ISOLATION OF OLIGOSACCHARIDES ENZYMATICALLY PRODUCED FROM HYALURONIC ACID*

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In earlier work application of carbon column chromatography to the products resulting from hydrolysis of umbilical cord hyaluronic acid by testicular hyaluronidase yielded fractions differing in average molecular weight (2). Monosaccharides were not detected in any of the digests, equivalence of hexosamine and uronic acid content was demonstrated in all fractions, and the constancy of certain end-group values was established. It was concluded that purified preparations of the enzyme hydrolyzed the polymer to oligosaccharides, in agreement with earlier workers, and that only the glucosaminidic linkages were affected, the uronidic linkages remaining intact.

The present report is concerned with the application of ion exchange and paper chromatography to the isolation of the individual oligosaccharides, and with the determination of their molecular sizes, as well as with the development of analytical methods suitable for their estimation in mixtures. The oligosaccharides isolated are designated oligosaccharides I to VII, in order of increasing size.

EXPERIMENTAL

Materials—The sodium hyaluronate, prepared from human umbilical cord, gave analytical values within the limits described elsewhere (3); the ethereal sulfate content was less than 0.5 per cent. The testicular hyaluronidase, a commercial preparation kindly supplied by Dr. Joseph Seifter of the Wyeth Institute of Applied Biochemistry, Philadelphia, assayed (4) at about 1500 turbidimetric reducing units per mg. The “24 hour digests,” used as the source of oligosaccharides II through VII in the paper and ion exchange isolations, were prepared by incubation at 37° of sodium hyaluronate at 20 to 50 mg. per ml. with enzyme at 0.5 mg. per ml. in 0.1 M acetate buffer, pH 5, 0.15 M in sodium chloride, containing 1 mg. per ml.

* This work was supported by grants from the National Institutes of Health and the Helen Hay Whitney Foundation. Part of the work has been reported in a preliminary communication (1).
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of gelatin. The "exhaustive digest," used as the source of oligosaccharides I through III in the alcohol fractionation, was incubated for 5 days (toluene), fresh enzyme additions of 0.5 mg. per ml. being made every day. (Tests of similar digests for aerobic or anaerobic bacterial contamination were negative.)

Analytical Methods—Most of the methods have been referred to previously (2, 3). The term Elson-Morgan color yield as used in this paper refers to the color given by saccharides having free reducing groups of the hexosamine or acetylhexosamine type with acetylacetone and dimethylaminobenzaldehyde (2). Acetylglucosamine is used as the color standard. For estimation of oligosaccharides in column work, the Brown modification (5) of the orcinol reaction was used, with a 30 minute boiling time and measurement at 660 m\(\mu\) (6). A dry 24 hour digest preparation (3) containing 41 per cent uronic acid was arbitrarily chosen as the standard substance, and results were reported as mg. of substance. Trials with the regular series of oligosaccharides showed that their individual color yields in this reaction, while considerably lower than that of the equivalent free uronic acid, nevertheless were proportional to their uronic acid content within precision of the method (about 5 per cent). Presence of formic acid in concentrations as high as 2 M introduced less than 10 per cent error.

The Willstätter-Schudel hypoiodite reducing sugar method generally gives stoichiometric oxidation of reducing oligosaccharides to the corresponding aldonic acids and is commonly used for estimating molecular weight. However, the iodine consumption of N-acetylhyalobiuronic acid (7) and N-acetylglucosamine, used as models, varied several fold with changes in the ratio of oxidant to sugar, the reaction time, and the rate of alkali addition. With the milder hypoiodite method of Macleod and Robison (8), in which sodium carbonate rather than sodium hydroxide is used, the results obtained varied less than 5 per cent for 2-fold increase of oxidant to compound ratio or reaction time for the conditions following. The sugar in 1 ml. of water was added to 1.00 ml. of 0.02 N iodine solution, the amount of sugar consumed not more than one-third of the iodine. With continuous stirring, 0.5 ml. of 0.1 M sodium carbonate solution was added dropwise over 1 to 1.5 minutes. The stoppered vessel was left in the dark for 30 minutes, and its contents were acidified and titrated with standard thiosulfate. N-Acetylglucosamine consumed 2.0 equivalents of iodine per mole, presumably yielding N-acetylglucosaminic acid. N-Acetylhyalobiuronic reproducibly consumed 3.2 equivalents, giving unknown products.1

1 The use of sodium carbonate-bicarbonate buffer (9) with the hypoiodite reaction over prolonged periods at 5°C also produced overoxidation with the disaccharide and afforded no advantage.
Paper Chromatography—Of the solvent mixtures tried, the one which gave most satisfactory resolution was butanol-acetic acid-water (10) in the ratios 50:15:35 and 44:16:40 (single phase systems), used with downward irrigation for 24 to 72 hours on Whatman No. 1 paper. Developer which contained less water (57:14:29) was too slow, although excellent for use with mono- and disaccharides. Smear results were frequently obtained with developer containing more water. The oligosaccharides all stained sensitively with the Elson-Morgan reagents (acetylacetone-dimethylamino-benzaldehyde) (10). No sugars having end-groups other than the acetyl-glucosamine type were detected with aniline trichloroacetate reagent (11). This reagent gave poor sensitivity with the oligosaccharides.

![Fig. 1. Paper chromatograms showing the type of results obtained on 24 hour digestion (A) and exhaustive digestion (B) of hyaluronic acid with testicular hyaluronidase. The intensity of the spots is indicated by shading. The spots correspond to individual oligosaccharides, designated by Roman numerals.](image)

Patterns yielded by a typical 24 hour digest and an exhaustive digest are illustrated in Fig. 1. The mobilities of the oligosaccharides which have been resolved are given in Table I.

In preparative paper work, a 24 hour digest at 50 mg. per ml. was applied twice along the starting line of thick filter paper sheets (Whatman No. 3 MM) in 10 μl. spots at 1 cm. intervals, and the papers were irrigated downward for 60 hours with the 44:16:40 developer. Narrow guide strips cut at close intervals were stained. The remaining paper was cut accordingly when resolution was sufficient (fair through oligosaccharide VII in some runs), and the bands were eluted with water. The eluates were lyophilized, redissolved, passed over Dowex 50 Na₂, treated with a little decolorizing carbon, and lyophilized again. The properties of the preparations (P-II through P-VII) are given in Table I.

Application of the paper technique for semiquantitative purposes was effected by running each mixture (usually 200 γ) in duplicate in adjacent lanes of a paper chromatogram. One lane was stained as a guide for cutting its duplicate. After air drying at least 24 hours, the piece of

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2 Sulfonated polystyrene resin, The Dow Chemical Company, Midland, Michigan.
paper containing each spot was eluted with water (2.5 ml.). Uronic acid in each eluate was determined by the Dische carbazole reaction. A piece of paper from a region containing no material (usually between oligosaccharides I and II) was also eluted and served as a blank correction. When the eluates were carefully freed of lint, this correction per spot was less than 3 per cent of the total uronic acid. Total recovery of uronic acid was usually 90 per cent. The proportion of each component was reported as the per cent of total recovered uronic acid. The accuracy of this method was limited for oligosaccharides larger than IV by the poor resolution of these when applied to paper in amounts exceeding 20 to 50 γ.

By this method, a typical 24 hour digest (Fig. 1, A) contained (in per cent), oligosaccharide I, 1; oligosaccharide II, 29; oligosaccharide III, 31; oligosaccharide IV, 22; oligosaccharide V and larger, 17. A typical exhaustive digest (Fig. 1, B) contained oligosaccharide I, 10; oligosaccharide II, 74; oligosaccharide III, 10; oligosaccharide IV, 6.

**Table I**

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Mobility on paper</th>
<th>Per cent uronic acid (Dische)</th>
<th>Reducing value</th>
<th>Elson-Morgan color, per cent acetylglucosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35 per cent H2O</td>
<td>10 per cent H2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.29</td>
<td>1.75</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>P-II</td>
<td>0.15 (1.00)</td>
<td>0.19 (1.00)</td>
<td>33.9</td>
<td>3.19 3.64 37 45</td>
</tr>
<tr>
<td>P-III</td>
<td>0.08 0.58</td>
<td>0.12 0.67</td>
<td>43.4</td>
<td>2.43 3.27 35 42</td>
</tr>
<tr>
<td>P-IV</td>
<td>0.28 0.07</td>
<td>0.41</td>
<td>41.1</td>
<td>1.71 3.23 28 35</td>
</tr>
<tr>
<td>P-V</td>
<td>0.13 0.09</td>
<td>0.26 37.1</td>
<td>40.3</td>
<td>1.23 3.00 22 25</td>
</tr>
<tr>
<td>P-VI</td>
<td></td>
<td>0.16</td>
<td>40.3</td>
<td>1.10 3.17 18 23</td>
</tr>
<tr>
<td>P-VII</td>
<td></td>
<td>0.09</td>
<td>37.1</td>
<td>0.93 3.40 14 19</td>
</tr>
</tbody>
</table>

* R₂ is the relative mobility on paper (based on assignment of R₂ 1.00 for oligosaccharide II) in chromatograms in which the solvent was run beyond the edge of the paper for greater resolution.
† For method of calculation, see foot-note 7.

was limited for oligosaccharides larger than IV by the poor resolution of these when applied to paper in amounts exceeding 20 to 50 γ.

By this method, a typical 24 hour digest (Fig. 1, A) contained (in per cent), oligosaccharide I, 1; oligosaccharide II, 29; oligosaccharide III, 31; oligosaccharide IV, 22; oligosaccharide V and larger, 17. A typical exhaustive digest (Fig. 1, B) contained oligosaccharide I, 10; oligosaccharide II, 74; oligosaccharide III, 10; oligosaccharide IV, 6.

**Ion Exchange Chromatography (12, 6, 13)**—Dowex 1 × 10,3 200 to 400 mesh, was suspended in water; resin of settling time 0.5 to 10 minutes was selected for the preparative columns below, and resin of settling time 10 to 30 minutes was selected for the analytical columns. The resin, after...
being washed with strong hydrochloric acid and water, was converted to the formate with sodium formate (13) and washed with water. Good resolution of the digest oligosaccharides was achieved on columns of this resin with dilute formic acid as developer. In Fig. 2 are presented the results obtained with a small preparative column, 0.64 cm.\(^2\) × 5.5 cm., to which were applied 5 mg. of sodium glucuronate, 10 mg. of N-acetylhyaluri-biuronic acid (oligosaccharide I; see below), and digest from 40 mg. of sodium hyaluronate. Negligible amounts of material were removed by washing with water (20 ml.); 270 ml. each of 0.015 M, 0.05 M, 0.15 M, 0.3 M, 0.5 M, and 0.8 M formic acid were used as developer; 10 ml. eluate fractions were collected and assayed by orcinol colorimetry. The material in each peak, pooled, concentrated, and examined by paper chromatography, showed only one spot per peak. Other experiments showed that oligosaccharide VI moved as a peak with 1.0 M formic acid.

Properties of oligosaccharides II through VI (Preparations R-II through R-VI) separated by larger scale application of this technique are given in Table II. Formic acid was carefully removed from these preparations by concentration \textit{in vacuo}, followed by repeated addition of water and concentration to dryness; the glass resulting from lyophilization, finely powdered, was dried for several weeks over sodium hydroxide at 0.1 mm. Finally, the preparations were redissolved, treated with decolorizing carbon, converted to sodium salts by passage over Amberlite IRC-50, Na\(^+\) lyophilized, powdered, and dried over phosphorus pentoxide. Oligosaccharide II was

\(^4\) Carboxylic acid exchange resin, Rohm and Haas Company, Philadelphia.
obtained pure (see below). The homogeneity of the remaining preparations was tested by the column technique described below. Preparation R-III contained 4 per cent of oligosaccharide IV as an impurity; Preparation R-IV contained 1 per cent oligosaccharide III, 10 per cent oligosaccharide V, Preparation R-V contained 3 per cent oligosaccharide VI; Preparation R-VI contained 2 per cent oligosaccharides IV and V, 5 per cent oligosaccharide VII.

Convenient operation and good reproducibility in analytical application of the ion exchange chromatography described above resulted from use of a gradient technique (i.e., continuous change of developer) and a siphon-operated fraction collector. Formic acid solution was allowed to flow from a reservoir into the inlet of a closed mixing vessel (14, 13) initially

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Optical rotation, $\alpha$ (c, 2.00)</th>
<th>Per cent uronic acid*</th>
<th>Per cent hexosamine*</th>
<th>Reducing value</th>
<th>Elson-Morgan color, per cent acetylglucosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-I†</td>
<td>-32 (48.9)</td>
<td>49.2 (48.6)</td>
<td>41 (45.1)</td>
<td>8.2</td>
<td>65 (47.8)</td>
</tr>
<tr>
<td>R-II‡</td>
<td>-51 (50.0)</td>
<td>49.5 (48.9)</td>
<td>43 (46.1)</td>
<td>4.42</td>
<td>53 (47.8)</td>
</tr>
<tr>
<td>R-III</td>
<td>-63.5 (47.7)</td>
<td>47.0 (46.4)</td>
<td>41 (44.0)</td>
<td>2.78</td>
<td>38 (47.9)</td>
</tr>
<tr>
<td>R-IV</td>
<td>-62 (47.8)</td>
<td>44.4 (42.9)</td>
<td>39 (44.1)</td>
<td>2.05</td>
<td>27 (47.9)</td>
</tr>
<tr>
<td>R-V</td>
<td>-68 (47.9)</td>
<td>48.2 (45.7)</td>
<td>41 (44.2)</td>
<td>1.74</td>
<td>22 (48.0)</td>
</tr>
<tr>
<td>R-VI</td>
<td>-65.5 (48.0)</td>
<td>45.8 (43.7)</td>
<td>39 (44.3)</td>
<td>1.28</td>
<td>15 (48.1)</td>
</tr>
<tr>
<td>E-I§</td>
<td>-34</td>
<td>44.0</td>
<td></td>
<td></td>
<td>58 (48.2)</td>
</tr>
<tr>
<td>E-II</td>
<td>-54</td>
<td>49.5</td>
<td></td>
<td>3.81</td>
<td>51 (48.3)</td>
</tr>
<tr>
<td>E-III</td>
<td></td>
<td>45.6</td>
<td></td>
<td>2.30</td>
<td>33 (48.4)</td>
</tr>
</tbody>
</table>

* The values in parentheses are calculated values (see "Discussion"). The preparations are sodium salts, except as indicated.
† N-Acetylhyalobiuronic acid prepared by ketene acetylation (7) of the crystalline disaccharide.
‡ Crystalline free acid. The optical rotation is an equilibrium value; the corresponding value for the pure sodium salt is $-58^\circ$.
§ Free acid.
containing a measured volume of water; the outlet of the mixing vessel led to the top of the column. The sharpness of the peaks and the degree of resolution obtained depended on the amount, granulation, and loading of the resin, the volume of liquid in the mixing vessel, and the rate of flow and concentration of the formic acid solution added from the reservoir. Good resolution through oligosaccharide IX was obtained with a set of conditions referred to as Standard Column A. In a typical run, the results of which are plotted in Fig. 3, a digest from 25 mg. of hyaluronate without removal of enzyme, salts, or gelatin was placed on a column containing 4 ml. settled volume of resin (4.0 gm. of moist resin from a stock container), and the column was washed with water (20 ml.). Gradient development was applied, 2.0 M formic acid being run into the mixing vessel which contained 500 ml. of water initially, and two 10 ml. fractions per hour were collected.

Collection of fewer fractions with some sacrifice of resolution above oligosaccharide VI was possible with a modified set of conditions, described as Standard Column B. Here, the settled resin volume was 2 ml. (2.00 gm. of moist resin), the mixing volume 350 ml., and 2.0 M formic acid was added to the mixing vessel; one 10 ml. fraction was collected per hour. In either column, the peaks did not shift by more than one tube from one run to another. In Standard Column B the peaks appeared as follows: oligosaccharide I, Tube 3; oligosaccharide II, Tube 8; oligosaccharide III, Tube 13; oligosaccharide IV, Tube 18; oligosaccharide V, Tube 23; oligosaccharide VI, Tube 27; oligosaccharide VII, Tube 32; oligosaccharide VIII, Tube 36; oligosaccharide IX, Tube 40.
Ethanol Fractionation—An exhaustive digest from 7.5 gm. of sodium hyaluronate was freed of enzyme and salts by alcohol precipitation and carbon adsorption as described earlier (3). By elution of the carbon column (40 gm. of Darco G-60) with 15 per cent aqueous ethanol (600 ml.), Fraction A (0.3 gm.) was obtained. Paper chromatography indicated that this fraction contained the major portion of oligosaccharide I present in the digest and also a considerable amount of oligosaccharide II. Further elution of the carbon by aqueous pyridine gave chiefly oligosaccharide II, 0.7 gm. Systematic fractionation of the 80 per cent ethanol precipitate (3), 6.1 gm., by aqueous ethanol after prior conversion to the barium salt gave an oligosaccharide II preparation (E-II, collected as the sodium salt, 2.5 gm.) and a small oligosaccharide III preparation (E-III, sodium salt, 0.06 gm.). The properties of these appear in Table II. Both were homogeneous to paper chromatography, and Preparation E-III was also homogeneous to ion exchange chromatography. Preparation E-II, however, contained a minor component, not yet investigated (oligosaccharide II-A) which had the same paper mobility as oligosaccharide II, but moved more slowly on the resin column. This component has been found in clearly detectable amount only in exhaustive digests.

Characterization of Oligosaccharide I (Disaccharide)—Further fractionation by aqueous ethanol of Fraction A as the barium salt gave material containing only little oligosaccharide II. This oligosaccharide I preparation (E-I, free acid, 0.08 gm.) had the properties shown in Table II, in fair agreement with those of N-acetylhyalobiuronic acid (Preparation S-I) from crystalline disaccharide (7), and had the same mobility on paper. When 50 mg. of Preparation E-I were allowed to stand in the cold with 0.03 M methanolic hydrogen chloride for 2 days, neutralized with pyridine, the solvent removed, and the residue acetylated in the cold with acetic anhydride and pyridine, there were isolated 46 mg. of heptaacetylhyalobiuronic acid methyl ester (ethanolate), having melting point, mixture melting point, and rotation identical with those previously reported (7).

Crystalline Oligosaccharide II (Tetrasaccharide)—On concentration in vacuo of the dilute formic acid eluate containing oligosaccharide II resulting from the preparative ion exchange column, some of the residue crystallized on the wall of the flask. When seeded, almost the entire batch slowly crystallized from water-ethanol. The sharp single needles were collected, washed with ethanol-water, methanol, ether, and air-dried. The compound decomposed above 200° without melting. Its solubility in water at room temperature was roughly 5 per cent; it was slightly soluble in methanol, insoluble in ethanol or ether. The crystals, when dried over phosphorus pentoxide at 25° or 60°, lost weight slowly, and at 110° rapidly; the loss, not determined with precision, corresponded to 3 molecules of
water. The rotation, analyses, and values in Table II (Preparation R-II) are reported on a dry basis.

\[ \text{C}_{38}\text{H}_{54}\text{O}_{38}\text{N}_{12} (776.68) } \]

Calculated. C 43.2, H 5.66, N 3.61, CH₂CO 11.1, neutral equivalent 388.3


The optical rotation was \([\alpha]_b^{27} -41^\circ \text{ (initial)} , -53^\circ \text{ (final, 5 hours)} \) (c 2.00; H₂O). Potentiometric titration indicated two poorly resolved constants, pK₁’ 2.8, pK₂’ 3.6.

**DISCUSSION**

For all oligosaccharide fractions, the molar ratio of uronic acid to hexosamine content is unity within experimental error (Table II). From this, and from the Elson-Morgan color reaction, it follows that all the species considered must consist of multiples of 1 disaccharide unit (2) as represented in Fig. 4.⁵ Oligosaccharide I is a disaccharide of demonstrated structure (Fig. 4, \( n = 1 \); (7)). Evidence has not yet been considered for the molecular sizes of oligosaccharide II, a crystalline compound of concordant analyses, and of the chromatographically homogeneous higher oligosaccharides.

When it is assumed that \( n \) in Fig. 4 has the regular values 1, 2, 3…7, a linear relationship is obtained between \( \log \alpha' \), a function of the paper mobility of the oligosaccharides and their degree of polymerization⁶ (Fig. 6). The position of the glucosaminic linkage in these oligosaccharides is arbitrarily represented and is not known, as most of these linkages have undoubtedly been formed during the enzymatic hydrolysis by a process of transglycosidation (1).

⁵ Since the \( R_F \) values of the higher oligosaccharides are so small as to make their direct measurement inaccurate, advantage has been taken of the well known proportionality between \( R_F \) and \( R_x \) (mobility relative to an arbitrarily chosen standard substance). The \( R_F \) values used in Fig. 5 have accordingly been computed from \( R_x \) values measured in chromatography of long duration in which the solvent front was allowed to run off the paper. Oligosaccharide II is the standard substance (\( R_x 1.00 \)), and its \( R_F \) value is used as the proportionality constant, \( (R_F)_x = (R_x)_x \times (R_F)_x \).
Such linearity prevails within each of the known polymer-homologous series of glucose and fructose oligosaccharides and has a basis in theory (15). Moreover, series of known oligosaccharides having the same components but differing in linkage type differ likewise in mobility (15). This would imply that the present oligosaccharides contain only one type of linkage and therefore constitute a series of pure chemical individuals. In the absence of additional evidence, however, such a conclusion must be approached with caution.

On the other hand, the conclusion that each fraction is of uniform and known molecular size may be considered to be safely established. Support for the molecular size assignment of greater generality than the paper mobility is given by the observed constancy of end-group values when computed on a molar basis (Table III). Except for irregularities between the di- and tetrasaccharides (oligosaccharides I and II), the molar Elson-Morgan color yield and ferricyanide reducing power are constant within reasonable limits. In both methods hot sodium carbonate solutions are used; the irregularity between di- and tetrasaccharide parallels the increase in molar reducing value in going from glucose to its disaccharide derivatives (16), and may reflect the increase in reducing value known to occur in hot alkaline solutions of testicular hyaluronidase digests (17). How-

There is apparently heavy contamination, as judged from low uronic acid values, of the preparations from paper chromatography (especially Preparations P-II and P-VII) with substances extracted from the paper itself. The ion exchange preparations have accordingly been selected as more suitable for end-group analysis. However, the trends of Table II are followed by those of Table I, perhaps even more faithfully than might be expected. The molar values of Table III are computed as follows, where \( n \) is the number of disaccharide units and 194 is the molecular weight of uronic acid: value per mole = \((100 \times 194n) \times \text{per cent uronic acid}\).
ever, the absence of a continued drift through the series seems to rule out alkali lability of glycosidic bonds as an explanation. Of perhaps greatest value are the results given by the Macleod-Robison reducing method (cold hypoiodite). The nature of the reaction in which 3.2 to 3.6 equivalents of oxidant are consumed is of course not clear; perhaps the reaction occurring consumes 4 equivalents and is incomplete. N-Acetylglucosamine, presumably a closely analogous model, consumes 2 equivalents of oxidant, as do the commoner aldoses. In any case, the constancy throughout a series of six members of the molar iodine consumption within the limits given is remarkable, and not only adequately confirms the assignment of molecular

Table III

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>No. of disaccharide units</th>
<th>Ferricyanide reducing value, * moles glucose equivalent</th>
<th>Elson-Morgan color, * moles acetylglucosamine equivalent</th>
<th>Macleod-Robison reducing value, * equivalents of hypoiodite</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-I</td>
<td>1</td>
<td>1.23</td>
<td>2.0</td>
<td>3.22</td>
</tr>
<tr>
<td>R-II</td>
<td>2</td>
<td>2.00</td>
<td>3.1</td>
<td>3.46</td>
</tr>
<tr>
<td>R-III</td>
<td>3</td>
<td>2.26</td>
<td>3.6</td>
<td>3.57</td>
</tr>
<tr>
<td>R-IV</td>
<td>4</td>
<td>2.32</td>
<td>3.4</td>
<td>3.58</td>
</tr>
<tr>
<td>R-V</td>
<td>5</td>
<td>2.13</td>
<td>3.7</td>
<td>3.50</td>
</tr>
<tr>
<td>R-VI</td>
<td>6</td>
<td>1.83</td>
<td>3.1</td>
<td>3.15</td>
</tr>
</tbody>
</table>

* Value per mole of substance, based on the gasometric uronic acid analysis (Table II) and degree of polymerization (number of disaccharide units). See footnote 7.

sizes, but also indicates similarity of the center (or centers) undergoing oxidation in the individual compounds.8

In the earlier studies of the action of testicular hyaluronidase on hyaluronic acid, the apparent cessation of reducing sugar production led to the erroneous conclusion that the 24 hour digest was essentially an end-product (2), while the unknown resolving power of the chromatographic system and the comparison of this product with that obtained with pneumococcal hyaluronidase led to the belief that it was predominantly a tetrasaccharide (19, 20). The present finding that 24 hour digests are indeed extremely heterogeneous and that they contain oligosaccharides having odd degrees of polymerization in amounts comparable to those of even number (Fig. 3)

8 In the numerous applications of end-group technique to molecular weight determination of mucopolysaccharides (see (9, 18)) neither the possibility of the departure from stoichiometry now shown in the oligosaccharide models nor the large errors which may result from presence of minor reducing impurities have been considered. The molecular weights reported in the literature are probably far too low.
removes the basis for this belief and for the related hypothesis (19) that half of the glucosaminidic linkages in the polymer are resistant to the enzyme and that the basic repeating unit is a tetrasaccharide. Rather, it can be concluded from the regularity of the distribution of size in digests that the repeating unit of the oligosaccharides and of hyaluronic acid must be a \textit{disaccharide}, glucuronido-acetylglucosamine. Also, the absence of any significant amount of branching in the polymer is indicated. That much more vigorous enzymatic treatment of the heterogeneous 24 hour digest than was applied heretofore does indeed lead to accumulation of large amounts of \textit{tetrasaccharide}, with production of only small amounts of disaccharide, is an apparent anomaly which it is hoped a forthcoming analysis of the process will resolve.

The oligosaccharides isolated here have, as anticipated, proved useful as model substrates in analysis of the action of a number of mucolytic enzyme systems. A result of one such study (1), which shows that testicular hyaluronidase is a transfer enzyme that forms as well as splits glucosaminidic linkages, prevents the use of these oligosaccharides for comparison of hyaluronic acids from various sources, another original motivation of this work. However, it is believed that the techniques described may be used for isolation of oligosaccharides from acid hydrolysates suitable for such comparisons and that these techniques will be valuable in problems connected with other acid polysaccharides such as the chondroitin sulfates, hemicelluloses, and pectins.

\textbf{SUMMARY}

A detailed examination has been made of the products of hydrolysis of umbilical cord hyaluronic acid by testicular hyaluronidase, by means of paper and ion exchange chromatographic techniques and alcohol fractionation. An analytical method utilizing Dowex 1 formate chromatography with gradient elution by dilute formic acid is described. The properties of seven oligosaccharide fractions (oligosaccharides I through VII) isolated from digests are given. End-group analysis and regularities of the mobilities on paper show that the molecular sizes of the oligosaccharide fractions form a regular series ranging from di- to tetradesaccharide in increments of 1 disaccharide unit. The repeating unit of hyaluronic acid, as of the oligosaccharides, is a disaccharide, glucuronido-acetylglucosamine. Oligosaccharide I is identical with \textit{N-acetylhyalobiuronic acid}, obtained by acetylation of the crystalline disaccharide from acid hydrolysis of hyaluronic acid. Oligosaccharide II, the tetrasaccharide, has been crystallized.

\textbf{BIBLIOGRAPHY}

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