ACTA CHEMICA SCANDINAVICA 7 (1953) 603–610

The Preparation of Hyaluronic Acid and the Determination of the Viscosity and Optical Rotation

C. E. JENSEN

Københavns universitets fysisk-kemiske institut, Copenhagen, Denmark

Several preparations of hyaluronic acid have been prepared which by analysis have been found to be rather pure. Some of these preparations exhibit unusually high viscosities in aqueous solution. The optical rotation of these highly viscous and pure preparations seems to be much smaller than that usually accepted.

The characteristics of the hyaluronic acid vary very much according to the sources and the methods of isolation (Meyer [1], Jensen [2]). The substance has been prepared from synovial fluid from cutting cattle, and from human umbilical cords, according partly to known methods and partly to methods modified by the author.

PREPARATION OF HYALURONIC ACID

A. From Umbilical Cords

The umbilical cords * free from placental tissue are stored under acetone after thorough rinsing with water so that all blood is removed. The cords are cut into small fragments (two to three cm), put into acetone and carefully minced in a Waring Blendor. On account of the production of heat the Waring Blendor must work only for a minute at a time, and it must be cooled constantly. After four to six treatments the pulp is extracted with acetone at room temperature for four days, the acetone being replaced every twenty-

* The author wishes to thank Prof. Erik Rydberg, M. D., Obstetrical Department of the Rigshospital, Copenhagen, for kindly placing extraordinary well rinsed human umbilical cords at his disposal.

four hours; then the pulp is filtered through gauze, and the rest of the acetone is removed in a vacuum desiccator containing silica gel. The dried powder is extracted with two to three volume glacial acetic acid at room temperature for a week under occasional stirring, the glacial acetic acid being replaced every twenty-four hours. By doing so remnants of blood are removed, and the protein to which the hyaluronic acid is attached is split off. The filtrate from the treatment with glacial acetic acid gives an ample precipitation upon boiling, and the protein-like precipitate also gives the ordinary reactions for proteins *e.g.* the biuret test, the test for sulphur and Millon's test. The bulk of the glacial acetic acid is removed by repeated quick rinsings with water through eight layers of gauze with suction, and the remainder is neutralized to pH 7 by adding saturated potassium hydroxide. Now the pulp is extracted with three to four volume water at about 4° C, and toluene is added in order to avoid bacterial growth. The first extraction lasted a week, and the next four weeks (occasional stirring). The hyaluronate is precipitated with one and a half volume alcohol. At this time the preparation is greyish; it is purified through ten rinsings (5 ml every time) with absolute alcohol on a Buchner funnel with weak suction. It is treated in the same way with ether distilled over sodium. This preparation is called Prep. 1.

Three preparations were prepared after the foregoing chemical procedure but at different pH values and a meatchopping machine for mincing the cords. Prep. 2a was precipitated with alcohol after extraction at pH 5, and Prep. 2 b and 2 c were precipitated with alcohol saturated with potassium acetate after extraction at pH 8.5 and pH 11, respectively.

B. From Synovial Fluid

The synovial fluid is withdrawn into a 30 ml syringe through a coarse canula from the ankle joints of the hindlimbs of cutting cattle, 15—20 minutes after slaughtering. The canula is provided with a stilett at the introducing. The canula is of the same kind as that used at bone marrow biopsy. If one introduces the canula into the lateral posterior tibio-tarsal joint capsule just anterior to the saphenous vein which is here crossing the capsule joint it is possible to obtain the synovial fluid as a colourless fluid without contamination with blood and tissue fragments. The withdrawing is done most easily when the animal is lying on its back and the Achilles tendon is cut. If the animal is suspended from hooks the withdrawing is difficult on account of the tightening of the joint capsule. The cows give about 15 ml, the bulls about 50 ml synovial fluid per ankle joint.

Just after the withdrawing the synovial fluid is cooled to 1° C, and the working up of the hyaluronic acid is started at once. The synovial fluid is diluted with about one volume of water, and the mucin is precipitated with about one volume per cent glacial acetic acid under vigorous stirring with a glass rod (Meyer [3], Blix [4], Lundquist [5]). The protein is separated from the hyaluronic acid after three different principles.

1. Enzymatic Digestion. The mucin is dissolved in water, and the pH is adjusted to about 8. Pancreatin (B.D.H.) is added together with 0.5 per cent sodium bicarbonate (Lundquist [5]). Incubation at 37° C for twenty-four hours destroys the protein. The subsequent filtration is rather difficult, preferably a folded filter or a layer of Hyflo-Super-Cel (Johns Manville) combined with weak suction is used. After cooling 10 per cent trichloroacetic acid is added, and the mixture is filtered again. The resulting solution is neutralized with potassium hydroxide and a saturated solution of potassium hydroxide in alcohol is added. This preparation is marked 3 A.

2. The Method of Sevag [6]. The aqueous mucin solution is shaken with ½ volume chloroform and 1/20 volume *iso*amylalcohol (Meyer [3], Blix [4]); we shook vigorously for half an hour by hand, in a nitrogen atmosphere to protect the hyaluronic acid against air (oxygen). After two shakings no protein is precipitated at the interface. The mixture is centrifuged at 0° C (3 500 r.p.m.), and the hyaluronic acid is now precipitated with alcohol saturated with potassium hydroxide. This preparation is marked 3 B.

3. The Glacial Acetic Acid Method. The mucinclot was chopped into small fragments and extracted with 4 volumes glacial acetic acid for eight days, the glacial acetic acid being replaced every twenty-four hours (Meyer and Palmer [7]). The glacial acetic acid is removed and the remainder neutralized to pH 7 with saturated potassium hydroxide. The pulp is extracted with water for a week, and the hyaluronic acid is precipitated with about 1 ½ volume alcohol. This preparation is marked 3 C.

All the preparations are nearly white or white and very hygroscopic, they dissolve quickly in water and the solutions are clear; they do not contain glycogen.

Nitrogen Determination

The nitrogen content was determined after Kjeldahl, modified by Blom [8]. The method of Blom is distinguished by requiring only one standard liquid, the acid. The ammonia set free is distilled into a receiver containing a nickel-ammoniumsulphate solution (for details see Blom in a forthcoming paper). The method was used as a micromethod. 8—10 mg hyaluronate were destruc-

Table 1. Nitrogen content and viscosity of diffe-

Source of hyaluronate	Method of preparation
Umbilical cords	Prep. 1. Mincing in a Waring Blendor, acetone + extraction with glacial acetic acid. Precipitation with ethanol at pH = 7.
	Prep. 2. Mincing with a chopping machine. Extraction with glacial acetic acid. Precipitations with a) ethanol at pH = 5 b) ethanol + potassium acetate at pH = 8.5 c) ethanol + potassium acetate at pH = 11
Synovial fluid	Prep. 3. Precipitation of mucin with glacial acetic acid. Separation of protein by A) Enzymatic digestion [1] B) Chloroform + *iso*amylalcohol [1] C) Treatment with glacial acetic acid [2]

[1] The synovial fluid contaminated with blood and tissue debris.
[2] The synovial fluid appeared to be pure, a preparation prepared in quite the same way showed a relative viscosity of 4.93, but the source of material was impure.

ted with 1 ml sulphuric acid, about 1 g potassium sulphate and about 100 mg mercuric oxide added. The ammonia is liberated with sodium hydroxide containing sodium sulfide and distilled into about 2 ml nickelammoniumsulphate solution. Eight nitrogen analyses on ammoniumchloride yielded the theoretical nitrogen content $\pm$ 1 %, and five analyses on pyridinhydrochloridezinc-chloride, which Dr. Blom kindly placed at our disposal, yielded nitrogen contents of 7.598, 7.606, 7.612, 7.601, 7.609 % respectively (theoretically 7.626%). The time of destruction of the hyaluronic acid was about two hours, the time of distillation about ten minutes. We titrated with a 0.1 *N* hydrochloric acid from a burette of 1 ml, divided into 0.01 ml. The colour change from green to purple was seen distinctly (indicator: a mixture of methyl red and methylene blue). All determinations were double determinations which agreed within a half per cent relatively. All volumetric flasks, the pipette and the burette had been standardized. The nitrogen per cents found are seen in Table 1. The theoretical nitrogen content calculated from the formula $(C_{14}H_{20}NO_{11}K)_n$ is 3.36 %.

rent preparations of potassium hyaluronate.

Yield	% N	Relative viscosity Conc. 1 g/l
From 12 cords		
1. extraction: 465 mg stringy hyaluronate	3.36	11.80
1. extraction: 480 » flocculent »	3.35	11.91
2. extraction: 145 » stringy »	3.31	12.19
2. extraction: 60 » flocculent »	3.34	12.12
From 3 × 6 cords		
60 mg hyaluronate	2.90	5.46
100 » »	2.88	68.46
745 » » [3]	2.82	5.29
From 3 × 500 ml synovial fluid		
205 mg stringy + 65 mg flocculent hyaluronate	1.16	5.75
460 » » + 250 » » »	5.78	(4.62)[4]
190 » hyaluronate	2.99	76.39

[3] The preparation was only partly soluble in water.
[4] The aqueous solution became clear only after centrifugation.

Determination of the Viscosity of Hyaluronic Acid

The relative viscosity, that is the viscosity of the solution divided by the viscosity of water, was determined by the Kvorning and Dalgaard-Mikkelsen [9] viscosimeter, a modification of the Ostwald viscosimeter. The apparatus has a capacity of 0.5 ml, the volume between the two marks being 0.2 ml. The flow time of our apparatus was 43.0 seconds at 20° C for distilled water. The concentration of hyaluronic acid was 100 mg in 100 ml solution. On account of its hygroscopicity the substance was weighed in a stoppered weighing bottle. The results of the measurements are in Table 1.

Measurement of the Optical Rotation of Hyaluronic Acid

The optical activity of hyaluronic acid was determined in a Schmidt and Haensch polariscope with a tubuslength of 4 dm. The readings were accurate to 0.01 angular degrees. The source of light was sodium light and the tem-

perature 20.0° C. The concentration of hyaluronic acid was 20 to 25 mg in 25 ml solution. The rotation angles are small, varying from 0.04° to 0.32°, and the corresponding $[\alpha]_D^{20}$-values are therefore to be considered with some reservation. When ferrous sulphate was added to the solutions to a concentration of 0.01 *M* the angles of rotation increased appreciably during six hours. The results of the measurements are in Table 2.

Table 2. The optical rotation of three preparations of potassium hyaluronate and its change by the influence of ferrous ions.

Time		0		1	2	6
Sample	Conc. mg/ml	α	$[\alpha]_D^{20}$	α	α	α
Prep 1	0.80	−0.08	− 25	−0.20	−0.24	−0.26
Prep 3 A	0.77	−0.32	− 104	−0.32	−0.32	−0.32
Prep 3 C	0.84	−0.04	− 12	−0.20	−0.24	−0.25

The times indicated are the times in hours from the addition of ferrous sulphate. The optical rotation, α, was measured in 4 dm tubes.

DISCUSSION

The great majority of viscosity determinations of hyaluronic acid vary from 1 to 8, most of them are beneath 4 as seen in a table by Hadidian and Pirie [10]. More recent reports indicate figures of a similar size (Jeanloz [11], Blix [12]). In the literature only one determination is rather high: 39 (Ogston [13]). This is a preparation from synovial fluid prepared by filtering the fluid or the dissolved mucin through membranes of collodion and filters of sintered glass into an evacuated reservoir. Ogston's preparation, however, contains protein, and the nitrogen content is about 7 %.

The measurements reported are of preparations of hyaluronic acid of different origin: umbilical cords, synovial fluid, exudates, the vitreous body, the cock's comb, peritoneal fluid and mesothelioma; the methods of preparation also differ considerably. The author is of the opinion that the much higher viscosity reported here is due to: 1) pure sources of material, 2) quick working

up, 3) effective treatment with glacial acetic acid and, as to the synovial fluid, 4) immediate cooling. Seeing that ferrous and ferric ions depolymerise hyaluronic acid [14] great care was taken to obtain human umbilical cords and synovial fluid free from blood, and by vigorous treatment with glacial acetic acid to remove every trace of blood from the material. The umbilical cords employed were rinsed so effectively that all the blood seemed to be removed. From the ankle joints of cutting cattle, just at the spot mentioned above, we succeeded in withdrawing a synovial fluid which was quite free from blood and tissue debris. Great importance was attached to this fact because the tissue round the synovial capsule contains relatively much ascorbic acid (Robertson [15], McClean [16], Daubenmerkl [17]) which also depolymerises the hyaluronic acid. The quick withdrawing and cooling of the synovial fluid certainly effect a decrease of the activity of depolymerising substances possibly present. The vigorous mechanical treatment in the Waring Blendor may be the reason why the viscosity of the preparation denoted Prep. 1 is lower than the highest ones obtained (see Mogilevskii and Klyuchareva [18]). The viscosity of the best preparations is stable in time.

Meyer [1] reports $[\alpha]_D^{20}$ to be $-70°$ and Rapport *et al.*[19] $[\alpha]_D^{25}$ to be $-74°$. Much the same ($-64°$) was found in preparations prepared after Meyer. As seen in Table 2, however, we find much smaller values in two preparations. It seems that the highly viscous preparations exhibit a small optical rotation and those of low viscosity an appreciably larger one.

CONCLUSION

As a result of the experiments the method of preparation denoted Prep. 1 is considered to be the better one. The yield was good, the nitrogen content equalled the theoretical content, and the viscosity was relatively high. The preparations were entirely white and very hygroscopic. They dissolved in water very quickly, and the solutions were quite clear.

Financial support from *Carlsbergfonden* is gratefully acknowledged. The author is greatly indebted to professor J. A. Christiansen, Ph. D., Institute of Physical Chemistry, Copenhagen, Denmark, for his invaluable help and great interest in this investigation.

REFERENCES

1. Meyer, K. *Physiol. Revs.* **27** (1947) 335.
2. Jensen, C. E. *Kemisk Maanedsblad. In print.*
3. Meyer, K. *J. Biol. Chem.* **128** (1939) 312.
4. Blix, G., and Snellman, O. *Arkiv Kemi, Mineral. Geol.* **19A** (1945) No. 32.

5. Lundquist, F. *Acta Physiol. Scand.* **17** (1949) 44.
6. Sevag, M. G. *Biochem. Z.* **273** (1934) 419.
7. Meyer, K., and Palmer, J. W. *J. Biol. Chem.* **114** (1936) 687.
8. Blom, J., and Schwarz, B. *Acta Chem. Scand.* **3** (1949) 1439.
9. Dalgaard-Mikkelsen, S. Kvorning, S. A., and Rasbech, N. O. *Skand. Veterinär tidskrift* **42** (1947) 661.
10. Hadidian, Z., and Pirie, N. W. *Biochem. J.* (*London*) **42** (1948) 260.
11. Jeanloz, Roger W., and Forchielli, E. *J. Biol. Chem.* **186** (1950) 495.
12. Blix, G. *Acta Chem. Scand.* **5** (1951) 981.
13. Ogston, A. G., and Stanier, J. E. *Biochem. J.* (*London*) **46** (1950) 364.
14. Jensen, C. E. *Acta Chem. Scand.* **3** (1949) 584.
15. Robertson, W. v. B., Ropes, M. W., and Baur, W. *Am. J. Physiol.* **126** (1939) 609.
16. McClean, D., and Hale, C. W. *Biochem. J.* (*London*) **35** (1941) 159.
17. Daubenmerkl, W. *Acta Pharmacol. Toxicol.* **7** (1951) 153.
18. Mogilevskii, M. Sh., and Klyuchareva, I. S. *Chem. Abstracts* **46** (1952) 11267, from *Doklady Akad. Nauk S.S.S.R.* **85** (1952) 185.
19. Rapport, M. M., Meyer, K., and Linker, A. *J. Am. Chem. Soc.* **73** (1951) 2416.

Received February 12, 1953.