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Hyaluronic Acid

VI. An Electron Microscope Study of Potassium Hyaluronate *

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An electron microscopical study of potassium hyaluronate shows that potassium hyaluronate molecules are filamentous with a length of several thousand Å and a width less than 30 Å. This agrees with other evidence concerning its molecular size.

The object of this investigation was to obtain visual evidence of the molecular configuration in potassium hyaluronate. Since electron microscopy is capable of revealing structures in colloidal range we tried to achieve our object by this technique. From the high viscosity of its dilute solutions, the streaming birefrigence, and its ability to spin into threads it was concluded that hyaluronic acid has a filamentous structure. Further evidence of this point of view has recently been furnished by one of us [1], as it was found that a graph giving corresponding values of specific viscosities and products of molecular weight and concentration was in agreement with Staudinger's empiric viscosity rule, which is valid for randomly coiled flexible long-chain molecules. In a forthcoming paper it will be stated that dilute solutions of potassium hyaluronate display elastic recoil, a property which may be attached to molecules of the above mentioned type.

From measurements of viscosity and double refraction of flow Meyer and Palmer [2], and Blix and Snellman [3] estimated mean particle lengths for different preparations of hyaluronic acid of 4 700 to 10 000 Å corresponding to molecular weights ranging from 2×10^5 to 5×10^5, the longest particles being found for umbilical cord hyaluronate. Recently we have determined the molecular weight of potassium hyaluronate of umbilical cord origin to be in the order of 5×10^5 [4].

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* Part I: *Acta Chem. Scand.* **3** (1949) 584; Part II: *Ibid.* **7** (1953) 603; Part III: *Ibid* **8** (1954) 292; Part IV: *Acta Pharmacol. Toxicol.* **10** (1954) 83; Part V: *Acta Chem. Scand.* **8** (1954) 937.

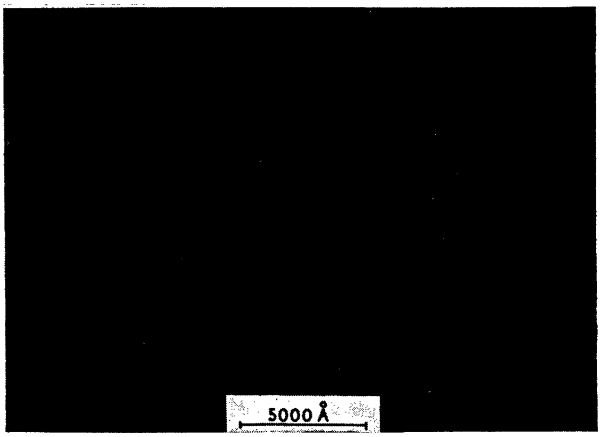

Fig. 1. Electron micrograph of potassium hyaluronate close to true focus. Explanation in text.

Assuming a length of 5 to 10 Å of the disaccharide unit, unbranched chains, and a degree of polymerisation of about 1 000, the length of this hyaluronate molecule would be 5 000 to 10 000 Å, and the transversal diameter about 5—10 Å. The present electron microscopy study substantiated this anticipation to a certain extent.

TECHNIQUE

For examination in the electron microscope, the sample must be placed on a membrane, only a few hundred Å in thickness, of a suitable material. It is normal practice to use a plastic film made from a solution of polyvinyl formal (Formvar) in ethylene dichloride placed on a supporting metal grid.

As the molecules seeked for are only about 5 to 10 Å in diameter, they would not be expected to yield sufficient contrast for detection in the electron microscope. We have therefore shadowed our specimens with palladium in a vacuum-evaporator at an angle of 1 : 3 by the technique of Wiliams and Wyckoff [5].

The easiest way to prepare a specimen from the potassium hyaluronate would be to place a droplet of a dilute solution of the material on the Formvar film and allow it to dry. During the drying proces, however, the dissolved molecules agglutinate so that the single molecules can not be detected. Other methods of preparation have been tried without success.

The Formvar film is usually slight hydrophobic; this sometimes causes the droplet to withdraw from areas, originally covered. These areas when inspected in the electron microscope do not usually display any structure but in our case on micrographs made from these parts some structural elements can be seen (Figs. 1 and 2). The pictures seem to agree well with those expected for threadlike molecules.

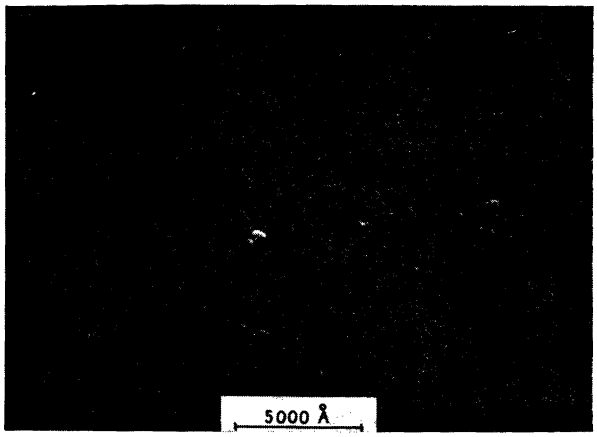

Fig. 2. Electron micrograph of potassium hyaluronate slightly underfocussed. Explanation in text.

This led us to the conclusion that some of the molecules, coming into close contact with the Formvar membrane, adhered so strongly to it, that they would be left on the membrane if the solvent was removed.

This assumption was confirmed by several experiments in which droplets of the solution were allowed to stay on the Formvar membrane for a short time only (60 sec.), and then removed completely. If allowed to stay for a considerable longer time, the membrane is rendered hydrophilic preventing a complete removal of the solvent.

The density of molecules on the membrane is a function of the concentration of the solution and the time the drop is left on the membrane, but in all cases the same filamentous structures were observed in the pictures.

By this method of preparation it is even possible to use solutions containing some amount of soluble salts because of the small amount of solvent and consequently of salt left on the membrane.

The electron micrographs shown here were taken of a highly purified, protein-free preparation from umbilical cords, the chemical composition of which corresponds closely to the theoretical values. The solutions were not dialysed. They contained about 0.01 % of the hyaluronate.

The electron microscope used in this study was an RCA type EMU 2B, capable of giving a resolution of about 20 Å. A focussing series of pictures were taken at an original magnification of 13 000, and the best focussed ones were later optically enlarged.

As seen from the micrographs * the molecules of potassium hyaluronate seem to form a dense network of anastomosing filaments, the individual fibers (molecules, it is supposed) having a relative smooth form and being fairly

* Unfortunately it was not to be avoided that many details disappeared in the reproduction.

constant in width all over their extent. Besides, all the threads are alike, a fact which really suggests that individual molecules, indeed, are observed on these micrographs. The observed patterns do not arise from the supporting film, as a micrograph of the latter taken under the same conditions as that of hyaluronate solutions, the only difference being the absence of hyaluronate, does not display structures.

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