	0.13	0.13	0.14	0.15	0.07	0.14	1.40	1.30	0.53	T_1
0.14	0.08	0.10	0.08	0.08	0.09	0.08	0.01	0.09	0.09	0.06	0.07	0.35	0.06	0.40	T_2
0.27	0.14	0.13	0.13	0.13	0.13	0.13	—	0.14	0.14	0.06	0.10	1.20	0.60	0.50	T_1
0.55	0.15	0.13	0.14	0.14	0.15	0.15	—	0.15	0.16	0.07	0.14	0.80	0.30	0.45	T_1
0.83	0.14	0.16	0.14	0.13	0.15	0.13	—	0.15	0.15	0.07	0.12	0.65	0.10	0.40	T_1

[a]Mean deviation ±5%.

TABLE II

EFFECT OF Cu^{2+} ON THE ^{13}C RELAXATION TIMES (s) OF D-GLUCURONIC ACID

mM Cu^{2+}	C-1β	C-1α	C-3β	C-5α	C-2β	C-3α	C-4α	C-4β	C-2α	C-5β	C-6β	C-6α	
—	1.00	1.00	0.95	0.97	0.99	1.00	1.00	0.97	1.00	1.00	1.37	1.28	T_1
—	0.87	0.74	0.74	0.70	0.69	0.88	0.81	0.86	0.81	0.70	1.64	1.79	T_2
0.049	0.95	0.90	0.87	0.87	0.89	0.92	0.91	0.88	0.91	0.88	7.22	6.73	T_1
0.098	0.94	0.92	0.90	0.85	0.89	0.96	0.91	0.88	0.90	0.87	3.20	3.30	T_1
0.147	0.92	0.88	0.90	0.83	0.90	0.91	0.90	0.85	0.92	0.81	2.68	2.80	T_1
0.147	0.65	0.67	0.65	0.05	0.73	0.71	0.80	0.72	0.73	0.04	0.24	0.20	T_2
0.196	0.94	0.92	0.92	0.82	0.91	0.93	0.89	0.87	0.93	0.83	2.00	2.10	T_1
0.245	0.91	0.89	0.92	—	0.89	0.91	0.87	0.85	0.92	—	1.60	1.80	T_1
0.345	0.93	0.88	0.90	—	0.90	0.90	0.85	0.82	0.89	—	1.40	1.35	T_1
0.539	0.92	0.86	0.89	—	0.85	0.88	0.83	0.83	0.87	—	—	—	T_1

TABLE III

EFFECT OF Cu^{2+} ON THE ^{13}C RELAXATION TIMES (s) OF 2-ACETAMIDO-2-DEOXY-D-GLUCOSE

mM Cu^{2+}	*C-1β*	*C-1α*	*C-3β*	*C-5α*	*C-3α*	*C-5β*	*C-4α,β*	*C-6α,β*	*C-2β*	*C-2α*	$CH_3\beta$	$CH_3\alpha$	*COβ*	*COα*	
—	0 50	0.45	0.49	0.47	0.47	0.47	0.47	0 26	0.43	0.43	0.90	0.87	7.64	7.59	T_1
—	0.50	0.50	0 48	0 46	0.51	0.46	0.50	0.30	0 48	0.42	1.10	1.10	2.31	2.58	T_2
0.049	0.50	0.46	0.45	0.47	0.47	0.47	0.47	0.28	0.42	0.43	0,90	—	6.90	7.90	T_1
0.098	0.51	0.46	0 46	0.44	0.47	0.47	0.47	0.26	0 44	0.44	0.90	0.90	6.21	6.47	T_1
0.200	0.54	0 50	0.51	0.51	0 55	0 49	0.51	0.28	0.50	0.50	1.10	1.10	5.47	5 87	T_1
0 290	0 57	0.49	0.55	0.52	0.49	0.52	0.51	0.27	0.49	0.48	1.10	1.10	5.21	5.76	T_1
0.400	0.53	0.51	0.52	0.51	0.52	0.52	0 51	0.29	0.49	0 48	1.08	1.05	4.18	5.34	T_1
0.400	0.52	0.50	0 52	0.55	0 49	0.52	0.48	0.30	0 44	0.49	0 90	0 90	1.43	1.69	T_2

TABLE IV

EFFECT OF Cu^{2+} ON ^1H RELAXATION TIMES (s)

Hyaluronic acid mM Cu^{2+}	*H-1,1'*	*H-5*	*H-2*	CH_3	*NH*
—	0.54	0.61	1.00	0.67	0.18
0.09	0.44	0.43	0.86	0.58	0.17
0.19	0.39	0.42	0.77	0.50	0.12
0.39	0.32	0.31	0.63	0.40	0.09
0.68	0.25	0.24	0.50	0.30	0.07
0.88	0.22	0.20	0.44	0.25	—
1.07	0.15	0.16	0.36	0.22	—

D-*Glucuronic acid* mM Cu^{2+}	*H-1α*	*H-1β*	*H-5α*	*H-5β*	*H-2α*	*H-2β*
—	2.17	1.32	2.24	1.16	2.35	1.74
0.21	0.78	0.95	0.73	0.60	1.12	1.18
0.42	0.47	0.73	0.43	0.41	0.71	0.89
0.58	0.41	0.70	0.38	0.36	0.56	0.85
0.81	0.25	0.51	0.24	0.24	0.40	0.57
1.37	0.16	0.40	0.16	0.18	0.34	0.44

2-Acetamido-2-deoxy-D-*glucose* mM Cu^{2+}	*H-1α*	*H-1β*	CH_3
—	2.11	1.12	0.87
0.19	1.77	1.06	0.85
0.39	1.150	0.94	0.80
0.69	1.23	0.83	0.72
0.98	1.05	0 79	0.65
1.47	0.88	—	0.58
1.97	0.73	—	0.51

correlation time of the bound paramagnetic ion, τ_s is the electron–nuclear relaxation time, and A/h is the electron–nuclear hyperfine coupling constant. R_{1A} is the outer-sphere relaxation rate, D is the diffusion coefficient, τ_D is the translational diffusion correlation time, and d is the closest distance between the bulk solution and the paramagnetic ion. N_s is the number of spins per mL of solution, p_m is the mole fraction of ligand nuclei bound to the ion, q is the coordination number, R_{1p} is the paramagnetic spin–lattice relaxation rate, R_{2p} is the paramagnetic spin–spin relaxation rate, and R_{1M} is the relaxation rate of a nucleus bound near a paramagnetic centre.

$$R_{(1,2)p} = p_{mq} \quad \frac{1}{p_{mq} T_{(1,2)A}} + \frac{1}{T_{(1,2)M} + \tau_m} \qquad 1$$

$$R_{1M} = 2/15 \cdot \gamma_I^2 g^2 S(S+1)\beta^2 r^{-6} \left(\frac{3\tau_c}{1 + w_I^2\tau_c^2} + \frac{7\tau_c}{1 + w_S^2\tau_c^2} \right)$$

$$+ 2/3 \cdot S(S+1)(A/h)^2 \frac{\tau_e}{1 + w_S^2\tau_c^2} \qquad 2$$

$$R_{2M} = 1/15 \cdot \gamma_I^2 g^2 S(S+1)\beta^2 r^{-6} \left(4\tau_c + \frac{3\tau_c}{1 + w_I^2\tau_c^2} + \frac{13\tau_c}{1 + w_S^2\tau_c^2} \right)$$

$$+ 1/3 \cdot S(S+1)(A/h)^2 \left(\tau_e + \frac{\tau_e}{1 + w_S^2\tau_e^2} \right) \qquad 3$$

$$R_{1A} = \frac{8}{225} \cdot \frac{N_S \gamma_I^2 \gamma_S^2 \hbar^2}{D.d} S(S+1)[7f(w_S\tau_D) + 3f(w_I\tau_D] \qquad 4$$

$$R_{2A} = \frac{8}{225} \cdot \frac{N_S \gamma_I^2 \gamma_S^2 \hbar^2}{D.d} S(S+1)[13/2 \cdot f(w_S\tau_D) + 3/2 \cdot f(w_I\tau_D) + 2] \qquad 5$$

$$\tau_c^{-1} = \tau_S^{-1} + \tau_m^{-1} + \tau_r^{-1} \qquad \tau_e^{-1} = \tau_S^{-1} + \tau_m^{-1}$$

In order to calculate the distances between the nucleus of the ligand and the paramagnetic center from the relaxation data, the assumption of fast exchange must be valid. This is guaranteed in our investigations since no exchange-broadening of the signals could be detected with variation of temperature, and individual chemical shifts of complexed and uncomplexed HA were not observed. Even in $(CD_3)_2SO$, where the exchange should be dramatically decreased, there was no evidence of individual chemical shifts of the two species. Also, measurements at various frequencies showed no alteration in the paramagnetic T_1 relaxation*. These findings accord with the results obtained for other Cu comlexes[16–23]. The assumption of fast-exchange conditions reduces the above equations to

$$R_{2M} = p_{mq}^{-1}(R_{2p} - R_{2A}) \qquad \text{and} \qquad R_{1M} = p_{mq}^{-1}(R_{1p} - R_{1A}).$$

The outer-sphere relaxation effects for all nuclei of a certain type are equal and depend only on the concentration of free Cu^{2+} *via* N_S (equations *4* and *5*). The ^{13}C data of Table I show that only those carbon atoms in the vicinity of the complexed paramagnetic ion experience increasing relaxation rates with increasing concentrations of Cu^{2+}, whereas all other ^{13}C nuclei remain unaffected. This finding indicates that the outer-sphere mechanism is of minor importance for the complex under investigation. In equations *2* and *3*, the scalar contribution to T_2 contains

*Due to the fact that the T_{1p} values at 200 and 90 MHz are equivalent, it may be argued that, for these frequencies, the extreme narrowing condition with respect to $w_I\tau_c$ is fulfilled.

one frequency-independent part, which is responsible for the fact that the quotient T_{1p}/T_{2p} differs from the theoretical value of 1.17 in the presence of a scalar contribution. Use of the above equations to analyse the ^{13}C relaxation process leads to the result that C-5 of GlcA experiences a strong scalar contribution (T_{1p}/T_{2p} 120). This mechanism is not that important for the carboxyl group (T_{1p}/T_{2p} 40). The carbonyl group of GlcNAc is influenced only by the dipolar mechanism of the paramagnetic Cu^{2+}. Dissolved O_2 occupies this position in the absence of Cu, which also leads to a strong scalar relaxation of C-5 due to the paramagnetic nature of O_2.

The ^1H relaxation rates measured for the HA–Cu complex decrease with increasing temperature* as expected for a dipolar relaxation mechanism in the fast-exchange limit. However, H-5 of GlcA experiences a small scalar relaxation contribution of Cu (T_{1p}/T_{2p} 3.3).

Complex formation. — Inspection of the relaxation data makes clear that one side of the bidentate ligand in the copper complex is formed by the carboxyl group. The second part of the ligand must be due to the glycosidic oxygen between GlcA and GlcNAc (*i.e.*, O-1 of GlcA). Complexation involving the ring oxygen in GlcA, as in the monomer, can be ruled out since all those protons which would be influenced by the copper are unaffected. However, for H-1′ and H-4, which must be affected by the complexation involving the glycosidic linkage, there is a paramagnetic influence on the relaxation rates (Fig. 1). Probably, there is additional stabilisation of the complex by overlap of the Cu electrons and the NAc-group (Fig. 2), since there was a remarkable decrease of the relaxation time of the NH proton as well as the ^{13}C of the acetyl group. The *trans* relationship between H-2′ and the NH proton, which gives rise[27] to a large J value (12 Hz), does not change on the addition of Cu^{2+}. Moreover, the binding of Cu^{2+} decreases the relaxation time of the methyl group. The relaxation data of those atoms that are affected by Cu^{2+} enable the distance between these atoms and the Cu^{2+} to be estimated approximately. The calculation is based on the fact that $(r_j/r_i)^6 = R_i/R_j$. Since the relaxation times of H-3 and C-3 were not influenced significantly by Cu^{2+}, they were taken as R_j. The differently affected atoms R_i then show the following quotients R_i/R_j: NH, 0.65; H-1′, 0.78; H-1, 0.93; H-5, 0.85; CH_3, 0.90; CO, 0.95; COO, 0.70. The decrease of the relaxation time of H-1′ can be visualised only when the measurements are done at higher temperatures. At room temperature, the signals for H-1′ and H-1 overlap. The partial overlap of the signals for other protons cannot be removed even at higher temperatures. Therefore, no exact T_1 values for H-4 and H-3 could be determined, but a rough estimate yielded T_1 1.2 s for each proton. Despite the overlapping, the behaviour of H-3 clearly indicates the absence of any Cu-dependent T_1 changes.

*The proton T_1 measurements at higher temperatures, by the decrease of the relaxation time[24] as well as by the changes in the chemical shift, also indicate the breaking of a H-bond. For H-1′, a coalescence of the lines associated with the coupling with H-2′ is seen at 95°. This finding demonstrates that the 2-acetamido-2-deoxy-D-glucose ring gains some twisting mobility at higher temperatures, which rapidly exchanges the axial and equatorial protons at position 2.

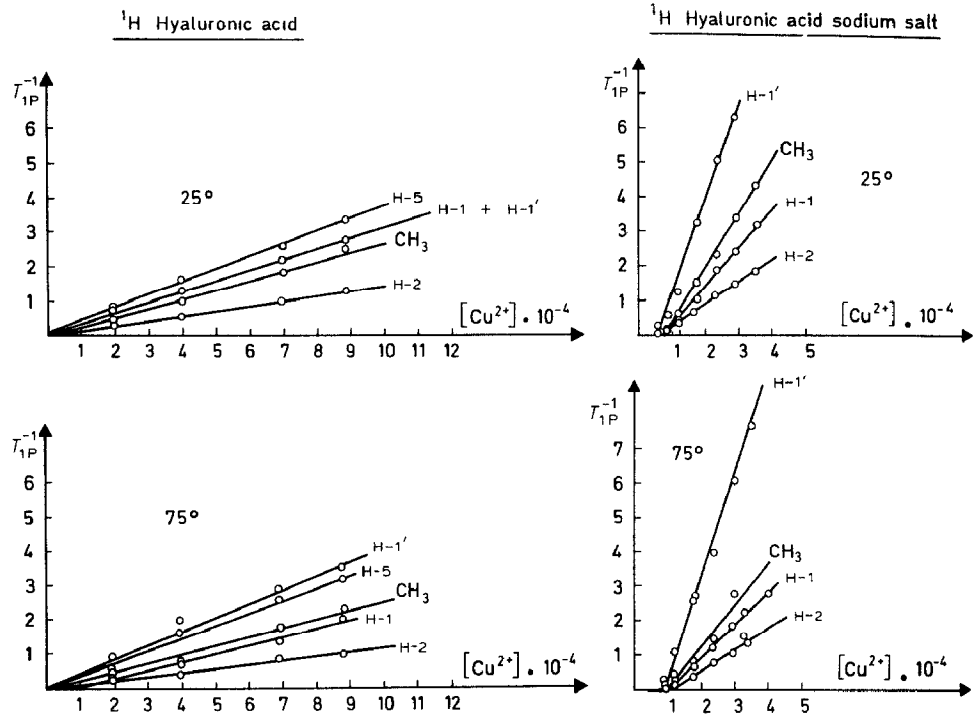

Fig. 1. Paramagnetic relaxation rates of HA (pH 2.6) and HA sodium salt (pH 7) at various temperatures and increasing amounts of Cu^{2+}.

From the measurements performed on α,β-GlcA, it can be deduced that the anomers have different locations of the Cu^{2+}. In the α anomer, the Cu is equidistant between H-1′ and H-5, whereas, in the β anomer, a rough estimation using the r^{-6} dependence of the dipolar paramagnetic relaxation behaviour leads to a Cu location which is ~0.02 nm closer to H-5. Although the stability of the Cu complexes of 2-hydroxycarboxylates is greater than in 2-alkoxycarboxylates[28], for GlcA the hydroxyl groups are in sterically unfavourable positions for the formation of bidentate Cu complexes.

The protons and carbon atoms of GlcNAc are not affected by Cu^{2+}, as can be seen from the relaxation data indicating that GlcNAc affords a complexing site for Cu only when incorporated into HA.

Comparison of our data with those in the literature[5,6,29–31], obtained by using different methods and compounds, shows that the complexation of the carboxyl group is well established. Different proposals with regard to the second part of the bidentate ligand have been made. Chakrabarti and co-workers[5,6,29] favoured the acetamido group as the second part of the ligand, whereas Balt *et al.*[31], for D-glucuronate and Yb^{3+}, postulated a complex containing two glucuronate moieties. Aruga[30] has discussed different ligand structures for the glucuronate ion, one of

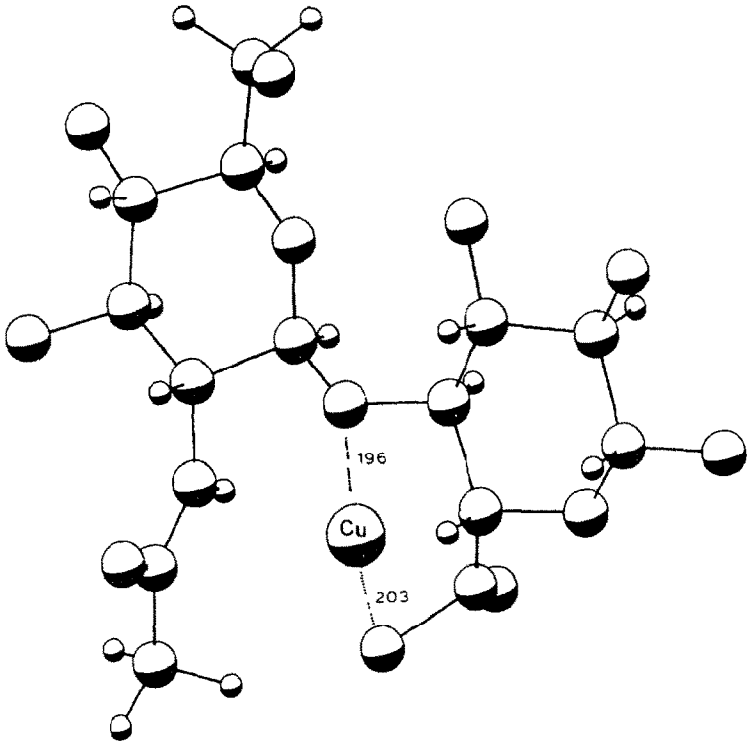

Fig. 2. Stereographic projection of the HA–Cu^{2+} complex. The bond lengths and bond angles are taken from ref. 25. The drawing was performed using a published[26] programme. The dashed lines indicate the proposed complexation and the distances in pm. According to the well-known preference of Cu^{2+} for tetragonal complex geometry, it is assumed that the two additional ligands (Cl, OH, or H_2O) are located above and below the Cu. The exact position of these ligands is probably moved a little towards C-5 for reasons of tetragonality.

which accords with our findings. The possibility that Cu^{2+} in its complex with HA is arranged between the carboxyl group and the GlcA–GlcNAc linkage-oxygen, and is also affected by the π-electrons of the *N*-acetyl group, has not been suggested before. Our results do not support other binding sites for Cu^{2+}. The NH group was suspected of forming a ligand for complexation[29], but investigations on aqueous solutions, using a hard-pulse Redfield sequence[32], show no outstanding influence on the NH relaxation time*. Thus, the NH group can be ruled out as playing an important role in Cu^{2+} complexation.

pH Dependence. — The interaction of HA and Cu^{2+} on the ^{13}C relaxation rates is not pH-dependent, whereas there is a marked increase in the relaxation rate of all protons due to changes in the range pH 2.6–7 (Fig. 1). The differences between the protons detected at pH 2.6, however, remain the same. This finding can be explained by assuming a stronger contribution of the outer-sphere relaxation

*Our analysis of the complex geometry based on the relaxation data of ^1H and ^{13}C clearly shows that NH–copper complexation (postulated in ref. 29) does not occur. We find that all the distances of influenced atoms are in the range 0.30–0.48 nm.

originating from Cu^{2+} in the neutral solution.

We believe that this phenomenon is based on the fact that, in solutions of pH 8, the copper exists[33,34] as the neutral $CuCl \cdot 3\,CuO$, or basic $Cu(OH)_2$ which shows a slightly reduced ability to form HA–Cu^{2+} complexes. On the other hand, the increase of the outer-sphere relaxation can be understood by assuming H-bonding interactions between the polysaccharide and the $Cu(OH)_2$. For $CuCl_2$ at pH 2.6, however, the active Cu^{2+} ion is the tetra-aqua complex, the complexation of which with HA is favoured[29,20].

EXPERIMENTAL

Native HA gives highly viscous, aqueous solutions and, consequently, broad resonances in the ^{1}H-n.m.r. spectra. Therefore depolymerised[24] HA was used. The n.m.r. measurements were performed on 2% solutions (in D_2O 99.9%) at 22° if not otherwise stated. The measurements of spin–lattice relaxation times (T_1) were performed by the inversion recovery method[35]. The spin–spin relaxation time (T_2) was measured by the published method[36,37]. A Varian XL 200 spectrometer was used, which was generously supplied by the "Fonds zur Förderung der wissenschaftlichen Forschung" (Projekt Nr. 3929). $CuCl_2$ and $Cu(CH_3COO)_2$ were used in the investigations, but no difference could be detected. For the ^{1}H and ^{13}C assignments of HA[24], the monomeric GlcA was assigned according to ref. 38. For the ^{13}C assignment of the monomeric GlcNAc, 2D techniques[39] were used.

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