# HYALURONIC ACID: STRUCTURE OF THE MACROMOLECULE IN THE CONNECTIVE TISSUE MATRIX

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## SUMMARY

Hyaluronic acid was extracted from the mucoid layer of rooster combs with limiting viscosity numbers as high as 13,000 ml per g. This material was shown to contain weak bonds, which were broken, irreversibly, by heating at neutral pH's at temperatures above 65°C. The activation energy of this reaction was 18Kcal per mole. Purified hyaluronic acid did not show this heat dependent irreversible degradation and protein did not seem to be a component of the structure formed by these bonds. These data suggest that the polysaccharide chains of the hyaluronic acid in the connective tissue matrix are organized into a macromolecular structure or network.

When hyaluronic acid from any source is purified by relatively mild procedures, the purified materials have lower viscosity values than the fluids or tissue extracts from which they were obtained (Ogston and Stanier, 1950; Balazs, 1958; Pigman, Rizvi and Holley, 1961; Swann, 1968; Silpananta, Dunstone and Ogston, 1968). This phenomena has been attributed to degradation and has usually been explained either by non-enzymatic oxidative reactions (Skanse and Sundblad, 1943; Blix and Snellman, 1945; Lundquist, 1949; Balazs and Sundblad, 1959), or by the disruption of protein-polysaccharide complexes, which, it has been assumed, occur in the connective tissue matrix (Ogston and Stanier, 1950; Silpananta, Dunstone and Ogston, 1968).

In an attempt to determine the structure of hyaluronic acid in

the connective tissue matrix the rooster comb has been examined. The results of studies on hyaluronic acid purified from this organ have already been described (Swann, 1968; 1968a). More than 90% of the hyaluronic acid in the comb had a molecular weight of $1.2 \times 10^6$ and an amino acid content of 0.35%. When the connective tissue of the mucoid layer of the comb was dissected free of other tissues and then washed free of all traces of blood, hyaluronic acid could be extracted with a limiting viscosity number as high as 13,000 ml per g. The present experiments were undertaken to characterize the structure(s) responsible for the extremely high viscosity of these extracts.

The connective tissue of the mucoid layer was obtained from White Leghorn rooster combs which had been blast-frozen immediately after death of the animal. The extractions were performed using distilled water at $4^{\circ}$C with thymol as a bacteriostatic agent. The initial washings and first two extracts were usually slightly colored; these were discarded. The subsequent extracts which were clear were then combined and filtered through a coarse glass filter to remove the comb pieces. These extracts had pH values in the range 6.4-6.7. This material (HAP) was dialyzed against distilled water or buffer and used for analysis or viscosity measurements without further treatment. The limiting viscosity values of many HAP preparations measured in a Cannon-Ubbelohde semi-micro dilution viscometer (shear rate range 60-240 $sec^{-1}$) at $25^{\circ}$C after dialysis against Tris buffer (0.05 M, pH 8.0; 0.2 M NaCl) varied between 8000-13000 ml per g. When HAP was incubated with pronase at $37^{\circ}$ for 16 hours (Grade B, Worthington Biochemical Company), the hyaluronic acid isolated by ethanol precipitation (HAPP) had limiting viscosity values of 5000-11000 ml per g. When HAP samples were treated in this way the HAPP always had lower viscosity values, but this decrease was caused by the ethanol precipitation procedure used to isolate the hyaluronic acid because pronase digestion alone failed to reduce the viscosity. It thus seemed

that protein was not a component of the structure(s) responsible for the extremely viscous nature of the HAP extracts. The results of analyses performed on HAP and HAPP are shown in Table 1. Hexuronic acid was determined by a modified carbazole method (Balazs et al., 1965) and glucosamine as described by Swann and Balazs (1966). Hyaluronic acid concentrations were obtained by multiplying the hexuronic acid value by a factor of 1.95. The amino acid contents were determined by ion exchange chromatography after HAP and HAPP samples had been hydrolyzed by refluxing with 6N HCl for 22 hours. Dry weight determinations were made by heating lyophilized samples at 40°C under vacuum and over $P_2O_5$ to constant weight. The ash content was determined after heating at 550°C until a constant weight was obtained. The amino acid content of isolated HAPP samples varied between 1.0 and 1.4% of the hyaluronic acid.

Viscosimetric measurements were then performed at 25°C in a Cannon-Manning semi-micro viscometer (solvent flow time 118.4 seconds) on samples containing 200 μg HAP per ml; 0.05M Tris pH 8.0 and 0.2M

TABLE 1

ANALYSIS OF HYALURONIC ACID PREPARATIONS BEFORE (HAP) AND AFTER (HAPP) TREATMENT WITH PRONASE, EXPRESSED AS A PERCENTAGE OF THE DRY WEIGHT

| | Hexuronic Acid | Glucosamine | Ash | Amino Acid |
|---|---|---|---|---|
| HAP | 38.0 | 34.9 | 9.8 | 20.0 |
| HAPP | 43.0 | 38.6 | -- | 1.4[x] |

[x]Calculated as a percentage of the hyaluronic acid determined by hexuronic acid analysis.

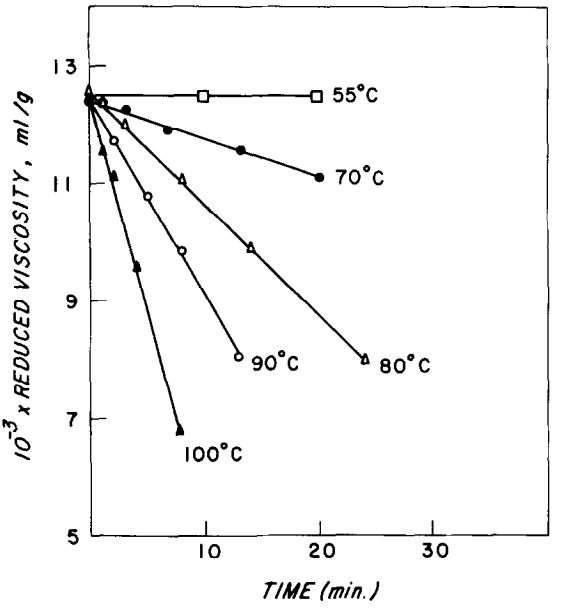


Figure 1. The decrease in reduced viscosity of samples containing 200 μg HAP per ml 0.05M Tris pH 8.0; 0.2M NaCl, after heating at various temperatures for increasing lengths of time.

NaCl, after this material had been heated at various temperatures for increasing lengths of time. At temperatures below 65°C reversible viscosity changes were observed, but above this temperature an irreversible change occurred. This is shown in Figure 1. When the rate constants of this reaction are plotted (Figure 2) against 1/T according to the equation: $\ln k = -\Delta S^{x}/R - \Delta H^{x}/R \cdot 1/T$ a value of 18Kcal per mole is obtained for the activation energy of reaction.

It is not possible to interpret the activation energy of this reaction in terms of the type or number of bonds that are disrupted; the above value for the activation energy may thus correspond to the cleavage of a large number of weak bonds, or a smaller number of stronger ones.

These data and the fact that purified hyaluronic acid preparations with limiting viscosity values in the range 2000-3000 ml per g (HAIIB,

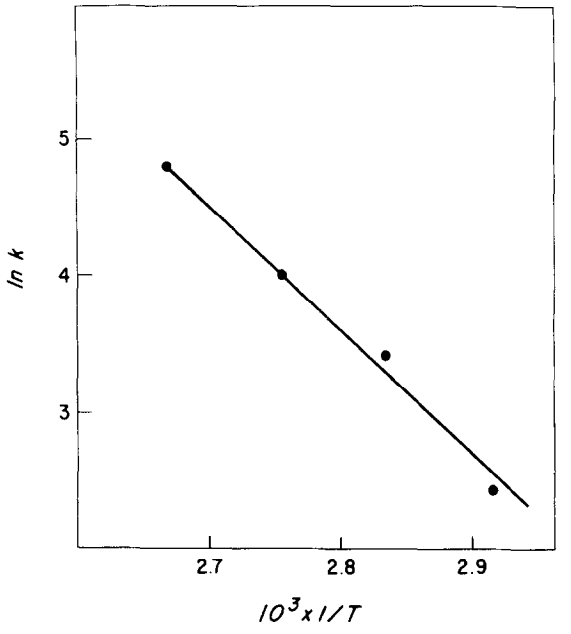


Figure 2. The temperature dependence of the rate constants for the reaction shown in Figure 2.

Swann, 1968a) did not show a temperature dependent irreversible degradation, suggest that the hyaluronic acid molecules in the samples of HAP are linked by weak bonds. The resistance of HAIIB to this temperature dependent degradation compared with the rapid decrease in the viscosity of this material that occurs when it is treated with ascorbic acid (Swann, 1967) clearly distinguish these two processes.

Although it is difficult to extrapolate in vitro observations to the in vivo circumstances, it is clear that the viscosity values reported here may not be the highest that can be obtained. If the polysaccharide chains of the hyaluronic acid molecules are linked by these bonds to form a three-dimensional network, then the viscosity value obtained will depend on the extent to which these bonds occur in the connective tissue matrices from different sources, and the extent to which they are disrupted during extraction.

These observations may have considerable importance for the biological function of this substance because cleavage of these bonds drastically altered the physical properties of hyaluronic acid solutions. It also seems reasonable to assume that when hyaluronic acid is present as a constituent in the connective tissue matrix, it would be affected in a similar manner.

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