The Mechanism of Action of Hyaluronidases*

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The term hyaluronidase has been used to denote a series of enzymes that depolymerize certain of the acid mucopolysaccharides. Earlier views that these enzymes possess a common enzymic activity are now obsolete as a result of the demonstration of differences in specificity and reaction products. Testicular hyaluronidase hydrolyzes the endo-β-acetylglucosaminic bond of hyaluronic acid, chondroitinsulfuric acid A, and chondroitinsulfuric acid C to a series of oligosaccharides that contain a preponderance of tetrasaccharides (1-4). Leech hyaluronidase also produces primarily tetrasaccharides but, in contrast, ruptures the endogluconic acid bond (5, 6). The bacterial hyaluronidases, which are inactive on chondroitinsulfuric acids A and C, degrade hyaluronic acid to a disaccharide (7-10).

EXPERIMENTAL PROCEDURE

Materials and Methods—Clostridium welchii hyaluronidase was prepared by the method of Becker et al. (12) from a stock strain of the Department of Microbiology of the University of Chicago. The purified enzyme contained 2000 to 3000 turbidity-reducing units per mg of protein. Crude streptococcal hyaluronidase containing 45 turbidity-reducing units per mg was obtained from the Wyeth Laboratories. Testicular hyaluronidase that contained 1600 turbidity-reducing units per mg was purified in this laboratory, according to the method of Freeman et al. (13), by Dr. Martin B. Mathews. Hyaluronic acid was prepared from the supernatant of a culture of Group A streptococcus, strain A111 (14). H3O* was obtained from the Weizmann Institute of Science, Rehovoth, Israel.

Enzyme activity was measured by the method of Dorfman, Ott, and Whitney (15). The enzymes were standardized by comparison with a standard preparation of testicular hyaluronidase prepared by Armour Laboratories (82 turbidity-reducing units per mg (lot $206-266E$)).

Uronic acid was determined by the method of Dische (16), total N by the micro-Kjeldahl method, and glucosamine by the method of Boas (17), omitting the Dowex treatment. N-Acetylgalactosamine was estimated by the method of Reissig et al. (18), total carbohydrate by the method of Dubois et al. (19), and reducing sugar by the method of Park (20).

The O18 concentration was determined in the mass spectrometer after combustion of the compounds with HgCl2 in an evacuated tube by the method of Rittenberg and Ponticorvo (21). This procedure gives a sample of all the oxygen in the compound in the form of CO2. The ratio, R, of CO2 with mass of 44 to CO2 with a mass of 46 was determined with a mass spectrometer. Atom % O18 was calculated from the following equation taken from Rittenberg (22):

\[
\text{Atom % O}^{18} = \frac{100}{2R + 1}
\]

Atom % excess O18 was obtained by subtracting normal O18 abundance as determined by analysis of unlabeled CO2 in a blank determination. Normal O18 abundance, determined with every set of analyses, was about 0.210 atom %. Samples of unlabeled disaccharide gave the same value for normal abundances as unlabeled CO2.

The unsaturated disaccharide formed by the action of bacterial hyaluronidase was isolated as described by Linker et al. (9), except that the ethanol elution was carried out at 4°C, and pyridine was not used. The compound was chromatographically pure. Its absorption spectrum was determined in aqueous solutions of pH ranging from 1.5 to 11. For these measurements, the compound was dissolved in 0.94 M KH2PO4. The pH was adjusted appropriately with NaOH or HCl immediately before recording the spectrum. The absorption spectra at pH 1.5 and pH 4 are presented in Fig. 1. Between pH 2 and pH 4, the maximum shifted from 235 μm to 232 μm and the absorbancy decreased.
extinction coefficients were calculated from the average of three separate determinations. Light path, 1 cm. The concentration of the unsaturated disaccharide in both solutions was 67.5 μg per ml on a weight basis and 0.153 pmole per ml on the basis of hexosamine analysis. The data illustrated in Table I.

Above pH 4.0, there was little change in the absorption spectrum. The molar extinction coefficients calculated on the basis of hexosamine analysis were εmax = 6.0 × 10^4 liter mole⁻¹ cm⁻¹ at pH 1.5 and εmax = 5.0 × 10^5 liter mole⁻¹ cm⁻¹ at pH 4.0.

**Experiments with Bacterial Hyaluronidase**

**Attempt to Demonstrate Saturated Intermediate**—There was a possibility that the depolymerization of hyaluronic acid by bacterial hyaluronidase might involve the initial formation of a saturated disaccharide which would lose water in a subsequent step to form the unsaturated disaccharide as a final product. If such a saturated intermediate should accumulate during the course of the enzyme reaction, there would be a greater initial increase in reducing power than in light absorption at 232 μm. In both cases, control experiments were performed in which the unsaturated disaccharide was incubated with the enzyme without added hyaluronic acid. In these control experiments, the reisolation of the disaccharide was carried out by the same procedure as that used for the isolation of the disaccharide from the enzymatic degradation.

**TABLE I**

<table>
<thead>
<tr>
<th>Chemical analysis of unsaturated disaccharide and hyaluronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molar ratio</strong></td>
</tr>
<tr>
<td>Glueuronic acid</td>
</tr>
<tr>
<td>Glucosamine</td>
</tr>
<tr>
<td>N-Acetylglicosamine</td>
</tr>
<tr>
<td>Reducing sugar (as glucose)</td>
</tr>
</tbody>
</table>

*Values are expressed as molar ratio with glucosamine taken as 1.00.*

1 Linker, Meyer and Hoffman reported a value of log ε = 3.75 (9). Dr. Meyer has informs us that this is a typographical error and should read log ε = 3.75. This would give ε = 5.5 × 10^4, in reasonable agreement with the value reported above.

**Absence of O18 Uptake during Bacterial Hyaluronidase Action**—The structure of the unsaturated disaccharide formed by bacterial hyaluronidase suggests that the enzyme action may be restricted to a cleavage of the bond from C-4 of the glucuronic acid moiety to the oxygen bridge, with accompanying loss of a proton from C-5. In this case, the reaction should proceed with no obligatory incorporation of oxygen from the water of the medium into the C-1 position of the acetylglucosamine moiety, or into any other position of the disaccharide. H2O18 was used to determine whether or not this was the case. Hyaluronic acid was depolymerized by bacterial hyaluronidase in a medium of heavy water, and the isolated disaccharide was analyzed for O18.

Because of the expense of the H2O18, the enzyme reactions were carried out in a minimal volume. Approximately 150 mg of hyaluronic acid were added directly to 2.5 ml of H2O18 containing 0.1 M acetate buffer at pH 5.0, 0.15 M NaCl, and the desired amount of enzyme. Solution of the substrate occurred within a few minutes. After incubation for 4 to 8 hours at 38°, the H2O18 was recovered by lyophilization and the residue was dissolved in approximately 3 ml of H2O. The disaccharide was absorbed on a column containing 5 g of Darco G-60-Celite (3:2). The column was washed with H2O until Cl⁻-free, and the unsaturated disaccharide was eluted with 20% ethanol. Washing required 2 days, whereas elution required 4 to 5 days. Both operations were carried out at 4°. The disaccharide was recovered by concentration in a vacuum followed by lyophilization. Of the hyaluronic acid, 60% was recovered as unsaturated disaccharide.

Samples of the disaccharide were prepared for O18 analysis by desiccation in a vacuum over P4O10 at 60° for 3 hours and at room temperature for 24 hours. Chemical analysis of the disaccharide product isolated from the experiments in heavy water yielded the results shown in Table I.

The results of the O18 analyses are shown in Table II. The values are given as the average atom % excess O18 of all the oxygen of the disaccharide. Separate experiments were carried out with streptococcal enzyme and with enzyme from C. welchii. In both cases, control experiments were performed in which the unsaturated disaccharide itself was incubated with the enzyme in H2O18 for the same period of time and under the same conditions as those used for the enzymatic degradation of the hyaluronic acid. In these control experiments, the reisolation of the disaccharide was carried out by the same procedure as that used for the isolation of the disaccharide from the enzymatic degradation.

![Absorption spectra of the unsaturated disaccharide.](image)

**FIG. 1.** Absorption spectra of the unsaturated disaccharide.

![Change in unsaturation and in reducing power during depolymerization of hyaluronic acid by bacterial hyaluronidase.](image)

**FIG. 2.** Change in unsaturation and in reducing power during depolymerization of hyaluronic acid by bacterial hyaluronidase. Reaction mixture contained 19 μmoles of hyaluronic acid per ml (concentration of hyaluronic acid was determined by glucuronidase acid analysis) in 0.1 M phosphate-0.15 M NaCl. The reaction was started by the addition of 1400 turbidity-reducing units of purified C. welchii enzyme. Aliquots were taken at the times indicated, frozen rapidly, and thawed just before analysis. O--O, change in light absorption at 232 μm, pH 5; O--O, reducing groups determined according to the ferricyanide method of Park (20), with glucose as a standard.
The data in Table II show that the net O\(^{18}\) content of the unsaturated disaccharide was trivial, far less than that expected for an obligatory incorporation of 1 atom of oxygen into each disaccharide molecule. The small amount of O\(^{18}\) incorporation occurred to about the same extent, whether the disaccharide was added at the beginning of the incubation or whether it was formed from hyaluronic acid. There was considerable variation in the control incorporation, for reasons which were not apparent, but variations several-fold larger than those observed would in no way affect the validity of the conclusions.

The small O\(^{18}\) incorporation observed was approximately of the magnitude expected from previous measurements of the rate of exchange of carboxyl groups and of sugar carboxyl groups with the oxygen of solvent water (23–26). The unsaturated disaccharide has a free reducing group; and both the disaccharide and hyaluronic acid contain carboxyl groups. Since O\(^{18}\) exchange might be expected in both the carboxyl group oxygen and in the hemiacetal oxygen, it seemed desirable to establish the relative contribution of the two groups. For this purpose, the rate of exchange of the disaccharide was compared with the rate of exchange of the corresponding alcohol obtained by NaBH\(_4\) reduction. The extent of incorporation of O\(^{18}\) into these two compounds after incubation with H\(_2\)O\(^{18}\) for 48 hours at 37° is indicated in Table III. There was a larger incorporation of O\(^{18}\) in the unreduced than in the reduced disaccharide, consistent with the presence of both hemiacetal and carboxyl groups in the former and only a carboxyl group in the latter. With the assumption that the carboxyl oxygen atoms exchange at the same rate in both compounds, the relative contributions of the hemiacetal and carboxyl groups may be calculated. Such a calculation at 48 hours indicates that 26% of the O\(^{18}\) is incorporated into the hemiacetal group and 74% into the carboxyl group.

In addition to the experiments carried out at 37°, detailed in Table III, measurements of isotope exchange rates at 4° were made by incubating the unsaturated disaccharide in H\(_2\)O containing 20 atom % excess O\(^{18}\) for 24, 48, and 96 hours. The respective values of 0.191, 0.302, and 0.560 atom % excess O\(^{18}\) were considerably lower than those obtained at 37°. The value obtained at 96 hours and 4° is a measure of the possible loss of oxygen during the isolation procedure used in the enzyme experiments. Since 3 of 11 oxygen atoms in the disaccharide can be labeled by exchange, complete exchange would give 20 × \(\frac{1}{11}\) or 5.6 atom % excess O\(^{18}\). The observed value of 0.56 atom % excess O\(^{18}\) therefore represents 10% exchange over a 96-hour period at 4°. This, then, represents the magnitude of the loss of labeled oxygen during the isolation procedure. A final control experiment was performed in which labeled disaccharide containing 0.95 atom % excess O\(^{18}\) was carried through the entire isolation procedure, which required 99 hours. The O\(^{18}\) content of the reisolated disaccharide was 0.84 atom % excess O\(^{18}\). The loss of 11% of the label agreed well with the observation that 10% labeling occurred when the compound was incubated in H\(_2\)O\(^{18}\) under the same conditions of time and temperature. These experiments showed that no large proportion of O\(^{18}\) could have been lost during the isolation procedure. They were performed in order to rule out the possibility that a large amount of O\(^{18}\) may have been incorporated during the enzyme reaction and then lost during the process of isolation of the disaccharide.

**Polarimetric Studies**—The direction of mutarotation after glycosidase action has been employed to ascertain the configuration of the reducing carbon atom liberated by the enzyme action

### Table II

**Bacterial hyaluronidase-catalyzed depolymerization of hyaluronic acid in H\(_2\)O\(^{18}\)**

<table>
<thead>
<tr>
<th>Enzyme Material analyzed</th>
<th>Concentration of O(^{18}) (atom % excess)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme control</td>
</tr>
<tr>
<td>streptococcus (^d)</td>
<td>Disaccharide</td>
</tr>
<tr>
<td>water</td>
<td>Disaccharide</td>
</tr>
<tr>
<td>C. welchii (^d)</td>
<td>Disaccharide</td>
</tr>
<tr>
<td>water</td>
<td>Disaccharide</td>
</tr>
<tr>
<td>water</td>
<td>Water</td>
</tr>
</tbody>
</table>

* The amount of O\(^{18}\) which would have been found in the product if one atom of oxygen were derived entirely from the medium. This number is calculated by dividing the atom % excess O\(^{18}\) of the medium by the number of oxygen atoms (11) present in the unsaturated disaccharide.
* Column 1 less column 3.
* The reaction mixture contained 3700 turbidity-reducing units of streptococcal hyaluronidase and incubation was for 8 hours.
* The reaction mixture contained 45,000 turbidity-reducing units of C. welchii hyaluronidase and incubation was for 4 hours.

### Table III

**Rate of oxygen exchange of unsaturated disaccharide and reduced unsaturated disaccharide in H\(_2\)O\(^{18}\) at 87°**

<table>
<thead>
<tr>
<th>Time</th>
<th>Concentration of O(^{18}) (atom % excess)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unsat. disaccharide</td>
</tr>
<tr>
<td>hrs</td>
<td>average</td>
</tr>
<tr>
<td>3/6</td>
<td>0.154</td>
</tr>
<tr>
<td>3</td>
<td>0.106</td>
</tr>
<tr>
<td>9/4</td>
<td>0.277</td>
</tr>
<tr>
<td>24</td>
<td>0.495</td>
</tr>
<tr>
<td>48</td>
<td>1.007</td>
</tr>
</tbody>
</table>

* The product obtained on reduction with borohydride contains 10 rather than 11 oxygen atoms per molecule (9). The values for the O\(^{18}\) contents obtained on analysis of the reduced unsaturated disaccharide were, therefore, multiplied by \(\frac{10}{11}\) in order to get numbers which could be compared directly with the values for the O\(^{18}\) contents of the unreduced disaccharide.

The solutions were made up to contain 100 mg of disaccharide in 0.5 ml of H\(_2\)O\(^{18}\) containing 20 atom % excess O\(^{18}\). The pH was adjusted to 5 with NaOH. Incubation was at 37°. At the time indicated, 0.1 ml aliquots were removed, dried, and analyzed for O\(^{18}\).

(27, 28). Similar measurements were performed with bacterial hyaluronidase to ascertain whether the results would be consistent with an enzyme mechanism involving inversion or retention of configuration at C-1 of the acetylglycosamine moiety.
Tests of the enzyme solution prepared as above showed that it contained 21.9 pmoles per ml of glucuronic acid and 12.3 turbidity-reducing units per mg sample. Analysis showed that it contained 21.9 pmoles per ml of glucuronic acid. To this was added an enzyme solution prepared by dissolving 8.5 mg of purified C. welchii hyaluronidase (1700 turbidity-reducing units per mg) in 4.0 ml of the phosphate-NaCl solution described above. This enzyme solution was clarified by centrifugation at 20,000 x g for 5 minutes at 4°C. The final reaction mixture contained 16 pmoles per ml of glucuronic acid and was incubated at 26°C. Polarimetric measurements were made with a Schmidt-Haensch polarimeter with tubes of 0.5-ml capacity and 1 cm length. The specific rotation, [α]", of the unsaturated disaccharide is -20°. The corresponding value for the hyaluronic acid was taken as -75°. The points on Curves a and b represent 

\[
\frac{100 \times (\text{observed specific rotation} - 20)}{75 - 20}
\]

In the calculations the observed specific rotation is taken as a positive number. The rotation actually observed before acid treatment ranged from \(\alpha = -0.450\) at 3 minutes to \(\alpha = -0.230\) at 95 minutes. After acid treatment, the rotation ranged from \(\alpha = -0.465\) at 1 minute to \(\alpha = -0.190\) at 64 minutes.

The optical rotation of hyaluronic acid has been reported as [α]₀, -70° (20). The value varies somewhat with the preparation. Because of the uncertain water content of solid hyaluronic acid, the specific rotation of the preparation employed in the present experiments was determined and calculated on the basis of the measured glucuronic acid content of an aqueous solution (prepared for the experiment of Fig. 3), and found to be [α]₀ = 75°. This value is considerably more negative than that of the unsaturated disaccharide, [α]₀ = -20° (9). (The latter value presumably represents the value for an equilibrium mixture of the α and β forms, but neither of these has been isolated separately). The combined results of hydrolysis and mutarotation must give a decrease in negative rotation. The results in Fig. 3 show clearly that the full decrease in negative rotation expected has not occurred at the time when hydrolysis is complete, as measured by the increase in ultraviolet absorption. The optical change continued for a considerable period after hydrolysis was complete. If an extra 6 hours were allowed for mutarotation at pH 3.6 in the absence of enzyme action, an additional decrease in negative rotation took place. The mutarotation at this pH was slow, but the results were clear cut.

The available evidence strongly suggests that the N-acetylglucosaminidic linkage of hyaluronic acid is β (6, 30). If the free reducing carbon atom were liberated in the β-configuration, then mutarotation would be associated with an increase in rotation (decrease in negative rotation) due to the formation of a certain proportion of the α-isomer. This is actually the direction of the change observed. The results are therefore completely consistent with an enzyme mechanism involving cleavage of hyaluronic acid at the bond between C-4 of the glucuronic acid moiety and the oxygen bridge to C-1 of the acetylglucosamine. Cleavage at this point could not lead to inversion of configuration at the C-1 position of glucosamine.

**Experiments with Testicular Hyaluronidase**

The product of the action of testicular hyaluronidase is primarily tetrasaccharide, with the structural formula shown in the second line of Fig. 4. Since conversion of hyaluronic acid to this product necessitates an uptake of water, the tetrasaccharide must acquire O³⁻ if the enzymic depolymerization is carried out in a medium of H₂O³⁺. There are two possible sites of incorporation of O³⁻. If the enzymic cleavage occurs between C-1 of the N-acetylglucosamine and the oxygen bridge, than O³⁻ should appear at Site a of Fig. 4. If the cleavage occurs between C-4 of glucuronic acid and the oxygen bridge, then O³⁻ should appear at Site b in Fig. 4. The two positions, at a and b, should differ markedly in their stability to exchange with solvent water. Thus, the O³⁻ in an alcohol group, at b, should be very stable,

![Diagram of enzymatic depolymerization of hyaluronic acid](image-url)
whereas the O\(^{18}\) in the hemiacetal position, as at \(a\), would undergo a measurable rate of exchange (23, 24, 31, 32). If the O\(^{18}\) were lost under conditions expected to cause such exchange, then the conclusion could be drawn that the site of O\(^{18}\) incorporation was at \(a\) rather than at \(b\).

Hyaluronic acid was depolymerized with testicular hyaluronidase in a medium of H\(_2\)O\(^{18}\). The basis of procedure adopted for the localization of the O\(^{18}\) is indicated in Fig. 4. The crude tetrasaccharide product (of the enzyme action) was hydrolyzed in unlabeled water to give an unsaturated and a saturated disaccharide which could be separated by chromatography on paper so that each of the products could be analyzed separately. Such separation seemed desirable in order to obtain O\(^{18}\) analyses on reasonably well defined compounds. The lengthy procedure involved extensive opportunity for loss of O\(^{18}\) by exchange from a labile position, but would not be expected to labelize any O\(^{18}\) at \(b\).

In two experiments, 500 mg of hyaluronic acid and 50 mg of testicular hyaluronidase (1000 turbidity-reducing units per mg) were added to 3.5 ml of H\(_2\)O\(^{18}\) containing 0.1 M phosphate buffer at pH 5.0. The pH was adjusted with sufficient alkali to give complete solution and an additional 50 mg of testicular hyaluronidase was added. During the incubation for 45 hours at 37\(^{\circ}\) under toluene, 60 mg of enzyme were added at the end of each 12-hour interval.

After the removal of the H\(_2\)O\(^{18}\), the residue was dissolved in 10 ml of H\(_2\)O, 10 ml of ethanol, and the mixture was placed for 1 minute in a boiling water bath. After 60 minutes at room temperature, the precipitate was separated by centrifugation. The precipitate was washed twice with 50% ethanol and the combined supernatant and washings were concentrated in a vacuum to 300 to 400 ml of 20% ethanol. The washing and elution were carried out at 4\(^{\circ}\) for a period of 1 week. Elution of the disaccharides was followed by measurement of absorption at 232 mp.

For the preparation of the disaccharides, 450 mg of the crude tetrasaccharide preparation were incubated at 37\(^{\circ}\) for 16 hours under toluene with 30 to 50 mg of purified C. velebit C. velebit hyaluronidase in 4 ml of 0.1 M acetate-phosphate buffer, pH 5.0, containing 0.15 M NaCl. After the incubation, the solution was poured through a column made with 7 g of Darco G-60-Celite (5:2) to absorb the disaccharides. After the column had been washed with H\(_2\)O until Cl\(^{-}\)-free, the disaccharides were eluted with 300 to 400 ml of 20% ethanol. The washing and elution were carried out at 4\(^{\circ}\) over a period of 1 week. Elution of the disaccharides was followed by measurement of absorption at 232 mp. After evaporation of the solvent in a vacuum, 140 mg of a white amorphous powder were obtained. On the basis of glucosamine analysis and 232 mp absorption, the disaccharide mixture contained 35% unsaturated disaccharide (Table IV). Chromatography on paper with the acid-butanol solvent showed two spots with the Partridge reagent (33). The more rapidly moving spot was identified as the unsaturated disaccharide by observation in ultraviolet light.

For separation of the disaccharides, 50 mg of the mixture were dissolved in 0.5 ml of H\(_2\)O and streaked on 9 sheets of Whatman No. 3 paper (12.5 \(\times\) 18 cm). Descending chromatograms with the acid-butanol solvent were carried out at 4\(^{\circ}\) for 24 hours. After drying for 2.5 hours at room temperature, the papers were cut and the disaccharides were eluted with H\(_2\)O. The eluates were treated with a small amount of Darco G-60, concentrated in a vacuum, and lyophilized. Approximately 20 mg of saturated disaccharide and 15 mg of the unsaturated disaccharide were obtained. Both compounds were amorphous but gave only one spot on chromatography on paper. Results of chemical analyses of the two compounds are given in Table V and show that the saturated disaccharide was contaminated by 3\% of the unsaturated disaccharide as indicated by absorption at 232 mp.

The results of analyses of the products for O\(^{18}\) are shown in Table VI. As in previous experiments, controls were carried out in which a tetrasaccharide preparation was incubated with testicular hyaluronidase under the same conditions as employed for the enzymatic degradation of hyaluronic acid in H\(_2\)O\(^{18}\). In Experiment 1 of Table VI, the disaccharide mixture was analyzed for O\(^{18}\) before separation by paper chromatography. This sample contained about 45\% of the O\(^{18}\) expected if 1 atom of O\(^{18}\) were incorporated per tetrasaccharide molecule. No appreciable amount of O\(^{18}\) above that in the controls was found in either the unsaturated or saturated disaccharide. Since the saturated disaccharide should not have lost any O\(^{18}\) incorporated at C-4 of its glucuronyl group, the conclusion was drawn that the O\(^{18}\) was incorporated at C-1 of the reducing acetylgalactosamine moiety of the tetrasaccharide.

**DISCUSSION**

The hyaluronidases have been termed glycosidases on the basis of the heteropolysaccharide nature of their substrates which are degraded to fragments of low molecular weight as a result of the hyaluronidase action. Linker et al. (9) have pointed out, however, that the bacterial hyaluronidase apparently catalyzes a simple elimination reaction. Their conclusion was based on the chemical structure of the product and on the demonstration that the tetrasaccharide product of testicular hyaluronidase action is converted by bacterial hyaluronidase into two disaccharides, one of which is saturated. Although the suggestion of Linker et al.
The formulation of the reaction as an elimination process precludes the possibility that there is an initial attack of the hydroxyl group on C-1 of the N-acetylgalactosamine moiety. This process would be associated with an incorporation of O18 in the O at C-1 if the process occurred in labeled water. The experiments with H2O18 described showed, in fact, that there was no obligatory incorporation of O18 from the medium into the unsaturated disaccharide during its formation by enzymatic action. In other words, the results show that the enzyme action does not involve a cleavage of the molecule between the C-1 of the N-acetylgalactosamine and the O attached to C-4 of the glucuronic acid moiety. The polarimetric studies likewise confirm this conclusion. They show that the first product of enzymatic action probably has the same B-configuration at the reducing group as the hyaluronic acid itself. Had an inversion of configuration accompanied hydrolysis, the results would have contradicted the data obtained with H2O18.

All of the results obtained with bacterial hyaluronidase are in keeping with a mechanism formulated as a simple elimination reaction. Bacterial hyaluronidase should almost undoubtedly be classified with enzymes such as fumarase and acetalase, and not with the glycosidases. It is of interest, however, that the ring structure of the uronic acid moiety of hyaluronic acid requires that the unsaturated disaccharide product be formed by the elimination of the H and OR groups, whereas the addition and elimination of water in both the fumarase and acetalase reactions has been shown to occur trans to the double bond (34–37).

The testicular hyaluronidase, unlike the bacterial enzyme, is a glycosidase with both hydrolytic and transglycosidase action (2, 35). From the structure of the product, it is apparent that addition of water is necessary in order to obtain a high yield of tetrasaccharide. It is not apparent, however, whether the hydroxyl group of water would attach to the C-1 or to the C-4 carbon atom of the carbohydrate and the oxygen bridge (39). Such use of labeled water was extended to the study of the site of cleavage of phosphate esters, including glucose 1-phosphate, by Cohn (40). The application of heavy water to the study of the enzymatic cleavage of glucoside bonds has also been employed and extensively discussed by Koshland (32, 41–43). He has pointed out that an enzyme reaction has the greatest specificity requirements for that part of the substrate structure which is closest to the position of the bond which is broken in the reaction. There is not much information regarding the specificity of testicular hyaluronidase for its substrate, but the small amount of information available suggests that the enzyme is specific for the uronic acid moiety. Thus, testicular hyaluronidase acts on chondroitin sulphuric acid A and C, but not on B. Chondroitin sulphuric acid B differs from other chondroitin sulphates in that it contains L-iduronic acid instead of D-glucuronic acid. From this, one might have expected the incorporation of O18 to occur at C-4 of the glucuronic acid moiety. The results show that this is actually not the case, but that the enzyme acts to cleave the bond between the carbonyl carbon atom and the oxygen bridge.

Seemed reasonable, it appeared to require more experimental evidence. Thus, there was a possibility that the reaction might occur in two steps involving an initial hydrolysis followed by elimination of water. If there was an initial displacement of OR from C-4 of the glucuronic acid moiety by the hydroxyl group of the medium, this might involve an inversion of configuration at C-4, which might be required for the subsequent elimination of water. The equivalent formation of reducing groups and double bonds during the entire course of the depolymerization provides evidence, however, that if there is an attack of the hydroxyl group at C-4 of glucuronic acid, the subsequent elimination of the same hydroxyl group proceeds so rapidly that no saturated intermediate accumulates in detectable quantities. It appears most likely that if the reaction proceeds as a displacement at C-4 of the glucuronate, the enzyme rather than water, would provide the attacking nucleophyllic group.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Material analyzed (before paper chromatography)</th>
<th>Concentration of O18 (atom % excess)</th>
<th>Enzyme reaction CONTROL</th>
<th>H2O18</th>
<th>H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Disaccharide mixture</td>
<td>0.238 0.117 0.132 0.106</td>
<td>0.225</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saturated disaccharide</td>
<td>0.110 0.064 0.062 0.048</td>
<td>0.02</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Unsaturated disaccharide</td>
<td>0.118 0.071 0.051 0.032</td>
<td>0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H2O</td>
<td>7.44 6.56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Saturated disaccharide</td>
<td>0.118 0.087 0.088 0.053</td>
<td>1.21</td>
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<td></td>
<td>Unsaturated disaccharide</td>
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<td>1.23</td>
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<tr>
<td></td>
<td>H2O18</td>
<td>14.6 13.7</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ a \] Corrected to the O18 concentration of the water used in the enzyme reaction.

\[ b \] Column 1 less Column 3.

\[ c \] The amount of O18 which would have been found in the product if 1 atom of oxygen were derived entirely from the medium. This number is calculated by dividing the atom % excess O18 of the medium by the number of oxygen atoms present, i.e., 12 for the saturated disaccharide, and 11 for the unsaturated disaccharide. For the disaccharide mixture, the expected amount is calculated on the assumption that there is 1 atom of O18 per molecule of unsaturated disaccharide only. The mixture analyzed contained 1.86 moles of saturated disaccharide per mole of unsaturated disaccharide. The number of oxygen atoms in which one O18 atom would be diluted is therefore 11 + 1.86 × 12 = 33.
SUMMARY

In the course of degradation of hyaluronic acid by bacterial hyaluronidase, the release of reducing groups was found to parallel the increase in ultraviolet absorption, thus affording no evidence for the formation of a saturated intermediate. When the reaction was carried out in H$_2$O$^{18}$, no O$_{18}$ of the medium was incorporated into the radioactive product. This is in contrast to other known glycosidases. It is suggested that this results from an attack by enzyme on the bond between O and the C-4 of uronic acid resulting in a simple elimination reaction such as occurs with fumarase and aconitase and not with glycosidases. This mechanism was supported by polarimetric studies which showed that the product first released has a $\beta$ configuration at the reducing end as is present in hyaluronic acid itself. In contrast to bacterial hyaluronidases, enzymic studies with testicular hyaluronidase in the presence of H$_2$O$^{18}$ suggest that the latter behaves as a typical glycosidase.

REFERENCES

The Mechanism of Action of Hyaluronidases
Julio Ludowieg, Birgit Vennesland and Albert Dorfman