Stoichiometric Depolymerization of Polyuronides and Glycosaminoglycuronans to Monosaccharides following Reduction of Their Carbodiimide-Activated Carboxyl Groups†

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ABSTRACT: Reaction of hydroxy acids with water-soluble carbodiimides leads to the formation of lactones which can be reduced with sodium borohydride to the corresponding alcohols. In a similar manner polyuronides and glycosaminoglycuronans react with water-soluble carbodiimides. The exact nature of the products formed from the polymers has not been established but they appear to be lactones or intramolecular esters. These products can be reduced with sodium borohydride to convert the uronic acid residues in the polymers to the corresponding neutral sugars. Reduction facilitates acid hydrolysis of the acid-resistant glycosyluronic acid bonds in the polymer by replacing them with the more acid-labile glycosyl bonds. When hyaluronic acid, chondroitin sulfate, and heparin are subjected to this reduction and acid hydrolysis reaction sequence, more than 90% of the original uronic acid glycosidic bonds are cleaved. The remaining acid-resistant amino sugar glycosidic linkages are cleaved with nitrous acid to complete the essentially quantitative, nondestructive depolymerization of these polymers.

Biologically important carbohydrate polymers occurring extracellularly and on the surfaces of cells often contain uronic acid residues which are ionized at physiological pH's. These and other charged residues in such polymers play an important role in determining the three dimensional structures of the polymers (Rees, 1967). The relationships between primary structure, polymer conformation, and biological properties of carbohydrate polymers are at present poorly understood but are gaining increasing attention as new experimental approaches are being developed (Lüderitz *et al.*, 1966; Marchessault and Sarko, 1967; Roseman, 1970).

Determination of primary sequences of polysaccharides containing uronic acids is often complicated by the unusual stability to acid hydrolysis of glycosidic bonds formed by these residues (for a review, see BeMiller, 1967). The most direct and obvious method to facilitate quantitative depolymerization of polymeric uronides under nondestructive hydrolysis conditions is to convert them to esters which in turn can be reduced to the more readily hydrolyzable neutral polymers. This approach (Aspinall, 1965) has had as its major shortcoming the difficulty in obtaining stoichiometric esterification of polyuronides in aqueous medium without repeated esterification steps. Recently, Hoare and Koshland (1967) have demonstrated that carboxylic amino acid side chains in proteins can be activated with water-soluble carbodiimides so that they will react with glycine methyl ester and give a stoichiometric yield of the amide. These authors suggest that in the presence of the carbodiimide carboxylic acids could be substituted by any good nucleophilic reagent. Thus, polyuronides might be esterified by reaction with an oxygen nucleophile in the presence of a water-soluble carbodiimide. The esterified polyuronide could then be reduced to the neutral polymer with sodium borohydride. This paper describes the development of this approach for essentially stoichiometric reduction of uronic acid containing polymers. Other uses of water-soluble carbodiimides in carbohydrate structural studies have been described by Saier and Ballou (1968) and by Danishefsky and Siskovic (1971).

Methods

Two different water-soluble carbodiimides were used in this work: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)[1] and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC). The former was obtained from Ott Chemical Co., the latter from Aldrich Chemical Co. Hyaluronic acid (K salt, grade III-P), heparin (Grade I), D-glucuronic acid, D-galacturonic acid, and β- and γ-hydroxybutyric acids were purchased from Sigma Chemical Co. [^{3}H]Sodium borohydride (25 mCi/mmole) was obtained from New England Nuclear Corp., and D-[^{14}C]glucose (261 mCi/mmole) from Mallinckrodt Nuclear.

Purified capsular polysaccharides from *Aerobacter aerogenes* strains A3(S1) (Conrad *et al.*, 1966) and NCTC 243 (Gahan *et al.*, 1967) were prepared as described previously. Chondroitin sulfate "C" (Na salt, B grade) and polygalacturonic acid (C grade) were obtained from Calbiochem. The latter was further purified as follows. A slightly alkaline 2% solution of the polymer was centrifuged to remove insoluble materials and the polygalacturonic acid was precipitated from the supernatant by dropwise addition of a saturated aqueous solution of barium acetate until no further material could be precipitated. The precipitate was centrifuged off and resuspended in water. Concentrated sulfuric acid was added dropwise to the vigorously stirred suspension until the pH was

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; CMC, 1-cyclohexyl-3-(2-morpholinolethyl)carbodiimide metho-*p*-toluenesulfonate; R_g, rate of chromatographic migration relative to the rate of [^{14}C]glucitol; EAFW, chromatography solvent composed of ethyl acetate–acetic acid–formic acid–water (18:3:1:4, v/v).

brought to 3. Insoluble barium sulfate was centrifuged off and the supernatant was dialyzed against distilled water. The polygalacturonic acid was then precipitated from solution by addition of two volumes of ethanol. The precipitate was washed with ethanol and ether and dried *in vacuo* at 50°.

Preparation of L-[6-^3H]*Gulonic Acid.* To 5 ml of 2 N sodium carbonate containing 200 μequiv of D-glucuronic acid was added 4 mg of [^{3}H]sodium borohydride (25 mCi/mmole). This solution was heated at 50° for one hour and then acidified by addition of 5 ml of 2 N HCl. The resulting L-[^{3}H]gulonic acid solution was used directly as a substrate for the carbodiimide reaction without removal of salts.

Reduction of Uronic Acids. To 10 ml of a solution containing 100 μequiv of carboxylic acid (in either monomeric or polymeric form) was added 1 mmole of solid EDC or CMC. As the reaction proceeded the pH of the reaction mixture was maintained at 4.75 by automatic titration with 0.1 N HCl using a Metrohm pH-Stat which plotted the total ml of acid added *vs.* time. All reactions were allowed to proceed for at least 2 hr. After hydrogen ion uptake had ceased an aqueous 2 M sodium borohydride solution was added slowly to the reaction mixture at room temperature. The pH rose rapidly to 7.0 as a result of destruction of borohydride at the acid pH and was maintained at this pH by automatic titration with 4 N HCl in the pH-Stat. The borohydride solution was added with a hypodermic syringe. The slow conversion of $NaBH_4$ to H_2 gas in the syringe gave a slow increase in pressure which forced the borohydride solution into the reaction mixture at an appropriate steady rate. A total of 15–25 ml of the borohydride solution was usually required for reduction which was complete in 60 min. A drop of 1-octanol was sometimes added as an antifoam agent. Modifications of this reduction procedure which were used for the glycosaminoglycuronans are described in the Results.

Determination of Per Cent Reduction. Prior to analysis reduced polyuronides were dialyzed overnight against glass distilled water and then concentrated to one-eighth their volume. Aliquots were mixed with one-half volumes of 3 N sulfuric acid, heated at 100° for 4 hr and analyzed by radiochromatography. This procedure, described elsewhere (Koeltzow *et al.*, 1968), involves mixing an aliquot of the hydrolysate with high specific activity [^{14}C]glucose (which serves as an internal standard used in quantitation and identification of the monosaccharides), neutralizing the mixture with sodium carbonate, and reducing it with [^{3}H]sodium borohydride. All hydrolysates, after the neutralization with sodium carbonate, were heated at 50° for 30 min to convert lactones to their corresponding acids prior to reduction with [^{3}H]sodium borohydride. This was necessary to avoid reduction of the carboxylic acids in the analysis step. The mixture of [^{3}H]-glycitols is separated on a paper chromatogram which is then cut into segments and counted to determine the sequence of peaks along the chromatogram. Using a predetermined value for the number of ^{3}H counts per minute obtained from a micromole of reducing sugar reduced with the [^{3}H]borohydride used in the analysis, the number of micromoles in any peak can be calculated from the number of ^{3}H counts per minute in that peak. The hydrolysates of the glycosaminoglycuronans were treated with $NaNO_2$ prior to radiochromatographic analysis to cleave the stable amino sugar glycosidic bonds not cleaved in the hydrolysis and to convert all amino sugars to anhydrohexoses which can be quantitated by radiochromatography (Shively and Conrad, 1970). In all of the radiochromatographic analyses in this work chromatograms were run on DEAE-cellulose paper (Shively and Conrad, 1970).

The data from the radiochromatograms are used to calculate the per cent of the original uronic acid which remains unreduced following the borohydride treatment of the carbodiimide reaction product according to

$$\%\ \text{unreduced uronic acid} = \frac{\mu\text{moles of unreduced uronic acid on the chromatogram}}{\text{total } \mu\text{moles of monosaccharide in all peaks on the chromatogram derived from the original uronic acid residues}} \times 100$$

Since all reducing sugars give the same counts per minute per micromole (Conrad *et al.*, 1966) calculations are made using total counts per minute in a peak instead of total micromoles in each peak.

The unreduced uronic acid appears on the radiochromatograms of hydrolysates of reduced polymers either as free uronic acid ($R_g \sim 0.7$) or as a disaccharide ($R_g \sim 0.3$) containing one (aldobiouronic acid) or two (digalacturonic acid) residues of uronic acid, depending upon the structure of the polymer. Thus, the total counts per minute in unreduced uronic acid on a chromatogram is equivalent to the sum of the counts per minute in the free uronic acid plus the counts per minute in the disaccharide (or, for digalacturonic acid, counts per minute in disaccharide peak × 2).

The total monosaccharide counts per minute in all peaks on the chromatogram derived from the original uronic acid residues in the polysaccharide is calculated in a similar way. The peaks derived from uronic acid will vary depending on the structure of the polymer analyzed. Thus, for polygalacturonic acid *all* of the monosaccharide equivalents on the chromatogram are derived from uronic acid. For the two capsular polysaccharides one-fourth of the total equivalents of monosaccharide are derived from uronic acid. For the mucopolysaccharides, total equivalents of original uronic acid is equal to the sum of all counts per minute in the peaks of hexose, uronic acid, and disaccharide (any disaccharide from these polymers would have a single unreduced uronic acid in it).

Results are expressed as per cent reduction of original uronic acid residues, which is equal to 100 minus the per cent unreduced uronic acid.

Results and Discussion

Reduction of Model Compounds. The reactions of carboxylic acids with carbodiimides in aqueous media in the presence and absence of a nucleophile (HX) are shown in Figure 1. Hoare and Koshland (1967) have shown that the formation of the *O*-acylisourea (reaction 1) proceeds rapidly with the uptake of from 1 to 2 moles of hydrogen ion per mole of acid, depending upon the degree of ionization of the acid at the pH of the reaction. When an appropriate nucleophile is present, reaction 2 proceeds with the release of 1 mole of hydrogen ion to the medium. Hoare and Koshland have shown that in the absence of a nucleophile the predominant reaction is the hydrolysis of the *O*-acylisourea (reaction 3) which proceeds with a first-order rate constant approximately 20 times that of the rearrangement to the *N*-acylurea (reaction 4). In the overall conversion of reaction 1 plus reaction 2 there is a net uptake of hydrogen ions which depends upon the degree of ionization of the acid, while in the only significant competing sequence, reaction 1 plus reaction 3, there is consumption of the carbodiimide but no net change in hydrogen ion concen-

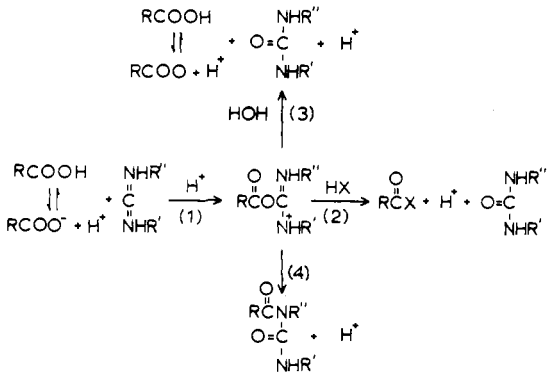

FIGURE 1: Reactions of carboxylic acids with carbodiimides (see Hoare and Koshland, 1967).

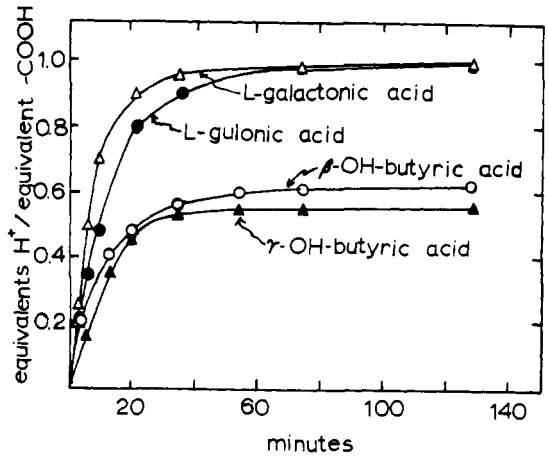

FIGURE 2: Acid uptake in the reaction of hydroxy acids with CMC at pH 4.75. Each reaction mixture had 0.2 mmole of the respective carboxylic acid and 2.0 mmoles of CMC in 20 ml of water. The pH was maintained at 4.75 during the course of the reaction by automatic titration with 0.1 M HCl.

tration. Thus, when the acid is reacted with excess carbodiimide and a nucleophile, the substitution of the carboxyl can be followed by observing the hydrogen ion uptake at constant pH.

Initial studies on the action of water-soluble carbodiimides showed that certain hydroxy acids, in the absence of added nucleophiles, could be converted quantitatively to lactones. This reaction was studied with β- and γ-hydroxybutyric acids and with L-gulonic acid and L-galactonic acid. Figure 2 shows the progress of hydrogen ion uptake in the reactions of these acids with carbodiimide at pH 4.75. In all cases the reaction is complete in 40 min. As shown in Table I the hydrogen ion uptakes measured in the carbodiimide reactions are consistent with the per cent ionizations of these acids calculated from their pK_a's. The products formed in these reactions all react with hydroxylamine to yield a hydroxamate which gives a characteristic red color with $FeCl_3$ as expected for lactones. Direct demonstration of the conversion of L-[^{3}H]gulonic acid to the lactone in the CMC reaction and of the subsequent reduction of the lactone with sodium borohydride is shown in the radiochromatographic profiles in Figure 3. The positions of migration are plotted relative to the position of the D-[^{14}C]glucitol internal standard. Initially the L-gulonic acid is retarded on the DEAE-cellulose chromatography strip (Shively and Conrad, 1970) but after conversion to the lactone in the CMC reaction, the ^{3}H-labeled product migrates slightly faster than D-glucitol. After reduction of the lactone there is a single symmetrical peak which coincides exactly with the position of D-[^{14}C]glucitol on the chromatogram.

Reaction of Polyuronides with Carbodiimides. For initial studies of the reaction of water-soluble carbodiimides with aqueous solutions of polyuronides, three polysaccharides with known structures were used: polygalacturonic acid and the capsular polysaccharides from *Aerobacter aerogenes* strains A3(S1) (Conrad *et al.*, 1966) and NCTC 243 (Gahan *et al.*, 1967). These structures are shown in Figure 4. Just as found with the monomeric hydroxy acids above, all of the polyuronides react with carbodiimides at pH 4.75 in the absence of added nucleophiles with the consumption of hydrogen ions and the generation of hydroxylamine-reactive

TABLE I: Correlation of Degree of Ionization of Hydroxy Acids at pH 4.75 and Hydrogen Ion Uptake in the Carbodiimide Reaction.[a]

Acid	pK_a	% Ionization at pH 4.75	Final H^+ Uptake (H^+/equiv of COOH)
L-Gulonic acid	3.20	94	0.99
L-Galactonic acid	3.42	93	0.98
β-Hydroxybutyric acid	4.70	58	0.61
γ-Hydroxybutyric acid	4.72	52	0.54

[a] CMC (2 mmoles) was incubated with 0.2 mmole of each of the hydroxy acids in 20 ml of H_2O at room temperature. The pH was maintained at 4.75 in a pH-Stat by addition of 0.1 N HCl and the final number of equivalents of H^+ added was recorded.

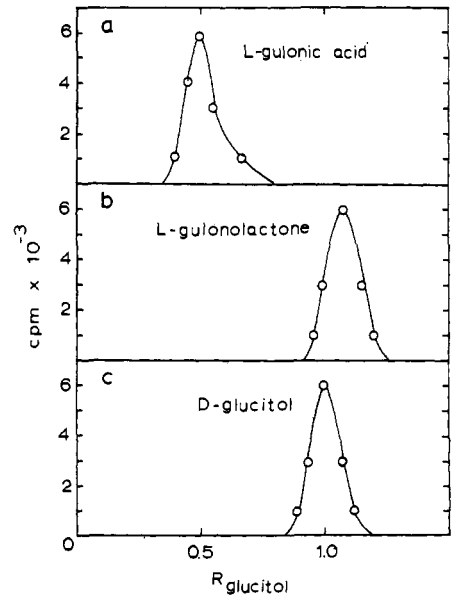

FIGURE 3: Paper chromatographic behavior of L-[^{3}H]gulonic acid in EAFW on DEAE-cellulose paper (a) before reaction with CMC, (b) after reaction with CMC, (c) after reduction of the CMC reaction product with sodium borohydride.

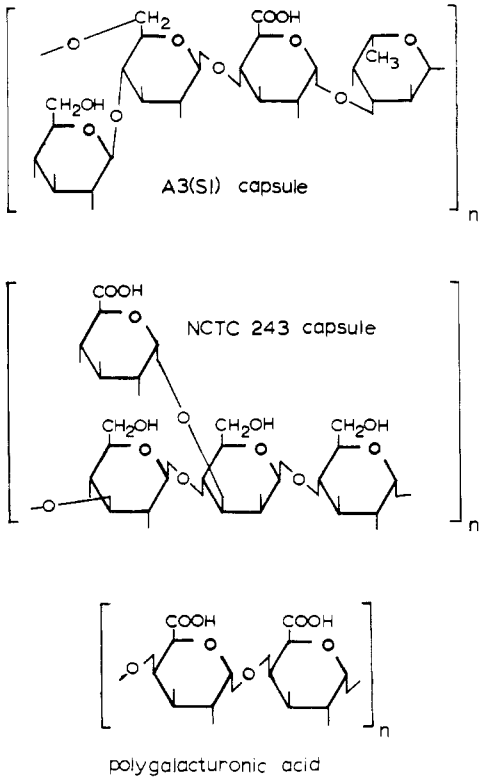

FIGURE 4: Structures of the polyuronides used in this study.

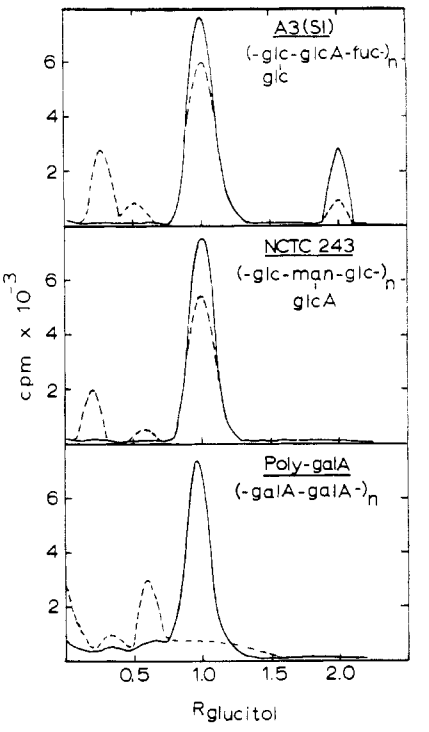

FIGURE 5: Radiochromatographic analysis of hydrolysates (1 N H_2SO_4, 100°, 4 hr) of polyuronides in EAFW on DEAE-cellulose paper before (- - - -) and after (—) reduction of the polymers in the EDC–borohydride reaction sequence. For identification of the peaks, see text.

products. For each of these polymers there is a consumption of approximately 0.75 mole of hydrogen ions/uronic acid residue indicating that the uronic acid carboxyls in these polymers are only 75% ionized at pH 4.75 (in contrast to carboxyl groups of monomeric L-gulonic acid and L-galactonic acid). There is no consumption of hydrogen ions when soluble starch is incubated with carbodiimide. The data suggest that the hydrogen ion consumption observed with the polyuronides is due to reaction of their carboxyl groups with carbodiimide followed by a nucleophilic attack by an OH group in the polysaccharide on the activated carboxyl. It is not clear whether the nucleophile is an OH on the uronic acid residue itself or one on an adjacent sugar residue in the same chain. Preliminary data suggest that it is not on an *adjacent* chain since varying the concentration of the A3(S1) polysaccharide from 2.5 to 30 mM (carboxyl equivalents) at a constant carbodiimide concentration does not alter the rate of hydrogen ion uptake. An alternate possibility, namely that the product of reaction 1 (Figure 1) accumulates in this reaction, is ruled out by the observed stoichiometry of hydrogen ion uptake. We conclude, therefore, that an intramolecular hydroxyl group serves as the nucleophile and that the product of the reaction is an ester.

Reduction of Polyuronide Reaction Products. Initial attempts to reduce the carbodiimide reaction products with sodium borohydride indicated that they are quite sensitive to alkaline pH's. Thus, in spite of the apparent quantitative conversion of carboxyl groups to esters, less than 50% of the uronic acid residues could be reduced when the reduction was carried out at pH's greater than 8.0. On the other hand, good results were obtained when an aqueous solution of sodium borohydride was added directly to the carbodiimide reaction mixture at pH 4.75. Under these conditions the reduction proceeds rapidly but is paralleled by a rapid destruction of borohydride which causes the pH to rise. If the pH is kept from rising above 7.0 in the pH-Stat, the polyuronide is maximally reduced to the neutral polysaccharide in 30–60 min at room temperature. Under these conditions more than 95% of the uronic acid residues in each of the capsular polysaccharides were reduced, while 88% of the acid residues in polygalacturonic acid were reduced. The latter is a minimum value since the disaccharide peak on the radiochromatogram of the reduced polymer was considered to be GalA → GalA and not GalA → Gal (see Methods). The radiochromatographic behavior of hydrolysates of the three polyuronides before and after reduction is illustrated in Figure 5. Under the hydrolysis conditions used here (1 N H_2SO_4, 100°) only a fraction of the glycosyluronic acid bonds in the unreduced polysaccharides are cleaved. In each of the capsular polysaccharides there is a relatively large aldobiouronic acid peak at an R_g value of approximately 0.3 and only a small amount of free D-glucuronic acid (R_g 0.55). Similarly, the unreduced polygalacturonic acid is incompletely hydrolyzed, yielding some free galacturonic acid but leaving the bulk of the carbohydrate as a mixture of oligogalacturonic acids that run together near the origin of the chromatogram. After reduction the hydrolysates show only traces of the free glycuronic acids and their oligomers, and there are corresponding increases in the expected neutral sugars (D-glucose and L-fucose from the A3(S1) polymer; D-glucose and D-mannose, which migrate together, from the 243 capsule; and D-galactose from the polygalacturonic acid).

Reduction of Glycosaminoglycuronans. The progress of hydrogen ion uptake in the reactions of EDC with hyaluronic acid, chondroitin sulfate, and heparin is shown in Figure 6. The final level of hydrogen ion uptake and the rates of uptake differ for the three polymers. Heparin reacts the fastest of all of the polyuronides described here while hyaluronic acid re-

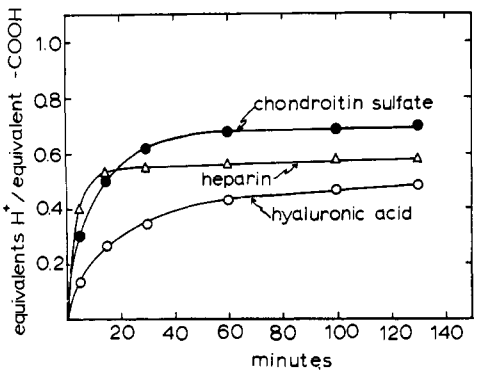

FIGURE 6: Acid uptake in the reaction of glycosaminoglycuronans with EDC at pH 4.75. For reaction conditions see legend to Figure 2.

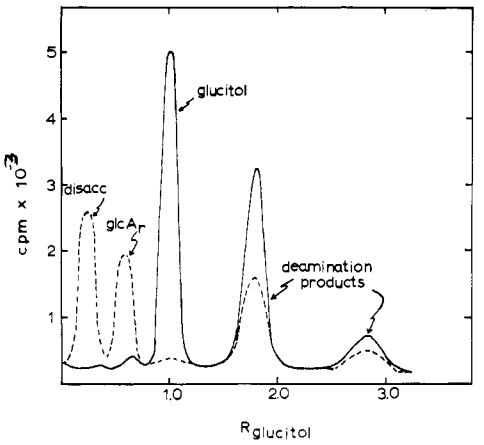

FIGURE 7: Radiochromatographic analysis of hydrolysates (1 N H_2SO_4, 100°, 6 hr) of hyaluronic acid in EAFW on DEAE-cellulose paper before (- - - -) and after (—) reduction of the polymer in the EDC–borohydride reaction sequence.

acts the slowest, perhaps because of the high viscosity of the hyaluronic acid solution used in this reaction. The reason for the differences in final hydrogen ion uptake is not clear. Addition of a second aliquot of carbodiimide does not give further reaction. If the sulfate residues in the chondroitin sulfate and heparin were esterified in this reaction, one would expect a much higher hydrogen ion uptake than observed. It may be pointed out that the equivalent weights assumed for calculations of the concentrations of carboxyl equivalents in these polysaccharide solutions may be in some error since no attempt was made to analyze or further purify these commercial products (Table II).

The radiochromatographic profiles of depolymerized hyaluronic acid and heparin are shown in Figures 7 and 8, respectively. These profiles are for the respective glycosaminoglycuronans following their depolymerization first by acid hydrolysis and then by deaminative cleavage with nitrous acid. Acid hydrolysis (1 N H_2SO_4, 100°, 6 hr) releases the *N*- and *O*-sulfates and the *N*-acetyl groups but leaves a high proportion of the glycosidic bonds intact because of the unusual stability of both the uronic acid glycosidic linkages and the glycosidic linkages of the N-deacylated and N-desulfated amino sugars. The latter are cleaved by addition of sodium nitrite directly to the hydrolysate (Shively and Conrad, 1970), a treatment which converts all of the amino sugar residues released by hydrolysis and deaminative cleavage to products which migrate faster than D-glucitol on these chromatograms. Since all of the glycosaminoglycans are polymers of disaccharides composed of one uronic acid residue and one amino sugar residue, this depolymerization procedure yields several characteristic peaks on radiochromatograms: (a) the monomeric deamination products of the amino sugars (mainly anhydro sugars) found at R_g's 1.8 and 2.8, (b) free glucuronic acid, R_g 0.55, and (c) the disaccharide which has an anhydro sugar at the reducing end and a uronic acid at the nonreducing end.

TABLE II: Effect of Reaction Conditions on the Degree of Reduction of Carbodiimide-Reacted Glycosaminoglycuronans.

Reduction Conditions[a]		% Reduction		
pH	Temp (°C)	Chondroitin SO_4	Hyaluronic Acid	Heparin
7.0	RT	59	78	15
8.0	RT	77	84	
9.0	RT			50
Uncontrolled	50	94	90[b]	85

[a] The carbodiimide reaction was carried out using EDC as described in Methods. Sodium borohydride (2 ml of a 2 M solution) was added dropwise at room temperature to 1 ml of the carbodiimide reaction mixture after acid uptake had ceased. All reductions were run 30 min. Those at stated pH's were controlled at that pH after destruction of the added borohydride brought the reaction to the desired pH. In reductions run without pH control, 1 ml of the carbodiimide reaction mixture was heated to 50° and 2 ml of 2 M $NaBH_4$ was added dropwise. Reaction was allowed to proceed for 30 min. [b] This reaction mixture was first reduced for 30 min at pH 8.0 as in footnote *a*; then 2 ml more of the 2 M sodium borohydride solution was added and the reaction was heated at 50° for an additional 30 min without pH control.

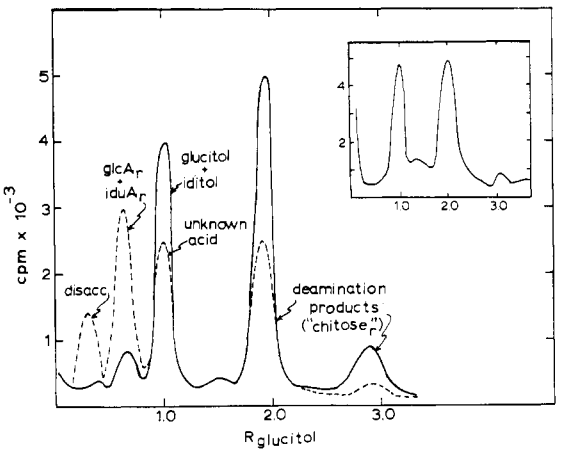

FIGURE 8: Radiochromatographic analysis of hydrolysates (in 1 N H_2SO_4, 100°, 6 hr) of heparin in EAFW on DEAE-cellulose paper before (- - - -) and after (—) reduction of the polymer in the EDC–borohydride reaction sequence. Inset shows analysis of the hydrolysate of the same reduced heparin sample in EAFW on Whatman No. 1 paper. For explanation, see text.

Figure 7 shows the peaks formed from unreduced hyaluronic acid in the dashed line. The profile in the solid line shows the peaks obtained after depolymerization of the *reduced* polysaccharide. Since reduction of the uronic acid residues yields neutral sugar glycosides which can be completely hydrolyzed by acid, the only stable glycosidic linkages which remain after acid hydrolysis are those of the N-deacylated amino sugars. Cleavage of these by the nitrous acid treatment then releases the deamination products of the amino sugars and their aglycons (the residues formed by reduction of the uronic acid residues in the polymer). Consequently, in the radiochromatographic profile for the reduced hyaluronic acid, the disaccharide and the uronic acid peaks seen in the profile of the unreduced polymer are essentially eliminated and the deamination products from D-glucosamine show a corresponding increase. In addition there is seen a large peak of D-glucose formed by the reduction of the D-glucosyluronic acid residues in the polymer. The molar ratio of hexose to total deamination products is 1.03, consistent with the previously established structure of hyaluronic acid. From the ratio of D-glucose to (D-glucose + D-glucuronic acid) it is calculated that 94% of the D-glucuronic acid residues in the polymer have been reduced (see Methods). Similarly, for chondroitin sulfate (data not shown) the molar ratio of hexose to total deamination products was 1.05 when 90% of the uronic acid residues was reduced.

The heparin profiles in Figure 8 show peaks analogous to those obtained from hyaluronic acid. In the profile for the unreduced heparin, the disaccharide peak is comparatively small, indicating that the uronic acid glycosidic linkages in heparin are more acid labile than those in hyaluronic acid. In addition to the peaks analogous to those in hyaluronic acid there is an additional peak in the unreduced heparin profile at R_g 1. Our previous analysis of a more highly purified heparin (Shively and Conrad, 1970) showed this same peak. It migrates with D-glucitol on DEAE-cellulose strips but at R_g 1.3 on Whatman No. 1 paper. This retardation of its migration on DEAE-cellulose suggested that it was an acidic component which interacted electrostatically with the cationic groups of the DEAE-cellulose. Other data were presented to show that the unknown was not derived from L-iduronic acid.

The profile for reduced heparin in Figure 8 is virtually identical with that for reduced hyaluronic acid. In the chromatographic solvent used for these analyses L-idonitol (derived from L-iduronic acid) does not separate from L-gulonitol (derived from D-glucuronic acid) nor does L-iditol separate from D-glucitol. When the reduced heparin profile on Whatman No. 1 paper is examined (inset in Figure 8), it is seen that the peak for the unknown acid is reduced almost to the background level of counts. This is further evidence that the unknown is an acidic component that can be reduced in the carbodiimide–borohydride reaction sequence. The ultimate fate of the unknown following reduction of the polymer is not established by these data, but comparisons of the data from the unreduced and the reduced profiles indicate that the reduced unknown migrates with the main deamination peak. Since the primary goal of the present work is to develop a useful general procedure for depolymerization of polyuronides, the nature of the unknown acid will be the subject of a future communication.

Since the structures and properties of hyaluronic acid, chondroitin sulfate, and heparin are representative of the glycosaminoglycuronans found in nature, it may be concluded that the approach described here is generally applicable for depolymerization of these structures. Furthermore, both the deaminative cleavage procedure and the reduction procedure can be manipulated to obtain partial depolymerization of either the original polymers or oligosaccharides derived from them. Use of these two procedures alone or in conjunction with each other thus offers an additional tool for study of structural features of these otherwise refractive polysaccharides.

References

Aspinall, G. O. (1965), *Methods Carbohyd. Chem. 5*, 397.

BeMiller, J. N. (1967), *Advan. Carbohyd. Chem. Biochem. 22*, 25.

Conrad, H. E., Bamburg, J. R., Epley, J. D., and Kindt, T. J. (1966), *Biochemistry 5*, 2808.

Danishefsky, I., and Siskovic, E. (1971), *Carbohyd. Res. 16*, 199.

Gahan, L. C., Sandford, P. A., and Conrad, H. E. (1967), *Biochemistry 6*, 2755.

Hoare, D. G., and Koshland, D. E. (1967), *J. Biol. Chem. 242*, 2447.

Koeltzow, D. E., Epley, J. D., and Conrad, H. E. (1968), *Biochemistry 7*, 2920.

Lüderitz, O., Staub, A. M., and Westphal, O. (1966), *Bacteriol. Rev. 30*, 192.

Marchessault, R. H., and Sarko, A. (1967), *Advan. Carbohyd. Chem. 22*, 421.

Rees, D. A. (1967), The Shapes of Molecules: Carbohydrate Polymers, Edinburgh, Oliver and Boyd.

Roseman, S. (1970), *Chem. Phys. Lipids 5*, 270.

Saier, M. H., Jr., and Ballou, C. E. (1968), *J. Biol. Chem. 243*, 992.

Shively, J. E., and Conrad, H. E. (1970), *Biochemistry 9*, 33.