Cu^{2+}-HYALURONIC ACID COMPLEX: SPECTROPHOTOMETRIC DETECTION

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Received November 8, 1976

Summary: Hyaluronic forms a complex with Cu^{2+} showing an absorption band at 238 nm. The results indicate a 2:1 polymer-Cu^{2+} ratio in the complex formation with an equilibrium constant, 3×10^3. Glucuronic acid, one of the monomers of hyaluronic acid, reacts with the cupric ion, showing a similar band at 235 nm, but the complex formation involves multiple equilibria. No complex formation was detected with N-acetylglucosamine--the other monomer of hyaluronic acid--and Cu^{2+}. The absorption bands of the copper complexes with the polymer and glucuronic acid are attributed to a charge-transfer involving ligand to the metal ion.

Hyaluronic acid, a glycosaminoglycan, is present in the intercellular matrix of most vertebrates. It is a linear polymer of alternating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid with a molecular size in the range of 5×10^4 -5×10^6 daltons. Previous investigations reported the catalytic role of Cu^{2+} and Fe^{3+} in the degradation of hyaluronic acid in the presence of ascorbic acid or other autoxidants (1-4). The proposed mechanism for the role of copper ion in this process was the reduction of Cu^{2+} to Cu^{+} by ascorbic acid and subsequent cleavage of the polymer by free radicals generated in a series of reactions (4). No direct interaction of either Cu^{2+} or Cu^{+} with hyaluronic acid has been suggested in this process. The interaction of copper ion with proteins and nucleic acids and the biological significance of this interaction have been extensively studied (5-9). The attention of many investigators has also been focused on studying the interaction of metal ions with another class of biopolymers, namely, glycosaminoglycans (10,11). This communication reports the results of our spectroscopic investigation of the interaction of copper ion with hyaluronic acid and its monomeric constituents, glucuronic acid and N-acetylglucosamine.

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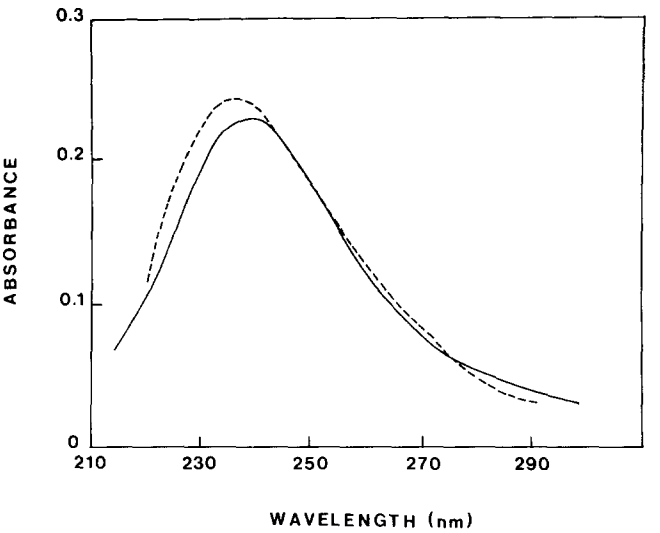

Fig. 1 DIFFERENCE SPECTRA OF CU^{2+} COMPLEXES OF HYALURONIC ACID AND GLUCURONIC ACID

Difference spectrum was measured with the use of two-compartment mixing cuvettes at room temperature and pH of the solution ~ 5. Absorbance is for 1 cm pathlength. Hyaluronic acid (Na-salt) $8x10^{-4}$M (Concentration of hyaluronic acid is expressed in terms of disaccharide units); glucuronic acid (Na-salt) $2.3x10^{-3}$M; $CuSO_4$ 10^{-3}M with each sample. Difference spectrum: ——, hyaluronic acid; -----, glucuronic acid. Concentrations of saccharides were chosen to yield the same absorbance value.

Materials and Methods: Solutions of hyaluronic acid prepared from rooster comb (12) were made by weighing the quantity of freeze-dried materials and the concentration of each sample was verified from hexuronic acid determination by the carbazole reaction method (13). Analytical grade hydrated copper sulphate, sodium-D-glucuronate and N-acetyl-D-glucosamine were obtained from Sigma Chemical Company. The difference spectra were recorded in a Cary-15 spectrophotometer using mixing cuvettes (14). Freshly prepared solutions, made with glass distilled water, were used in all experiments.

Results and Discussion: Figure 1 shows the spectra of copper complexes with glucuronic and hyaluronic acid with the respective band maxima at 235 and 238 nm. N-acetylglucosamine does not exhibit any complex band with copper ion in the wavelength range of our study. With an equimolar mixture of glucuronic acid and N-acetylglucosamine, copper ion shows a band similar to the Cu^{2+}-glucuronate complex. This indicates that the observed Cu^{2+}-hyaluronic acid band at 238 nm must involve the carboxylic groups of the polymer. Using

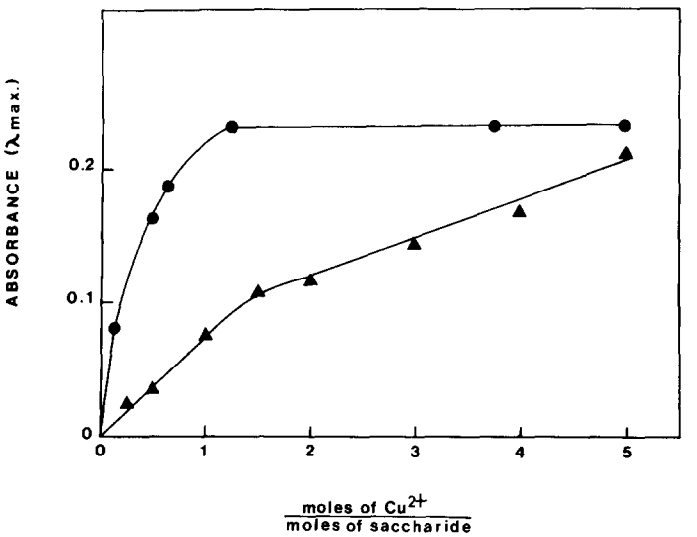

Fig. 2 TITRATION OF HYALURONIC ACID AND GLUCURONIC ACID WITH CU^{2+}

●—● is λ_{max} for hyaluronic acid; ▲—▲ is λ_{max} for glucuronic acid. $CuSO_4$ was added to 8×10^{-4}M of either saccharide. Other conditions as in Fig. 1.

the absorption values at 238 nm, titrations of hyaluronic acid-Cu^{2+} complex were made varying either of the constituents. In the titration curve of hyaluronic acid with copper ion, a plateau is reached when the polymer-Cu^{2+} ratio is 2:1 (Fig. 2). On the other hand, with glucuronic acid there is a continuous increase in the magnitude of the Cu^{2+}-complex band with the increase in cupric ion concentration > 1:1 ratio. In this case, several equilibria involving more than one carboxyl group are expected to occur and the situation is comparable to that reported for the acetate-Cu^{2+} complex (15). Since the ligand groups are well separated from each other along the polymer chain, intramolecular complex formation and multiple equilibria are not expected in a linear form (15,16).

The Scatchard plot (Fig. 3), obtained from the results of titration curves, was analyzed using the least square linear regression method. The straight line intercepts the x coordinate at 0.57 of Cu^{2+}: hyaluronate and the correlation coefficient is 0.937. This agrees reasonably with the results from Fig. 2 where the polymer-Cu^{2+} ratio in the complex formation is 2:1. The binding constant calculated from the slope is 3×10^3. However, we have observed that in the titration curve, when hyaluronic acid is the variant, the ratio of

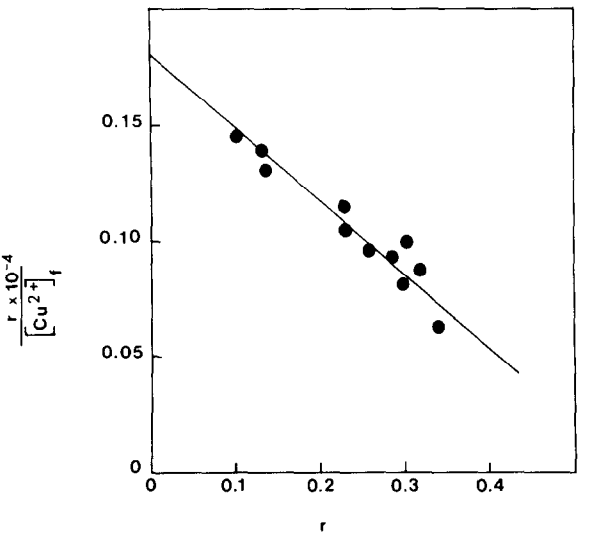

Fig. 3 A SCATCHARD-TYPE GRAPH FOR BINDING OF CU^{2+} BY HYALURONIC ACID

The equation used for the graph is: $r/[Cu^{2+}]_f = kn - kr$ where r is the moles of bound Cu^{2+} per total moles of hyaluronic acid; $[Cu^{2+}]_f$ is the molar concentration of unbound Cu^{2+}; n is the number of binding sites; and k is an intrinsic binding constant. Bound Cu^{2+} was determined by using: $\epsilon^{Cu^{2+}}_{238} = 870$, obtained as a saturation absorbance value of Cu^{2+}, in excess of hyaluronic acid; then $[Cu^{2+}]_{bound} = A_{238}/\epsilon^{Cu^{2+}}_{238}$ and $[Cu^{2+}]_f = [Cu^{2+}]_{total} - [Cu^{2+}]_{bound}$.

saturation of the polymer to Cu^{2+} is more than one. It is likely that in the presence of a large excess of hyaluronic acid, further replacement of water molecules from the coordination square (x-y plane) of copper by two more oxygen molecules can take place. Hence, neither the value of the binding constant nor the stoichiometric ratio of the complex formation can be ascertained conclusively.

Conformational studies of hyaluronic acid in solution (17, 18) and in the solid state (19, 20) indicated a helical structure of the polymer. In the X-ray diffraction studies (19) of sodium hyaluronate film it was reported that three non-coaxial helical chains of the polymer are stabilized by a network of hydrogen bonds and O...Na...O bridges involving one oxygen

atom of the carboxylate and another of the acetamide group. This conformation allows a coordination complex with copper ions involving two oxygen atoms. Since the viscosity of hyaluronic acid does not change appreciably (not shown) on binding with Cu^{2+}, intermolecular crosslinking with multiple chains of the polymer can be ruled out. It is not evident from our experiments that any oxygen atom of the acetamido group is involved in the Cu^{2+}-hyaluronic acid complex formation although this possibility cannot be excluded. Both the coordinating oxygen atoms may also be of two carboxylates. In either case, a preferred conformational order is necessary to form such a complex.

We interpret the ultraviolet absorption band in the 238 nm region of both glucuronic and hyaluronic acid with Cu^{2+} in terms of charge-transfer involving ligand to the metal ion. Since the ligand in this case does not possess any acceptor orbital, metal to ligand charge-transfer can be safely excluded.

The decrease in viscosity of hyaluronic acid in the presence of cuprous ion has been reported, but no such change was observed with cupric ion (21). An EPR measurement of Cu^{2+}-hyaluronic acid complex indicates that the copper ion is indeed in a bivalent state. The depolymerization of hyaluronic acid by Cu^{2+} and Fe^{3+} in the presence of ascorbic acid and any change of conformation of the polymer is of major biological importance. The role played by the metal ion-polymer complex in this process has yet to be assessed. Studies in this regard are in progress, and will be published elsewhere.

Acknowledgements: This investigation was supported by NSF Grant 38380, NSF Grant BMS 75-15316 and PHS Grant EY01760-01. The authors wish to thank Dr. David Swann of the Harvard Medical School for providing the hyaluronic acid, and Ms. S. Flavia Blackwell for editorial assistance.

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