HYALURONIC ACID AND THE ORD REACTION

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Hyaluronic acid undergoes a depolymerization reaction which will be described as the ORD reaction (oxidative-reductive depolymerization). Since hyaluronic acid, a major component of ground substance, seems to be a principal factor in the permeability of connective tissue, the biological significance of this reaction in health and disease is potentially high. The ORD reaction or a similar one may be general for nucleic acids and polysaccharides (Skanse & Sundblad, 1943; Gilbert et al., 1957; Machida et al., 1959).

Although some aspects have been previously described (Hale, 1944; Skanse & Sundblad, 1943; v. Robertson et al., 1941; Jensen, 1949), the reaction was encountered in the present work in studies of some unexplained degradations of hyaluronic acid and synovial fluids. These included degradations by merthiolate (used for preservation), by lyophilization in the presence of salts, and by weak acids in the preparation of synovial mucin (Pigman et al., 1959).

The basic features of the ORD reaction involve several interesting variables. The degradation of hyaluronic acid proceeds best in the presence of a "reducing substance" such as ferrous or cuprous ions, ascorbic acid or cysteine. Phosphate ions greatly speed up the reaction compared to chloride ions. Oxygen is necessary. The reaction is inhibited by glucose and completely repressed by high concentration of alcohol. In this system at 30° and pH 7.3, the specific viscosity of hyaluronic acid dropped to less than 10 per cent of the initial viscosity at times varying from 5 min. to several hours. Synovial fluids showed similar degradations. Detailed results will be given below.

Experimental

The reactions were carried out generally as follows: A 0.5 ml. solution of "reducing agent" was added to 1.0 ml. of hyaluronic acid solution in phosphate buffer at 30°, pH 7.3. In the final solution, the hyaluronic acid concentration was 0.03% and the sodium phosphate buffer was 0.2M. The concentration of "reducing agent" is given with the data. At intervals up to 6 hrs., aliquots were removed, and the viscosity was measured at 30° in semimicro capillary viscometers (Cannon-Manning type). Comparisons were made of the percentage decrease of specific viscosity from the initial viscosity or that of controls.

The hyaluronic acid was prepared from cattle synovial fluid by the electrodialysis procedure repeated five times (Roseman & Watson, 1957). The products contained no detectable amount of protein, less than 4%, and had an intrinsic viscosity of about 40 in 0.2M phosphate buffer at pH 7.3 and 30°.

When inhibitors were tested, these were incorporated in the hyaluronic acid solution before mixing with the "reducing substance".

For tests of the effect of nitrogen and hydrogen, a special apparatus was designed; all transfers took place in the presence of these gases.

Results and Discussion

Typical results are given in Table I. Ferrous ions were most effective, but cuprous ions, ascorbic acid, several SH compounds and other substances were active. The oxidized forms of these substances showed little or no activity (except at elevated temperatures). Hale (1944) found cuprous ions or ascorbic acid to be effective, whereas Skanse and Sundblad (1943) and v. Robertson et al. (1941), used more complex systems of hydrogen peroxide or cupric ions and ascorbic acid. Jensen (1949) found both ferrous and ferric ions effective but carried out the reaction with ferric ions in the presence of hydrazine.

The reaction was carried out in presence of "repurified" nitrogen (20 ppm. of oxygen) or of catalytically purified hydrogen (less than 5 ppm. of oxygen), with 0.2 mM./l. solutions of ferrous sulfate, ascorbic acid or cysteine. As indicated previously by Hale (1944) and Skanse and Sundblad (1943), the reaction

was greatly inhibited. After one hour, the ferrous sulfate system with hydrogen had 75% of its initial viscosity and with 0.2M sodium chloride instead of phosphate, 92%.

Table I

Reduction in the viscosity of hyaluronic acid (HA) and cattle synovial fluid (SF) at 3 hrs. in phosphate buffer (0.2M) at pH 7.3 and 30° exposed to air.

"Reducing agent"	Concn.[a] (mM./1.)	% Initial viscosity[b]		"Reducing agent"	Concn.[a] (mM./1.)	% Initial viscosity[b]	
		HA	SF			HA	SF
None (blank)	0	99	98	Hydroquinone	.55	41	65[d]
$FeSO_4$[c]	.12	7	7[d]	Glutathione	1.4	84	84
$FeCl_3$	.89	82	-	Leuco-methylene blue	.55	64	87
CuCl	.21	28	21	Methylene blue	.55	94	98
$CuSO_4$	.53	84	-	H_2O_2	1.0	54	76
Cysteine	.55	31	10[d]	Bromine	5.4	46	97
Cystine	.55	98	99	Merthiolate	.50	55	96
Ascorbic acid	.56	31	10[d]	Serum albumin	(670)[e]	84	-

a. Concn. of hyaluronic acid .03-.04 mg./ml. (.79-1.06 mM./1. as glucurone).
b. Reduction of specific viscosity from blank without "reducing agent".
c. Reduction of viscosity at 5 min.
d. For the synovial fluid the concn. of reducing agent was ten times greater.
e. Concn. as mg./100 ml. and for a time of 6 hours.

Phosphate ions were much more effective than the same molar concentrations of sodium chloride in enhancing the degradation. In 30 min., ferrous sulfate (1.25 mM./1.) reduced the specific viscosity to 51% of the initial value in 0.2M NaCl (no phosphate) and to 9% in 0.2M phosphate buffer. With ascorbic acid (0.63 mM./1.), the corresponding values at 30 min. were 72 and 6%. Even without "reducing agent", solutions of hyaluronic acid in 0.2M phosphate showed slow reductions of viscosity, not evident in water. Solutions of hyaluronic acid lyophilized in the presence of phosphate buffer (and also of sodium chloride) underwent considerable reduction of viscosity as compared with dialyzed solutions.

Visible light did not affect the reaction. Under conditions similar to those of Table I with ferrous sulfate, ascorbic acid or cysteine, the extent of degradation was the same when the reaction was carried out in the dark, with room illumination or with a 500 w. incandescent lamp at one foot.

Several inhibitors for the reaction were found, under conditions such as

those for Table I, with ascorbic acid, cysteine, ferrous sulfate or hydroquinone as "reducing agent". In the presence of about 16% (by vol.) ethanol, no degradation was detected under these conditions (and at 100° without reducing agent). Solutions saturated with toluene showed reductions to 58% of the initial viscosity, whereas in the absence of toluene the final viscosity was 12% (with ferrous sulfate as reducing agent). In the presence of D-glucose (133 mg./100 ml.), the final viscosity was 20% of the original compared with 10% for the control (with ascorbic acid as reducing agent). Serum albumin also showed some inhibition, although by itself acted as a "reducing agent" (Table I).

No detectable loss of glucuronic acid and hexosamine components could be found after the reaction with ascorbic acid (5.2 mM./l.) or ferrous sulfate (3.3 mM./l.) under the conditions of Table I for dialyzed solution which retained only 1-2% of the initial viscosity. Losses of about 5% would not have been detected. These results are different from those of Skanse and Sundblad (1943) and Machida et al. (1959) for more complex systems who reported the production of carboxyl groups or carbon dioxide.

These depolymerized solutions were dialyzed and examined in the ultracentrifuge and by Tiselius electrophoresis. The patterns appeared generally like hyaluronic acid after mild treatment with hyaluronidase and showed broad diffuse peaks. The electrophoretic mobility was not changed.

These studies are being continued. A free radical mechanism seems likely. Although the ORD reaction seems general for polysaccharides, present evidence suggests that a glycosidic linkage near a carboxyl group of a uronic acid polymer may be particularly susceptible.

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