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The Influence of Metallic Ions on the Viscosity of Hyaluronic Acid Solutions

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Blix and Snellman[1] mention the possibility that heavy metals cause a depolymerisation of aqueous hyaluronic acid solutions. The purpose of the present work was to prepare hyaluronic acid, which was stable with regard to viscosity in aqueous solution and to examine the influence of some metallic ions on the viscosity.

PREPARATION OF HYALURONIC ACID

Two sources have been used for the preparation: human umbilical cords and synovial fluid from cattle.

The umbilical cords which had been collected under alcohol were carefully minced, the pulp was extracted with 90 per cent actic acid according to Meyer and Palmer[2]. The bulk of the acetic acid was removed by washing with water, and the rest was neutralised to pH 7 by adding saturated potassium hydroxide. After extraction with water the hyaluronic acid was precipitated by 1 ½ volume alcohol. The precipitate was dissolved in water, precipitated by alcoholic potassium acetate (McClean[3]), washed with alcohol and ether, and dried in vacuo over phosphorus pentoxide. 3 to 20 umbilical cords were worked up at the same time. 3 umbilical cords treated in this way yielded about one half gram.

800 ml synovial fluid was filtered through gauze, and 1 per cent acetic acid was added. The precipitate was extracted by glacial acetic acid. The product was worked up as outlined above.

The preparations showed the same rather high viscosity in aqueous solutions for several days. A solution which was 118 mg % (mg per 100 grams) showed a relative viscosity of 4.93, and the same value was found after a fortnight a room temperature. The reason may be that the iron has been effectively removed by the acetic acid. As will be shown later in the present paper iron has a definite viscosity decreasing effect on hyaluronic acid solutions even if present in very small amounts only.

It was possible to precipitate the potassium hyaluronate by 1 ½ volume alcohol directly from the aqueous extract. The treatment with acetic acid and the strong alcohol has probably denatured the protein in the mucin complex as the precipitate apparently does not contain protein. *Cf.* the relative low nitrogen per cents in Table 1.

It proved impossible to increase the yield by adding serum to the aqueous extract of umbilical cords acidified by acetic acid, as no mucin clot was formed; probably hyaluronic acid is linked only to certain proteins.

It was observed that when hyaluronic acid is precipitated by alcoholic potassium acetate, one part of the product formed is present as a stringy clot, the other as a flocculent precipitate. If the solutions are very dilute the last modification is by far the most prominent.

A very important stage in the preparation is the preliminary mincing of the starting material, which ought to be very careful in order to obtain maximal yield. One method of obtaining this is repeated chopping, another freezing by liquid air, but here we meet with the difficulties of crushing the glasslike material.

Table 1. Nitrogen content of some potassium hyaluronate preparations.

Source of hyaluronate	Nitrogen per cent
Umbilical cords	3.60
Synovial fluid	2.65
Umbilical cords frozen in liquid air	2.71

The preparations did not contain glycogen.

INFLUENCE OF METALLIC IONS ON THE VISCOSITY OF HYALURONIC ACID SOLUTIONS

For this study Ostwald viscosimeters with a capacity of 3 ml have been used, the flowtime for water varies from 12.0 to 65.4 seconds. The composition of the fluids used has been as follows: To 4 ml potassium hyaluronate the concentration of which was about 230 mg % is added 2 ml of buffer, respectively water, plus 2 ml of the metal salt, the influence of which was to be examined. In case buffer solutions have been employed their pH have been about 7 and

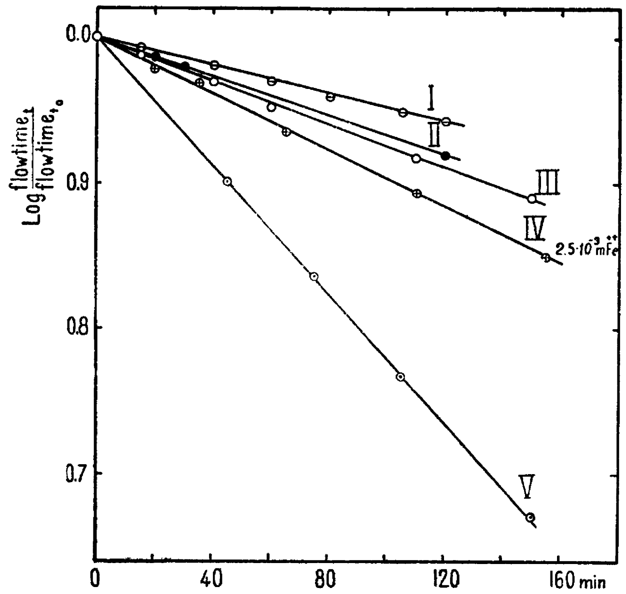

Fig. 1. The logarithm of the flowtime as a function of the time elapsed from the addition of iron; as ordinates are used the difference between the logarithm initial flowtime and the logarithm of the flowtime measured.

I. $2.5 \cdot 10^{-5}$ M Fe^{++}
II. $2.5 \cdot 10^{-4}$ M Fe^{++}
III. $5.0 \cdot 10^{-4}$ M Fe^{++}
IV. $2.5 \cdot 10^{-3}$ M Fe^{++}
V. $2.5 \cdot 10^{-2}$ M Fe^{++}

their molarity 0.1. If nothing else is mentioned the temperature has been 34° C in all experiments.

The following metal salts showed no measurable effect during 24 hours in 0.025 molar solution: Zinc sulfate, mercury chloride, cobalt nitrate, silver nitrate, lead nitrate, magnesium sulfate, beryllium chloride, nickel nitrate *, and chromic chloride. Experiments were also made at a molarity of 0.0025 with regard to the three last named salts. The measurements were made both in water and buffer solution.

The only cations which displayed any influence on the viscosity were iron and copper.

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* At the low pH values which are necessary to keep certain salts in solution a low initial viscosity is observed.

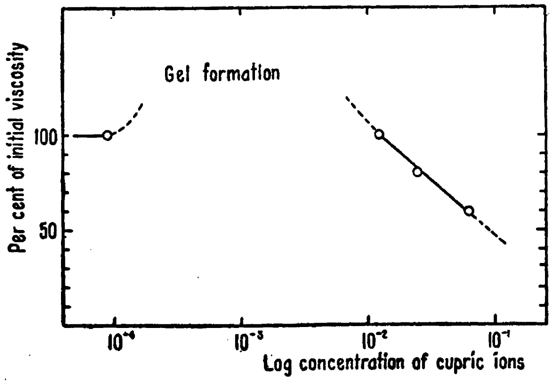

Fig. 2. The relation between viscosity and concentration of copper.

THE INFLUENCE OF IRON

Iron was found to cause a continuous decrease of the viscosity even at a concentration of about 2.5×10^{-5} molar, thus confirming the assumption of Lundquist[4]; the measurements were carried out in maleic acid buffer.

The iron salt used was ferrous sulfate. The solutions were prepared just before use in order to avoid too extensive transformation of ferrous into ferric salt, which is precipitated when present in larger amounts, but the effect of ferric ion is equal to that of ferrous ion as has been observed in case of very dilute solutions. To avoid formation of ferric precipitates in the solution 10 mg hydrazin sulfate may be added to 3 ml hyaluronate solution without influencing the results. Addition of 20 mg hydrazine sulfate to a similar hyaluronate solution causes a decrease of only about 2 per cent in the viscosity in 4 hours.

In Fig. 1 the logarithm of the flowtime is plotted against the duration of the experiment. For further details concerning this technique *cf.* Lundquist[4]. The inclination of the lines is a measure of the influence of the iron salt.

The finding of McClean and Hale[5] that ascorbic acid in very low concentration causes a depolymerisation of hyaluronic acid solutions has been confirmed. Ferricyanide does not change the viscosity of hyaluronate solutions.

THE INFLUENCE OF COPPER

The influence of cupric ions (cupric sulphate was used) on the viscosity of hyaluronate solutions depends on the concentration. Concentrations above 10^{-2} molar cause a decrease, smaller concentrations a gel formation which is detectable even at a concentration of about 10^{-4} molar.

Fig. 2 shows graphically the influence of cupric ions on hyaluronate solutions. The duration of the experiments was 3 hours. If the hyaluronate is dissolved in maleic or phtalic acid buffer the gel formation is observed at a considerably higher copper concentration. Other substances, *e. g.* several dicarboxylic acids have been applied but without effect. The reason for that may be that a complex formation takes place between maleic, respectively phthalic acid and copper thus causing a decrease in the concentration of cupric ions. When a 0.5 molar maleate solution is mixed with cupric sulfate solution a dark blue complex crystallizes on cooling. Photometric measurements indicate that at most one third of the copper is present in ionic form under the experimental conditions employed. It may be mentioned that the gel formation was more prominent after 4 than after 24 hours. Vigorous shaking apparently destroys the gel. Attempts were made to regenerate the hyaluronate from the gel by addition of alcoholic potassium acetate, the product evidently contained copper and displayed a gel-like appearance when treated with water.

It is of some interest to notice that the hyaluronic acid is found in the vitreous humor in a gelatinised form much resembling that produced by the influence of copper ions on potassium hyaluronate solutions.

SUMMARY

Potassium hyaluronate has been prepared which appears to be stable in aqueous solutions.

The influence of some cations on the viscosity has been examined.

Ferrous and ferric ions have been found to cause a decrease in the viscosity of aqueous hyaluronate solutions.

Small cupric ion concentrations cause a gel formation, concentrations above 10^{-2} molar a decrease in the viscosity.

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