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Hyaluronic Acid

III. On Potassium Hyaluronate from the Endolymph of Sharks *

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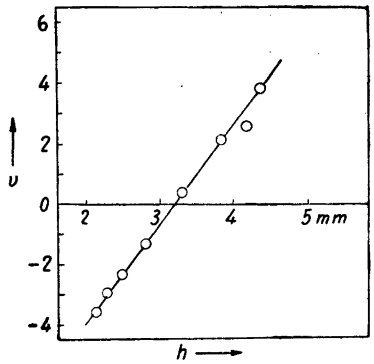
Potassium hyaluronate was isolated from the endolymph of shark ears. The nitrogen and potassium contents agreed with the theoretical values. From its osmotic pressure the molecular weight was calculated at 7.8×10^5. The substance is disaggregated by hyaluronidase.

As previously reported by Vilstrup and the author [1] it appeared likely that the endolymph of sharks contains hyaluronic acid, but the scarcity of the material prevented any attempt to its isolation. In the meantime, however, we have succeeded in collecting the endolymph from the ears of 150 sharks *(Acanthias vulgaris)*. This animal was employed as its labyrinths are especially easy of access, the skeleton is cartilageneous, and therefore technically easily manipulated. Moreover, the labyrinths of the sharks are relatively the largest known in the vertebrate system.

By a vertical section just behind the blow-holes the endolymphatic space, which contains the endolymph, is opened and the content, which is a translucent jelly, is withdrawn by gentle suction through a glass tube. By this technique 50 ml endolymph was obtained from 300 ears. The endolymph was apparently free from blood and cellular debris. The single endolymphatic clots merged into a homogeneous mass.

Immediately after withdrawing the endolymph it is cooled to —10° C, and the working up of the hyaluronic acid is started at once. During this investigation it became apparent that immediate cooling and quick working up of the starting material is very essential for the isolation of hyaluronic acid. The mucin is precipitated with about one volume per cent glacial acetic acid with vigorous stirring with a glass rod. As the endolymph is not miscible with water this precipitation must take place without the usual dilution with water. The mucin clot is washed quickly but thoroughly with water and dried in vacuum over sulphuric acid and silica gel. The dried powder is finely ground in a mortar and extracted with two or three volumes of glacial acetic acid at room temperature for three days with occasional stirring, the acid being replaced every

* Part I: *Acta Chem. Scand.* 3 (1949) 584, part II: *ibid.* 7 (1953) 603.



Fi. 1. The graph shows the relation between the height of the air column (abscissae), and the rate of movement of the upper meniscus of the air column (ordinates) in micrometer divisions in ten minutes.

twenty-four hours. By this procedure remnants of blood are removed, and the protein to which the hyaluronic acid is attached is split off. The acetic acid is now removed and the residue neutralized to pH 6.8 with a saturated potassium hydroxide solution (Glass electrode). The pulp is extracted with 4 volumes of water for three days, and the potassium hyaluronate is precipitated as a stringy mass with 4 volumes of alcohol. By adding more than the customary 1 1/2 volume of alcohol a further precipitate was formed. No flocculent precipitate was observed on standing. The preparation was purified according to Meyer [2]. The yield was 32 mg. The preparation was white and very hygroscopic; it dissolved in water in few hours, giving a clear solution.

The nitrogen content was determined by the method of Kjeldahl, modified by Blom [3]; (see Table 1). All volumetric flasks, the pipette and the burette

Table 1. The content of nitrogen and potassium, the viscosity and molecular weight of the potassium hyaluronate preparation.

% N*	% K**	Relative viscosity Conc. 1 g/l	Molecular weight $\times 10^{-5}$
3.33	9.01	24.24	7.79

* Calculated: 3.36.
** Calculated: 9.37

had been standardized. The potassium was determined as potassium sulphate; (see Table 1). The relative viscosity ($\eta_{rel} = \frac{t_H}{t_w}$, where t_H = flowtime of hyaluronate solution and t_w = flowtime of distilled water) was determined by a Kvorning and Dalgaard-Mikkelsen [4] viscosimeter; (see Table 1). Temperature 20° C (Variations about 0.01° C). The molecular weight (see Table 1) was determined by the inverted microosmometer recently described by Christiansen and the author [5]; temperature 20° C (Variations about 0.01 °C); the semipermeable testtube-shaped membrane used was a Kapcello cap. Fig. 1 shows the results of the single measurements.

As hyaluronidase is known to be highly specific towards hyaluronic acid (Chain [6], McClean [7], Robertson [8]) we tried to disaggregate our preparation

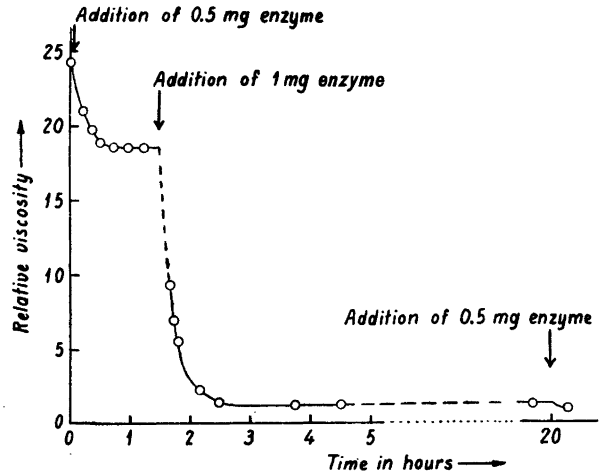


Fig. 2. Decrease in viscosity of a potassium hyaluronate solution caused by the action of testicular hyaluronidase.

by the action of a commercial (Schering) preparation of testicular hyaluronidase. When hyaluronic acid is disaggregated by hyaluronidase the viscosity of the aqueous solution is lowered.

Three millilitres of a 1 % aqueous solution of hyaluronate was incubated with 0.5 mg hyaluronidase at 20° C. The relative viscosity (measured in the viscosimeter mentioned above) which prior to the addition of enzyme was 24.24 decreased appreciably in the course of an hour. After that time the viscosity did not change, but after adding further 1 mg enzyme the viscosity decreased again and after a third addition of enzyme it equalled that of pure water. The results of the measurements are shown in Fig. 2. The diagram shows that the activity of the enzyme is rapidly inhibited. Whether this is due to destruction or adsorption of the enzyme, or the presence of inhibitory agents we do not know as yet.

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REFERENCES

1. Jensen, C. E., and Vilstrup, Th. *Acta Chem. Scand.* **7** (1953) 1128.
2. Meyer, K., *Physiol. Revs.* **27** (1947) 335.
3. Blom, J., and Schwarz, B. *Acta Chem. Scand.* **3** (1949) 1439.
4. Dalgaard-Mikkelsen, S., Kvorning, S. A., and Rasbech, N. O., *Skand. Veterinär-tidsskrift* **42** (1947) 661.
5. Christiansen, J. A., and Jensen, C. E. *Acta Chem. Scand.* **7** (1953) 1247.
6. Chain, E., and Duthie, E. S. *Brit. J. Exptl. Pathol.* **21** (1940) 324.
7. Mc.Clean, D., and Hale, C. W. *Nature* **149** (1940) 867.
8. Robertson, W. B., Ropes, M. W., and Bauer, W. *J. Biol. Chem.* **133** (1940) 261.

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