Stoichiometry of the Nitrous Acid Deaminative Cleavage of Model Amino Sugar Glycosides and Glycosaminoglycuronans*

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ABSTRACT: Treatment of N-deacylated amino sugars or their glycosides with nitrous acid yields a mixture of deaminated monomeric products at the aldehyde or ketone oxidation level. The products are readily quantitated by radiochromatography (Koeltzow, D. E., Epley, J. D., and Conrad, H. E. (1968), *Biochemistry 7*, 2920). The composition of the deaminated mixture varies with the amino sugar in question and with the anomeric configuration of the amino sugar glycoside.

For D-glucosamine and D-galactosamine the sum of the reducing equivalents in all of the deamination products is equal to the number of reducing equivalents of amino sugar present before nitrous acid treatment.

Hydrolysis of model acetamino sugar glycosides and glycosaminoglycuronans with 1 N sulfuric acid at 100° gives quantitative N deacylation in several hours but incomplete cleavage of the glycosidic linkages. Treatment of the N-deacylated hydrolysis mixture with sodium nitrite results in quantitative cleavage of the remaining amino sugar glycosides and converts the amino sugars into products which can be measured by radiochromatography. Application of this approach in the depolymerization of the chondroitin sulfates, dermatan sulfate, hyaluronic acid, heparin, and heparan sulfate is described.

The acid-catalyzed depolymerization of glycosidically bound amino sugars, which usually are substituted by acid-labile *N*-acyl or *N*-sulfo groups, is complicated by the fact that hydrolysis may proceed by two pathways, shown in Figure 1; one (reactions b plus d) in which the *N*-acyl linkage is hydrolyzed before the glycosidic bond, and a second (reactions a plus c) in which the glycosidic bond is cleaved before the *N*-acyl bond (Moggridge and Neuberger, 1938). When a significant fraction of the N-substituted amino glycosyl residues (I) in a polysaccharide is hydrolyzed by the first pathway, the electrostatic (Moggridge and Neuberger, 1938) and inductive (Marshall, 1963) effects of the alkylammonium group on the residue that is generated (III) markedly stabilize the glycosidic linkage. Consequently, to obtain quantitative release of the free amino sugar (IV) and the aglycon to which it is linked one must use high concentrations of acid and elevated temperatures (Adams, 1965; Spiro, 1966; Davidson, 1966), which favor reaction a over reaction b, but which cause significant destruction both of amino sugars and of any uronic acids and neutral sugars that may be present in the polymer (Boas, 1953; Belcher *et al.*, 1954; Smith and Zwartouw, 1956; Exley, 1957; Foster *et al.*, 1957; Lee and Montgomery, 1961).

In contrast, the cleavage of amino sugar glycosides by nitrous acid (reaction e, Figure 1) proceeds readily at room temperature with deaminative loss of nitrogen (reactions e and f). The deamination reaction proceeds with both free and glycosidically bound amino sugars only when the amino groups are unsubstituted (or sulfated, see Lagunoff and Warren, 1962; Cifonelli, 1965) and yields a mixture of products, the content and complexity of which vary with the amino sugar in question (Shafizadeh, 1958). 2-Amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose—the most common amino sugars in biopolymers—are converted mainly into 2,5-anhydrohexoses (*e.g.*, V in Figure 1) while 2-amino-2-deoxy-D-mannose yields a more complex mixture (Shafizadeh, 1958). Thus, acid hydrolysis and nitrous acid deamination are complementary approaches to depolymerization of amino sugar glycosides, the former proceeding readily only when the amino groups are substituted, the latter only when amino groups are free. Therefore, if mucopolysaccharides or glycoproteins are hydrolyzed with acid until all of the amino sugars (both the monosaccharides and those still glycosidically bound) have free amino groups and all of the neutral glycosidic bonds are split, treatment with nitrous acid should cleave all remaining glycosidic bonds and simultaneously convert 2-amino-2-deoxy-D-glucose or 2-amino-2-deoxy-D-galactose into 2,5-anhydro sugars. If deamination is in any sense stoichiometric, and if the resulting product(s) can be measured quantitatively, this sequence of reactions should offer an approach to glycosaminoglycan depolymerization relatively unencumbered by the difficulties associated with forcing conditions of hydrolysis.

The present work was undertaken to determine whether the stoichiometry of the nitrous acid deamination reaction is adequate for its use in quantitation, and takes advantage of the radiochromatographic method for quantitation of reducing carbohydrates (Koeltzow *et al.*, 1968) following reduction with [^{3}H]sodium borohydride (reaction g, Figure 1). Since the radiochromatographic method of analysis permits direct determination of all reducing compounds (free and acetylated amino sugars and deamination products) and indirect determination of the amounts of unhydrolyzed glycosides, it is readily adapted for following hydrolysis through the intermediate stages. Consequently, the rates of the individual reac-

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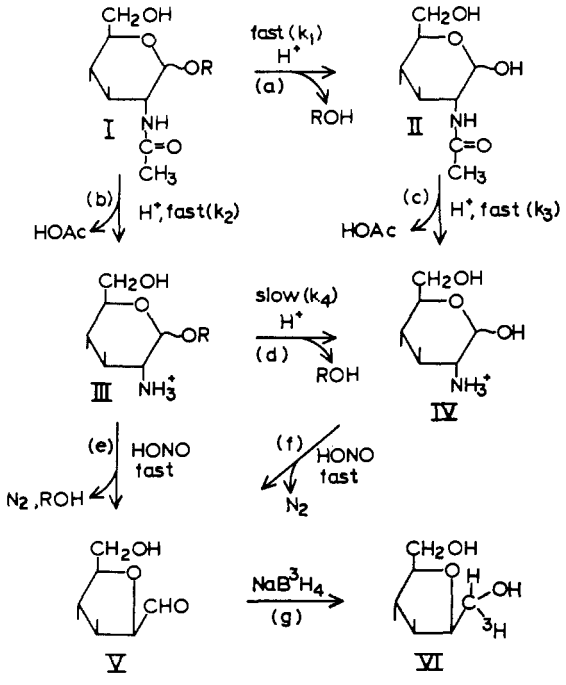

FIGURE 1: Pathways for hydrolysis and deaminative cleavage of amino sugar glycosides.

tions, a, b, c, and d in 1 N sulfuric acid at 100° have been measured and found to be consistent with the values for the overall rate of conversion of I into IV reported in the literature (Moggridge and Neuberger, 1938; Foster *et al.*, 1957; Marshall, 1963). The data show that when the rate constants for deacylation and for glycoside hydrolysis are similar, as found for methyl 2-acetamido-2-deoxy-α-D-glucoside, up to 30% of the glycoside may remain after hydrolysis for 6 hr in 1 N sulfuric acid at 100°. In such cases the nitrous acid treatment is essential for complete cleavage of the 2-amino-2-deoxyglycosidic bond. Application of the combined hydrolysis–deamination approach for depolymerization of a number of glycosaminoglycuronans is also described.

Methods

2-Amino-2-deoxy-D-glucose hydrochloride, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-mannose, and chitin (poly β1→4GlcNAc*p*[1]) were obtained from Sigma Chemical Co. Chitobiose (GlcN*p*β1→4GlcN) and chitotriose (GlcN*p*β1→4GlcN*p*β1→4-GlcN) were generously supplied by Dr. Saul Roseman, di-*N*-acetylchitobiose by Dr. J. A. Rupley, and sodium idonate by Dr. J. A. Cifonelli. Chitosan (poly β1→4GlcN*p*) was prepared by three treatments of chitin with sodium hydroxide as described by Horton and Lineback (1965). The product was dialyzed exhaustively and lyophilized (N, 6.7%; theory, N, 7.1%). Dermatan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate, hyaluronic acid, heparin, and heparan sulfate were supplied by Dr. Martin B. Mathews.

Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside, prepared by the method of Zillikin *et al.* (1957), gave $[\alpha]_D^{25}$ +131° and mp 187–189° (lit. $[\alpha]_D^{25}$ +131°, mp 187°). Methyl 2-amino-2-deoxy-α-D-glucopyranoside was prepared by the action of barium hydroxide on methyl 2-acetamido-2-deoxy-α-D-glucopyranoside (McEvoy *et al.*, 1960) and gave $[\alpha]_D^{25}$ +127° (*c*, 2% in water) (lit. $[\alpha]_D^{25}$ +127° (Neuberger and Rivers, 1939)). Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside, prepared from 3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-glucopyranosyl chloride as described by Conchie and Levy (1965), gave $[\alpha]_D^{25}$ −43° and mp 202–204° (lit. $[\alpha]_D^{25}$ −43°, mp 204°). Methyl 2-amino-2-deoxy-β-D-glucopyranoside, $[\alpha]_D^{25}$ −24° (lit. (Irvine and Hynd, 1912) $[\alpha]_D^{25}$ −24°) was prepared by N deacylation of methyl tri-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucoside with triethyloxonium fluoroborate (Hanessian, 1967). Ethyl 2-(2,4-dinitrophenyl)-2-deoxy-α-D-glucoside was prepared by the method of Lloyd *et al.* (1969) and gave $[\alpha]_D^{25}$ +22° and mp 193–194° (lit. $[\alpha]_D^{25}$ +23°, mp 193–194°). It was converted into ethyl 2-amino-2-deoxy-α-D-glucoside, $[\alpha]_D^{25}$ +105° (lit. $[\alpha]_D^{25}$ +129°), as described by Lloyd and Stacey (1960).

Hydrolysis. All hydrolyses were performed as follows: the carbohydrate (2 mg) was dissolved in 25 μl of water and 25 μl of an aqueous solution of uniformly labeled D-[^{14}C]glucose (20 μCi/ml, 200 mCi/mmole, New England Nuclear Corp.) was added. Sulfuric acid was then added to obtain a 2% carbohydrate solution in 1 N sulfuric acid. Using a Hamilton syringe this solution was transferred to a melting point tube and covered with 3–5 μl of mineral oil. The tube was immersed in a sand bath at 100° and at appropriate intervals 2-μl aliquots were withdrawn for analysis.

Analysis of Hydrolysates. The progress of hydrolysis was followed by the radiochromatographic method described earlier (Koeltzow *et al.*, 1968) wherein aliquots are transferred to a microreduction vial, neutralized with an equal volume of 1 M sodium carbonate, and reduced with 5 μl of 0.5 M [^{3}H]sodium borohydride (15 mCi/mmole, New England Nuclear Corp.) in 0.1 N sodium hydroxide. The reduced samples were chromatographed in ethyl acetate–acetic acid–formic acid–water (18:3:1:4) and counted as described previously.

For the nitrous acid deamination 2-μl aliquots of the hydrolysate were transferred to a microreduction vial and treated with 5 μl of 5.5 M sodium nitrite. After 10 min at room temperature the sample was neutralized with 2 μl of 1 M sodium carbonate and reduced, chromatographed, and counted as above.

The amount of D-[^{14}C]glucitol appearing on the chromatogram was used to indicate the volume of the hydrolysate that was represented on the chromatogram. The ^{3}H counts per minute in each peak were summed and converted into micromoles per milliliter of hydrolysate using a computer program developed for processing the counting data.[2] With the [^{3}H]-borohydride used in this work the glycitols formed on reduction gave 4.165×10^5 cpm/μmole (counted at 5% efficiency on paper chromatograms). The position of the D-[^{14}C]glucitol on

[1] Abbreviations used are: R_{Gl}, rate of chromatographic migration relative to the rate of [^{14}C]glucitol; GlcN, 2-amino-2-deoxy-D-glucose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; GalN, 2-amino-2-deoxy-D-galactose; GalNAc, 2-acetamido-2-deoxy-D-galactose; ManN, 2-amino-2-deoxy-D-mannose; ManNAc, 2-acetamido-2-deoxy-D-mannose; βMeGlcN, me-β-GlcN; αMeGlcN, me-α-GlcN; αEtGlcN, Et-α-GlcN; GlcUA, D-glucuronic acid; IdUA, L-iduronic acid; HexN, hexosamine; HexA, hexuronic acid.

[2] D. E. Koeltzow, unpublished data.

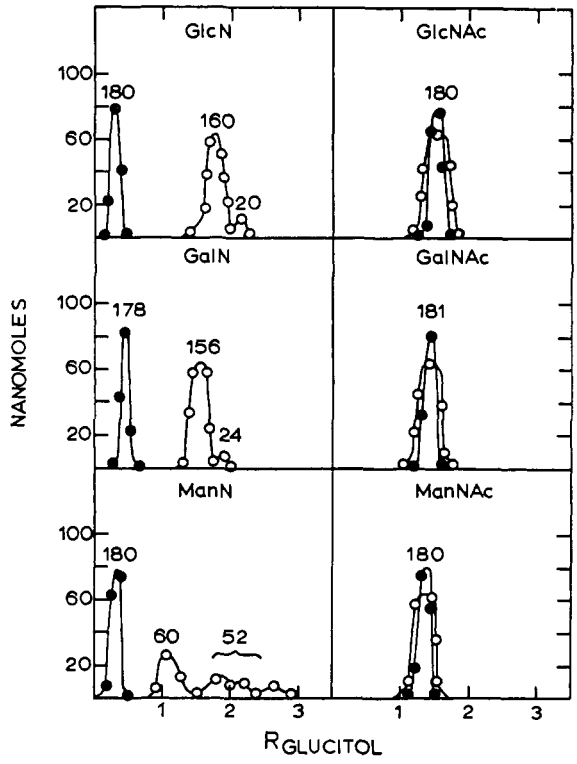

FIGURE 2: Radiochromatography of amino sugars before (-●-●-) and after (-○-○-) nitrous acid treatment. Procedures for deamination and radiochromatography are described in Methods. The numbers above each peak indicate the total number of nanomoles in the peak.

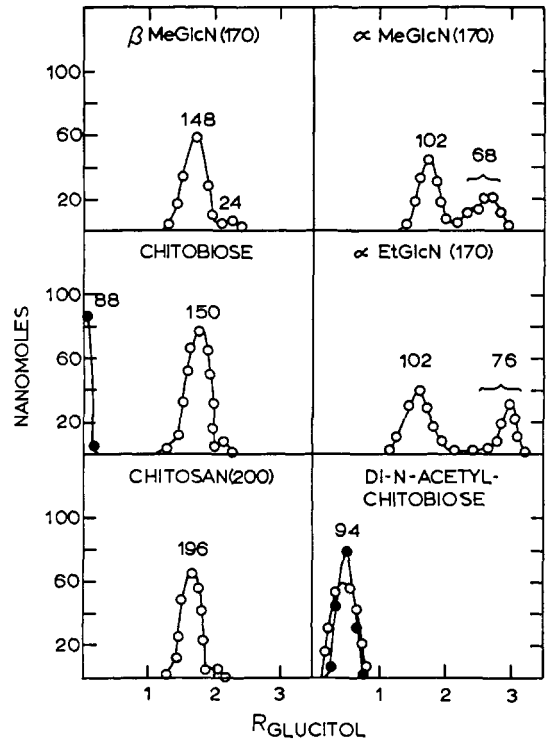

FIGURE 3: Radiochromatography of amino sugar glycosides before (-●-●-) and after (-○-○-) nitrous acid treatment. Procedures for deamination and radiochromatography are described in Methods. The numbers above each peak indicate the total number of nanomoles in the peak. For nonreducing substrates the number of nanomoles deaminated is indicated in parentheses.

the chromatogram was used to identify peaks on the basis of their R_{Gl}[3] values.

Results

Effect of Nitrous Acid on Radiochromatographic Behavior of Amino Sugars. Figure 2 shows the positions of migration of hexosamines on chromatograms before and after nitrous acid treatment. It is seen that the relatively slow movement of the amino sugars is markedly altered by reaction with nitrous acid which yields faster moving products. 2-Amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose each yield a major peak with an R_{Gl} greater than 1, and one or two minor peaks which migrate somewhat faster. Deamination of 2-amino-2-deoxy-D-mannose gives a more complex profile of products. While it has not been the purpose of this work to characterize these products, it is presumed on the basis of literature reports that the major products from 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose are 2,5-anhydro-D-mannose and 2,5-anhydrotalose (Shafizadeh, 1958), respectively. The product obtained in highest yield from 2-amino-2-deoxy-D-mannose behaves chromatographically like a hexose. A further comparison of the radiochromatographic behavior of the deamination products of 2-amino-2-deoxy-D-glucose with compounds reported in the literature to be products has shown that there is no detectable D-arabinose (Schorigin and Makarova-Semiljanskaja, 1935) or 2-deoxy-D-glucose (Matsushima, 1951) formed in these experiments. It is of interest to note that when 2-amino-2-deoxy-D-glucose is deaminated and chromatographed *without borohydride reduction*, the major deamination product migrates with glucose (unreduced).

Figure 2 shows also that the only effect of nitrous acid on the N-acetylated amino sugars is a slight broadening of the chromatographic peaks. This is presumably due to the effect of the salts from the reaction mixture on the chromatographic migration. No difference in the radiochromatographic profiles is observed when the nitrous acid treatment is carried out at 100°. The R_{Gl} values and profiles of the deamination products of the amino sugars are sufficiently different so that they may be used to distinguish these compounds for purposes of identification.

Effect of Nitrous Acid on Radiochromatographic Behavior of Glycosides of 2-Amino-2-deoxy-D-glucose. The radiochromatography of the anomeric methyl 2-amino-2-deoxy-D-glucopyranosides, ethyl 2-amino-2-deoxy-α-D-glucopyranoside, chitobiose, chitosan, and di-*N*-acetylchitobiose before and after deamination is shown in Figure 3. Chitobiose, chitotriose (not shown), chitosan, and methyl 2-amino-2-deoxy-β-D-glucopyranoside all react with nitrous acid to give a chromatographic profile very similar to that obtained with 2-amino-2-deoxy-D-glucose. Both methyl and ethyl 2-amino-2-deoxy-α-

[3] Throughout this manuscript observations made concerning the rates of chromatographic migration refer to borohydride-reduced products. Since the behavior referred to is that of the parent sugar treated by the standard procedure described here, the chromatographic peak is described as that of the parent sugar rather than its glycitol.

TABLE I: Stoichiometry of Deamination.

Substrate	Deamination Products (μmoles/μmole of substrate)
GlcN	0.99–1.02[a]
GalN	1.00
ManN	0.65
αMeGlcN	1.00
βMeGlcN	1.01
αEtGlcN	0.99
Chitobiose	0.90[b]
Chitotriose	0.87[b]
Chitosan	0.98

[a] Range of yields obtained in more than ten experiments. [b] Substrate samples contained 10% of nonreducing material as determined by radiochromatography of the sample without nitrous acid cleavage.

D-glucopyranoside, on the other hand, yield, in addition to the peak which appears to be identical with that obtained from the β-linked glucosides, significant amounts of two faster moving peaks that migrate together. As expected, di-*N*-acetylchitobiose does not react with nitrous acid. Thus, all β-linked glycosides of 2-amino-2-deoxy-D-glucose yield the same characteristic radiochromatographic profile of products which differs from that found for the α-linked glycosides. The data suggest that for glycosides of 2-amino-2-deoxy-D-glucose at least, the nitrous acid deamination products reflect the configuration at the anomeric carbon of the glycoside. However, in the studies with heparin and heparan sulfate described below the α-linked amino sugars do not yield the faster moving peak following depolymerization with nitrous acid.

Effect of Conditions on the Deamination Reaction. The method of *in situ* generation of nitrous acid used in this work was devised so that it could be readily adapted to the procedures used in this laboratory for analysis of polysaccharide hydrolysates by radiochromatography. Consequently, aliquots taken from hydrolysates in 1 N sulfuric acid were mixed directly with 5.5 M aqueous solutions of sodium nitrite at room temperature and the reaction was allowed to proceed 10 min before neutralizing and reducing the sample. Only when a sufficient volume of the sodium nitrite solution to raise the pH of the solution above 3 was added did complete conversion of 2-amino-2-deoxy-D-glucose occur. The reaction mixture was strongly buffered and further addition of sodium nitrite did not increase the pH above 4 and had no effect on the course of the reaction. These results are consistent with previous reports (Smith and Baer, 1960) that the active species in the deamination is N_2O_3 which exists in equilibrium with nitrous acid at pH 3–7. In the standard conditions adopted for this study a 2-μl aliquot of a 2% solution of hexosamine is mixed with 5 μl of 5.5 M sodium nitrite at room temperature to give a 150 molar excess of sodium nitrite over hexosamine. Under these conditions deamination of all substrates tested here is complete in less than 5 min. When the 1 N sulfuric acid is replaced with equal volumes of 1 N acetic or 1 N hydrochloric acid in the deamination of chitosan, results are both qualitatively and quantitatively identical with these obtained with sulfuric acid. The latter conditions are more typical of those described previously in the literature.

The Stoichiometry of the Deamination Reaction. Table I shows the stoichiometry of the nitrous acid conversion of amino sugars and their derivatives. It can be seen that, in spite of the diversity of products that may be formed (Figures 2 and 3), the sum of all [^{3}H]borohydride reducing equivalents in the products is equal to the number of [^{3}H]borohydride reducing equivalents in the reactants. This is true for free and for glycosidically bound amino sugars. 2-Amino-2-deoxy-D-mannose is an exception. The two most widely occurring amino sugars, 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose, can thus be depolymerized, identified, and quantitated by coupling nitrous acid deamination with radiochromatography. The position of migration of the deamination products is in an area of the chromatogram where very few other monosaccharides appear. In the event that *N*-acetyl amino sugars or deoxy sugars (which migrate at rates similar to those of the deamination products and thus interfere with quantitation) are present they may be determined by radiochromatography without deamination and their values substracted out of a parallel chromatogram of the nitrous acid treated sample. The [^{14}C]glucitol present on the chromatograms allows an exact comparison of the parallel chromatograms. Since the radiochromatographic behavior of amino sugars is specifically altered by nitrous acid treatment, examination of chromatograms of a sample before and after nitrous acid treatment serves to identify a slow-moving peak in the untreated sample as an amino sugar or an amino derivative (see data for glycosaminoglycuronans below).

Coupling of Hydrolysis and Deamination for Depolymerization. The applicability of depolymerization by combined hydrolysis and deamination is illustrated with model compounds in Figure 4 in which the progress of hydrolysis in 1 N sulfuric acid at 100° is followed by radiochromatographic analysis of aliquots with and without nitrous acid treatment. In these studies 2-amino-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-glucose, and the reducing oligosaccharides are measured directly; the amounts of methyl 2-amino-2-deoxy-D-glucopyranosides are measured by the amount of deamination products formed in excess of those expected from the free glucosamine found in a duplicate sample not treated with nitrous acid; and the amounts of methyl 2-acetamido-2-deoxy-D-glucosides remaining are calculated by subtracting the total moles of hydrolysis products from the number of moles of pure starting material present initially. From the data thus obtained for these and several other model compounds, rate constants for the individual steps in Figure 1 have been calculated and are given in Table II. The method used for calculation of rate constants k_1 and k_2 is described in the Appendix to this paper.

The α- and β-methyl glycosides of 2-acetamido-2-deoxy-D-glucose present an interesting contrast in their hydrolysis patterns. For the glycoside, the rate constants for glycoside and *N*-acetyl hydrolysis are very similar and as a consequence approximately one-third of the starting material is converted into the very stable methyl 2-amino-2-deoxy-α-D-glucopyranoside. The remainder is converted into 2-acetamido-2-deoxy-D-glucose which is further hydrolyzed to 2-amino-2-deoxy-D-glucose. Because of the stability of the methyl 2-amino-2-deoxy-α-D-glucopyranoside to hydrolysis, only 65% of the start-

TABLE II: Hydrolysis Rate Constants for Model Compounds.

Reaction	Hydrolysis Rate Constant[a] ($k \times 10^4$ sec^{-1})
Amide hydrolysis	
ManNAc → ManN + HOAc	7.65
GalNAc → GlaN + HOAc	2.62
GlcNAc → GlcN + HOAc	2.10
αMeGlcNAc → αMeGlcN + HOAc	1.44
βMeGlcNAc → βMeGlcN + HOAc	*b*
Glycoside hydrolysis	
βMeGlcNAc → GlcNAc + MeOH	29.9
βMeGlcN → GlcN + MeOH	0.0043
αMeGlcNAc → GlcNAc + MeOH	1.85
αMeGlcN → GlcN + MeOH	0.0021
$GlcNAc\overset{1}{\underset{\beta}{\rightarrow}}{}^{4}GlcNAc$ → 2GlcNAc	6.4
$GlcN\overset{1}{\underset{\beta}{\rightarrow}}{}^{4}GlcN$ → 2GlcN	0.038
$GlcN\overset{1}{\underset{\beta}{\rightarrow}}{}^{4}GlcN\overset{1}{\underset{\beta}{\rightarrow}}{}^{4}GlcN$ → 3GlcN	0.012

[a] 1 N sulfuric acid, 100°. [b] Too low relative to glycoside hydrolysis to measure.

ing material is eventually converted by acid into the free amino sugar. If, however, the hydrolysate is treated with nitrous acid, 100% of the starting 2-amino-2-deoxy-D-glucose appears in the deamination products.

The rate constant for hydrolysis of β-methyl glycosidic bond, on the other hand, is 16 times than that for the α-glycoside. Therefore, the starting material is converted almost quantitatively into 2-acetamido-2-deoxy-D-glucose which is then readily hydrolyzed further to 2-amino-2-deoxy-D-glucose. In this case quantitation of total amino sugar is only slightly improved through use of the deamination reaction. A similar result is obtained with di-*N*-acetylchitobiose, suggesting that this is the general pattern that may be expected for β-linked acetamido sugar glycosides.

The rates for *N*-acetyl hydrolysis shown in Table II are very similar for 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactose. For the mannose derivative, however, the rate constant is approximately three times as large. This might be explained in terms of neighboring group participation (Goodman, 1967) in *N*-acetyl hydrolysis. In 2-acetamido-2-deoxy-D-mannose the C_3 hydroxyl is *cis* to the acetamido group and thus can participate more readily to assist in hydrolysis than can the *trans* C_3 hydroxyls in the glucose and galactose derivatives. It may also be noted that when there is substitution of the anomeric OH the rate constant for amide hydrolysis is lowered. Although the effect is not large, it might conceivably be an important factor in amino sugar containing polysaccharides in which the aglycon is larger residue than the methyl group described here. Other data not shown suggest that substitution of the C_3 hydroxyl of 2-acetamido-2-deoxy-D-glycosides, as found in a number of glycosaminoglycuronans, may also result in lower rate constants for hydrolysis of the amide.

The data for glycoside hydrolysis given in Table II are, in general, consistent with previous data in the literature (Moggridge and Neuberger, 1938; Foster *et al.*, 1957; Onodera and Komano, 1961; Marshall, 1963). The rate constants found for the glycosides of 2-amino-2-deoxy-D-glucose are an order of magnitude lower than some of the earlier values. The progress of these hydrolyses is so slow that data must be collected over a several-day period.

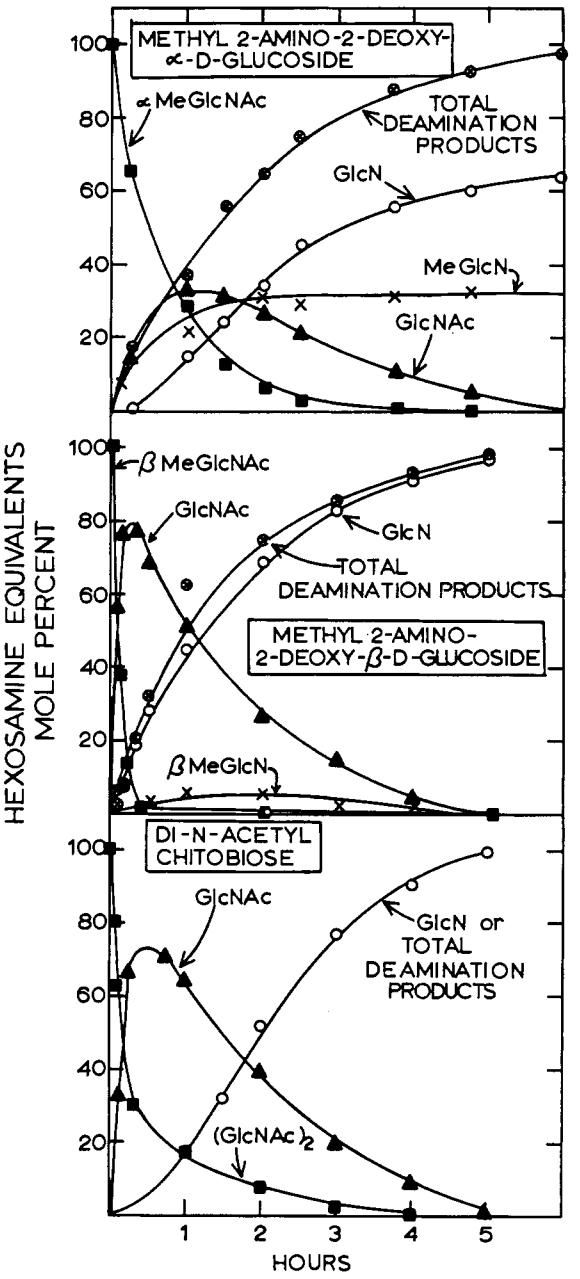

FIGURE 4: Hydrolysis of model amino sugar glycosides in 1 N sulfuric acid at 100°. Aliquots were analyzed as described in Methods.

Analysis of Glycosaminoglycuronans, Identification of Radiochromatographic Peaks. The utility of the hydrolysis–deamination approach for study of complex polymeric structures is evaluated here by its application to a number of glycosaminoglycuronans for which analytical data have been obtained by more classical approaches. The purified polymers

TABLE III: Analysis of Glycosaminoglycuronans.

Polymer	Repeating Unit[a]	$[\alpha]_D$[b] (deg)	Hexosamine (%)[c]			Hexuronic Acid (%)[c]			Molar Ratios (HexN:HexA)	
			Calcd[d]	Found EM[b,e]	Found RC[f]	Calcd[d]	Found Carb[b,g]	Found RC[f]	RC: RC	EM: Carb
Chondroitin 4-sulfate	$\rightarrow^{4}_{\beta}GlcA^{1}\rightarrow^{3}_{\beta}GalNAc(4\text{-}SO_4)^{1}\rightarrow$	−25	32.0	27.2	28.2	35.0	34.1	33.0	0.99	0.92
Chondroitin-6 sulfate	$\rightarrow^{4}_{\beta}GlcA^{1}\rightarrow^{3}_{\beta}GalNAc(6\text{-}SO_4)^{1}\rightarrow$	−11	32.0	26.2	24.4	35.0	34.6	28.2	1.00	0.88
Dermatan sulfate	$\rightarrow^{4}_{\alpha}IdUA^{1}\rightarrow^{3}_{\beta}GalNAc(4\text{-}SO_4)^{1}\rightarrow$	−70	30.2	23.0	32.6	33.0	12.8	18.2	1.99	2.00
Hyaluronic acid	$\rightarrow^{4}_{\beta}GlcA^{1}\rightarrow^{3}_{\beta}GlcNAc^{1}\rightarrow$	−69	40.3	38.3	24.3	44.0	47.2	27.9	1.01	0.95
Heparin	$\rightarrow^{4}_{\alpha}GlcA^{1}\rightarrow^{4}_{\alpha}GlcNSO_3H(SO_4)^{1}\rightarrow$	+52	26.9	23.8	23.1	29.4	38.7	34.0	0.80	0.62
Heparan sulfate	$\rightarrow^{4}_{\alpha}GlcA^{1}\rightarrow^{4}_{\alpha}GlcN\{Ac\ \text{or}\ SO_3H\}^{1}\rightarrow$	+73	32.2	24.6	24.8	35.2	44.1	34.4	0.75	0.68

[a] See Jeanloz (1963). [b] Data supplied by Drs. Mathews, Cifonelli, and Rodén. [c] Per cent by weight. [d] Values calculated for sodium salt (except for heparan sulfate in which case the calcium salt was analyzed) using data supplied for moles of sulfate per mole of hexosamine. [e] By the method of Elson and Morgan (1933) with correction for loss on hydrolysis for hyaluronic acid, chondroitin 4-sulfate, and chondroitin 6-sulfate only. [f] By radiochromatography. [g] By the carbazole method (Dische, 1947).

were supplied together with their analyses by Drs. M. B. Mathews, J. A. Cifonelli, and L. Rodén of the LaRabida-University of Chicago Institute. The gross structural features of the glycosaminoglycuronans (Cifonelli, 1968a; Jeanloz, 1963) are given in Table III. The calculated per cents of total weight represented by hexosamine and hexuronic acid are based on the sodium salts of the structures given (except for heparan sulfate, which is the calcium salt) using the sulfate analyses supplied with the samples. These values might be significantly altered by the still evolving structural features of heparin (Wolfrom *et al.*, 1969; Yamauchi *et al.*, 1968), heparan sulfate (Cifonelli, 1968b), and dermatan sulfate (Fransson and Rodén, 1967). However, a legitimate comparison can be made of the radiochromatographic analyses with the analytical data or hexosamine and hexuronic acid obtained by the Elson–Morgan and carbazole procedures.

For radiochromatography mucopolysaccharides were hydrolyzed for 24 hr at 100° in 1 N sulfuric acid. Maximum yields of hexosamine and hexuronic acid were obtained at this time (except for dermatan sulfate, see below) and the amount of degradation of monomers was not significant as indicated by the small degree of browning and by the fact that the [^{14}C]-glucose added at the start of hydrolysis was recovered quantitatively in a single symmetrical glucitol peak on the radiochromatograms. Furthermore, the analytical data described below show that recoveries of hexosamine and hexuronic acid are, in most cases, very close to the theoretical values. Under the hydrolysis conditions used a soluble polymer of neutral sugars would be hydrolyzed completely in 3–5 hr.

Chromatographic profiles are presented for heparin and heparan sulfate in Figure 5, for chondroitin 4-sulfate and chondroitin 6-sulfate in Figure 6, and for dermatan sulfate and hyaluronic acid in Figure 7. Because of the similarities of the polymer structures and the chromatographic profiles, identification of peaks is discussed here for Figures 5–7 together. Based on (1) the established structures of these polymers (Jeanloz, 1963), (2) the radiochromatographic behavior of model compounds, and (3) the R_{G1} values of standards, it is possible to make quite logical peak assingments. Thus, a deacylated monomeric amino sugar is recognizable by its R_{G1} value (approximately 0.3) before nitrous acid treatment and its complete conversion into deamination products when treated with nitrous acid. Standard glucuronic, iduronic, and galacturonic acids appear at $R_{G1} \sim 0.9$–0.95. The peak at R_{G1} 1.3 is tentatively assigned as a monomeric uronic acid (see below). With the exception of dermatan sulfate the hydrolysis procedure alone yields relatively little monosaccharide; the bulk of the reducing sugars migrate more slowly than the hexosamine and are therefore oligosaccharides. All residues should be completely N deacetylated and N and O desulfated after the 24-hr hydrolysis period (Meyer and Schwartz, 1950). Since all of these polymers are made up of alternating glycuronic acid and hexosamine residues, the oligosaccharides remaining after 24-hr hydrolysis should be subject to complete

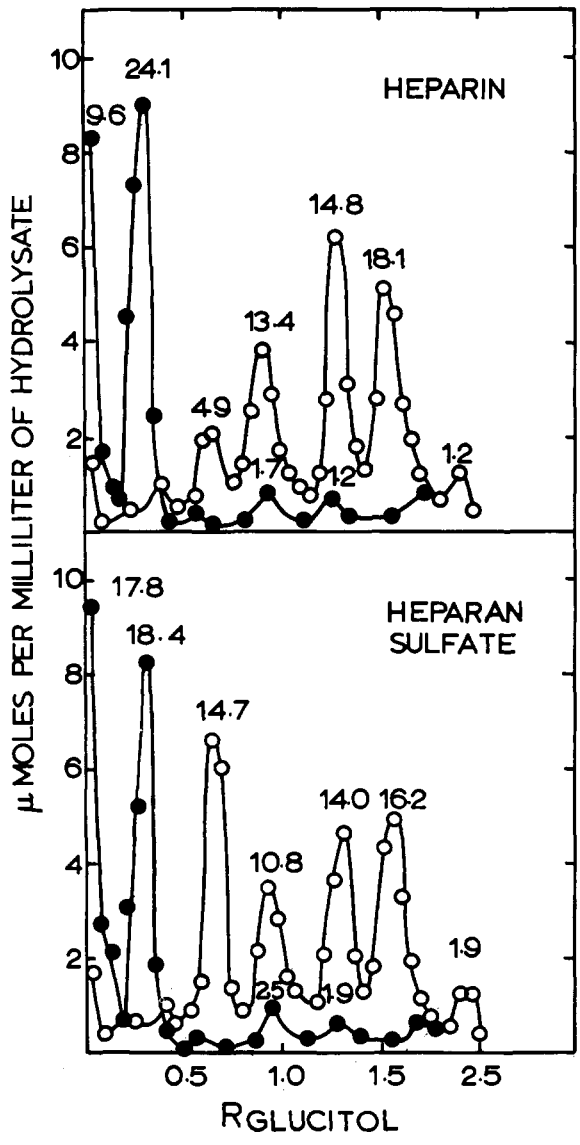

FIGURE 5: Radiochromatographic analysis of heparin and heparan sulfate. A 2% solution of the vacuum-dried (60°, 24 hr) mucopolysaccharide was hydrolyzed in 1 N sulfuric acid at 100° for 24 hr and then analyzed by radiochromatography before (-●-●-) and after (-○-○-) nitrous acid treatment. The numbers directly above, or below, each peak indicate the total micromoles of that component per milliliter of hydrolysate. For identification of peaks, see Results.

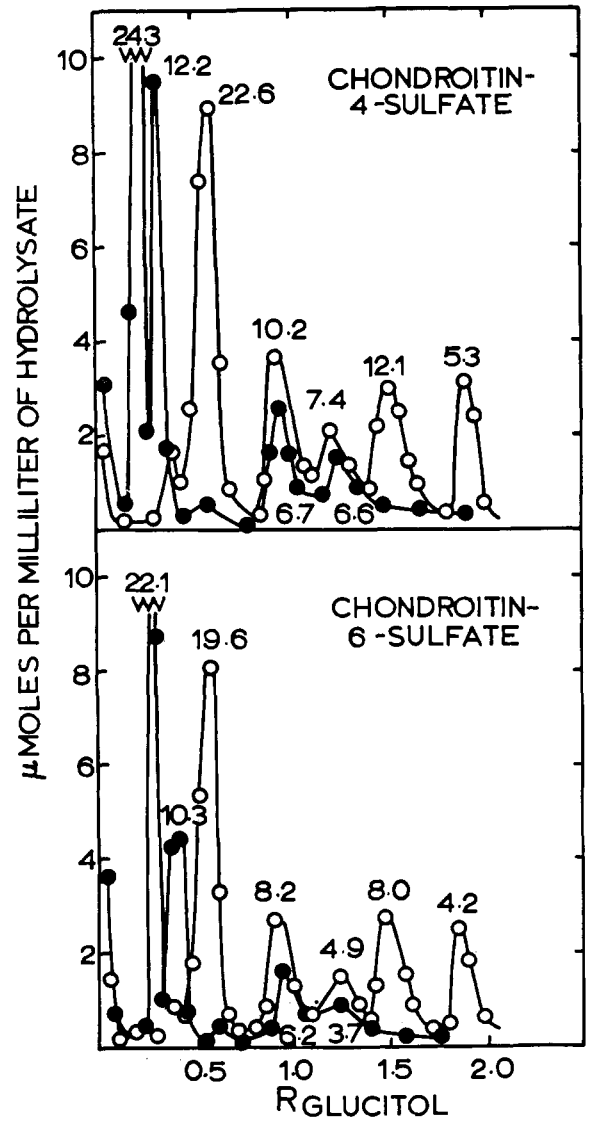

FIGURE 6: Radiochromatographic analysis of chondroitin 4-sulfate and chondroitin 6-sulfate. See legend to Figure 5.

conversion by nitrous acid either into monomers or into disaccharides composed of glycuronosyl residues at the nonreducing terminals and anhydro sugars at the reducing ends. An examination of Figures 5–7 shows that all of the deamination profiles have a peak in the disaccharide region at R_{G1} 0.6–0.7 consistent with that anticipated for the glycuronosyl → "anhydro sugar."

Since it will be a critical point in the discussion below, further comment is in order concerning the assignment of the R_{G1} 1.3 component as a monomeric carbohydrate acid. Following nitrous acid treatment each radiochromatogram shows 3–4 major peaks. There are no peaks beyond R_{G1} 2.0 in any of the chromatograms; therefore, none of these polymers yield the fast-moving deamination peak found with the α-linked model compounds, nor is there any significant fraction of the reduced uronic acid which travels as a lactone. The disaccharide (R_{G1} 0.6–0.7), the glycuronic acid (R_{G1} 0.9), and the main deamination peak (R_{G1} 1.6) are readily identifiable. Of these three only two, the disaccharide and the glycuronic acid, contain carboxyl groups and should behave like acidic saccharides on chromatography. This is confirmed by substituting DEAE-cellulose paper (Whatman) for regular filter paper in the radiochromatography. In such a comparison the same components on the two chromatograms are recognized by the number of micromoles of [^{3}H]borohydride reducing equivalents in the peaks. As shown in Figure 8 only the deamination peak migrates with the same R_{G1} on both Whatman No. 1 paper and DEAE-cellulose strips. This is taken as evidence that *all* of the retarded components, including the R_{G1} 1.3 compound, are acids. Identical results were obtained for the corresponding peaks from the mucopolysaccharides not shown in Figure 8. Also, when radiochromatograms are run in ethyl acetate–

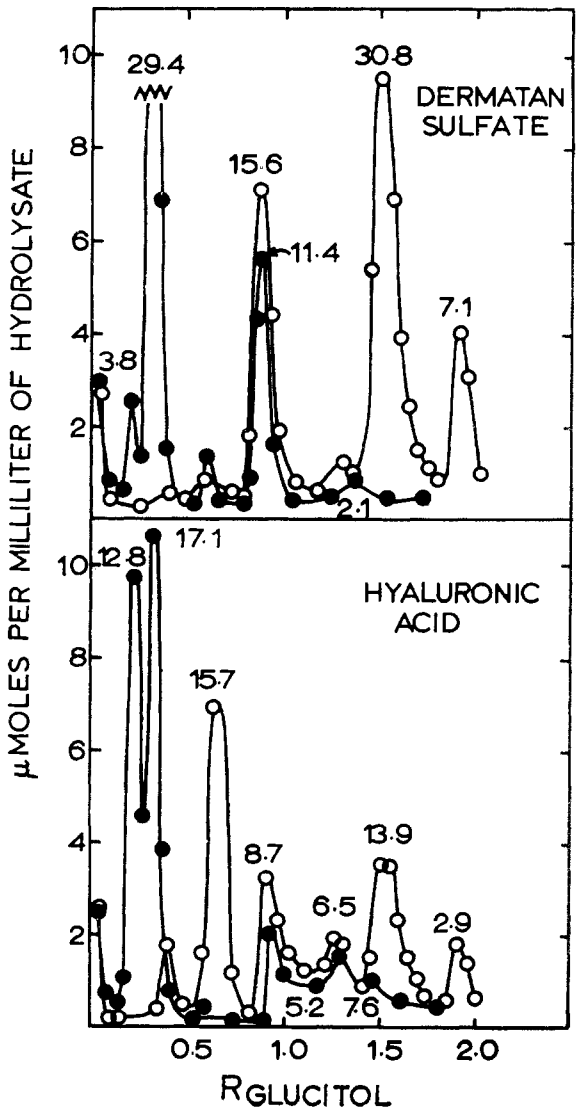

FIGURE 7: Radiochromatographic analysis of dermatan sulfate and hyaluronic acid. See legend to Figure 5.

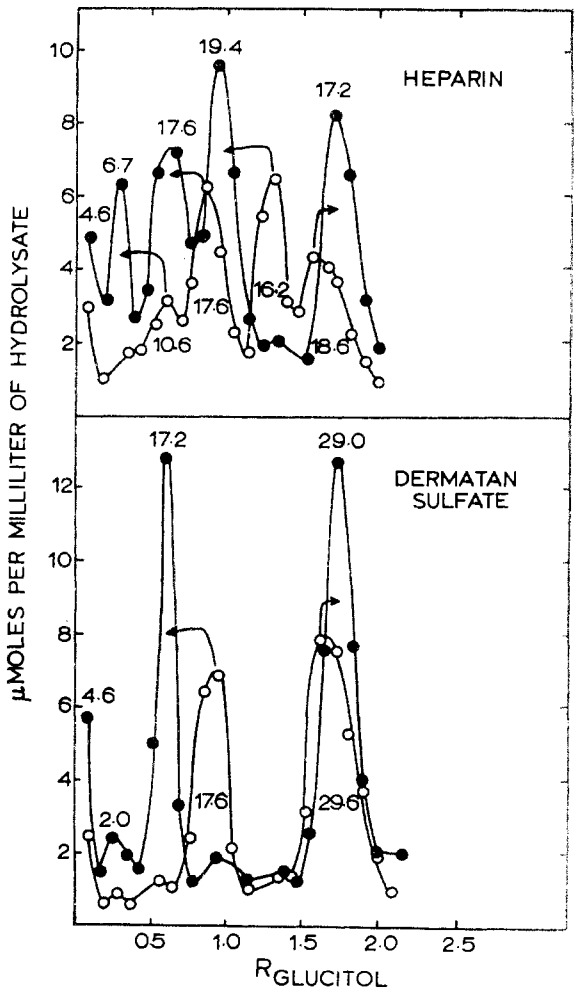

FIGURE 8: Comparison of radiochromatograms of hydrolyzed (24 hr) and deaminated heparin and dermatan sulfate on paper (-○-○-) and DEAE-cellulose (-●-●-) strips. Numbers directly above, or below, each peak indicate the total micromoles of that component per milliliter of hydrolysate and serve to identify the components which are the same on the two chromatograms. Arrows show shifts in migration rates.

pyridine–water (8:2:1), only the deamination peaks migrate significantly, while the components having R_{Gl} values of 0.6, 0.9, and 1.3 in the acetic acid containing solvent remain at the origin in the pyridine solvent. Thus it is concluded that the R_{Gl} 1.3 component so prominent in the heparin and heparan sulfate profiles is an acidic reducing sugar distinct from D-glucuronic, D-galacturonic, or L-iduronic acid.

Quantitation of the Components of Glycosaminoglycuronans. For obtaining quantitative values, the number of moles of amino sugar in a polymer is obtained by taking the sum of the total molar equivalents of deamination products plus 1 mole for each mole of disaccharide. Total uronic acid is taken as the sum of the moles of free uronic acid plus 1 mole for each mole of disaccharide. In neither case are any corrections made for loss on hydrolysis. From the data in Table III, it is seen that the radiochromatographic method gives hexosamine values very close to the Elson–Morgan analyses for the chondroitin sulfates and for heparin and heparan sulfate, but marked deviations are observed for dermatan sulfate and for hyaluronic acid. The radiochromatographic values for hexuronic acid are similar to those obtained by the carbazole method but are somewhat lower. Again, the dermatan sulfate is an exception, but it is known that the carbazole method gives low values for iduronic acid (Hoffman *et al.*, 1956). In the present work the progress of the glycosaminoglycuronan depolymerization was followed at intervals throughout the hydrolysis period. The values obtained for hexosamine and hexuronic acid at the end of 24 hr were equivalent to the maximum values attained in the hydrolysis with the single exception of the uronic acid from dermatan sulfate. This value was only 56% of the maximum value which was observed at 3 hr. It appears, therefore, that the iduronic acid in dermatan sulfate is much more labile to acid than glucuronic acid which is the major uronic acid in the other polymers. It may be noted that the assignment of the R_{Gl} 1.3 material as a uronic acid, as suggested by its chromatographic behavior, is necessary in order to obtain agreement in the analytical data in Table III. Its alternative assignment—as a previously unobserved deamination product from an amino

sugar—would increase the values for hexosamine above the theoretical values in most of the samples and simultaneously give a marked decrease in the value for hexuronic acid.

Molar ratios of hexosamine to hexuronic acid, calculated both from the radiochromatographic data and from the Elson–Morgan and carbazole data, are also given in Table III. In this comparison reasonable agreement in the ratios is observed. The values obtained from the radiochromatographic data are remarkably precise for the accepted structures of the chondroitin sulfates and hyaluronic acid but somewhat off for heparin and heparan sulfate. The ratio of hexosamine to hexuronic acid in dermatan sulfate when calculated by both sets of data is 2 instead of 1 as required by the presently accepted structure for dermatan sulfate. In the colorimetric data this discrepancy is a result of the low color yield for iduronic acid in the carbazole analysis (Hoffman *et al.*, 1966); in the data obtained by radiochromatography the high ratio is due to destruction of approximately one-half of the uronic acid under the hydrolysis conditions. The acid lability of the L-iduronosyl bond has been noted earlier by Cifonelli and Dorfman (1962) and by Wolfrom *et al.* (1969); the latter workers have suggested also that the usual conditions for hydrolysis of heparin have destroyed iduronic acid and thus delayed its recognition as a component of heparin (see Discussion).

Discussion

All of the present methods for quantitation of amino sugars in polymers require their prior depolymerization to monosaccharides. As described by Johansen *et al.* (1960) the usual conditions for hydrolysis of amino sugar containing polymers are chosen to take advantage of the marked differences in activation energy for hydrolysis of amides and amino sugar glycosides in order to favor reaction a over reaction b (Figure 1), thus maximizing hexosamine release. However, these authors, as well as others (Belcher *et al.*, 1954; Smith and Zwartouw, 1956; Exley, 1957; Boas, 1953; Lee and Montgomery, 1961), find that in the course of complete hydrolysis, 10–20% of the amino sugar is destroyed. Under the relatively mild depolymerization conditions used in the present work the [^{14}C]glucose added as an internal standard at the beginning of hydrolysis is recovered quantitatively in a symmetrical peak on radiochromatograms even after hydrolysis at 100° for 24 hr. Thus, destruction of sugars *via* reactions at their reducing groups would appear to be minimal. This is substantiated further for amino sugars (1) by the accumulation and disappearance patterns of intermediates and products in the hydrolysis of model glycosides and (2) by measurement of total recovery of products. Both observations indicate that the rate of destruction of amino sugars under these conditions is insignificant. The analytical values for uronic acid content of chondroitin 4-sulfate, heparin, and heparan sulfate are very close to the calculated values, indicating that in these polymers, at least, D-glucuronic acid and L-iduronic acids are quite stable to acid. In following the progress of these hydrolyses at 1-hr intervals, we have observed that, except in the case of dermatan sulfate, the value for uronic acid content at 24 hr is identical with the value obtained after 8–10 hr, at which time the uronic acid released by nitrous acid reaches a maximum.

The use of lower acid concentrations, in addition to minimizing destruction, results in two further possible advantages in the study of amino sugar containing polymers. By favoring selective N deacylation the milder acid hydrolysis results in a much greater difference in the percentage of amino sugar glycosidic bonds hydrolyzed in the α- and β-linked compounds. Consequently, determination of the final yield of amino sugar before treatment with nitrous acid and after (*i.e.*, deamination products) can serve as an indicator of anomeric configuration of the amino sugar glycoside. In addition, it is anticipated that, if such selective N deacylation is followed by nitrous acid cleavage, it may be possible to obtain from amino sugar containing polymers oligosaccharides not obtainable by other approaches.

It has been recognized that the deamination of free amino sugars yields a mixture of products in which the major product is an anhydrohexose (Shafizadeh, 1958). The unanticipated result in this work is that with the α- and β-linked 2-amino-2-deoxy-D-glucosides grossly different chromatographic profiles are obtained. This result is observed with the simple model compounds but not with complex polymers containing α-linked 2-amino-2-deoxy-D-glucosides. Furthermore, the deamination profiles from the hydrolyzed mucopolysaccharides showed qualitative differences from each other and from the model compounds. Clearly, this reaction with amino sugars is quite complex and its course is affected by structural features in a way that is poorly understood. The variability of the composition of the deamination mixture found with model compounds suggests that use of nitrous acid in structural studies to obtain oligosaccharides may yield a *mixture* of oligosaccharides upon cleavage of a polysaccharide at any given position. The products of these reactions are not identified but apparently are all at the aldehyde or ketone oxidation level.

The radiochemical approach in the study of amino sugars offers a sensitivity and a simplicity of operation similar to that of the colorimetric methods and avoids a number of the difficulties. It is recognized that the colorimetric reactions yield mixtures of chromophores, the compositions of which are highly dependent on the structures of the sugars present and the nature of other components in the samples. The [^{3}H]borohydride, on the other hand, reacts quite specifically with reducing carbohydrates in the presence of most other natural products and gives identical molar ^{3}H incorporation into all reducing carbohydrates. Thus, free and acetylated amino sugars, uronic acids, and neutral sugars can be measured simultaneously with a minimum of sample manipulation, using one set of reagents and a single sugar standard to determine the specific activity of the [^{3}H]borohydride. Analyses by this method require prior hydrolysis of polymeric carbohydrates and are therefore subject to the difficulties associated with quantitative, nondestructive depolymerization. With the [^{3}H]-sodium borohydride used in this study there was a very low-background level of ^{3}H along the chromatogram (50–150 cmp/0.5-in. segment); however, if the specific activity of the [^{3}H]borohydride is increased tenfold to increase the sensitivity of the method, backgrounds may increase to 500–1500 cpm and corrections for background must be applied. Some of the glycitols are poorly separated on the chromatogram and so cannot be determined individually. For example, we have not been able to separate D-glucuronic and L-iduronic acids in this work. Thus, we have not considered the question of the possible epimerization of D-glucuronic acid to L-iduronic acid in the deamination reaction (Yamauchi *et al.*, 1968) and have not shed any light on the relative amounts of these two uronic acids in heparin (Wolfrom *et al.*, 1969; Perlin *et al.*, 1969).

The inclusion of a ^{14}C internal standard in the hydrolysis, confined to [^{14}C]glucose in this work, can be extended to include mixtures of ^{14}C-labeled D-glucose, 2-amino-2 deoxy-D-glucose, and/or D-glucuronic acid in any hydrolysate to obtain an accurate evaluation of the relative losses of individual components as hydrolysis proceeds.

The glycosaminoglycuronan data reported here present several interesting points. First, the radiochromatographic profiles before and after deamination are uniquely characteristic for each polymer and serve to distinguish each polymer (with the exception of the chondroitin sulfates, which give practically identical profiles) from all of the others. Also, the data permit an approximation of the percentages of the total hexosamine and hexuronic acid bonds which are cleaved in the hydrolysis. The percentage of intact hexuronosyl bonds *remaining* after the hydrolysis period is equal to the percentage of hexuronic acid which remains in the disaccharide after nitrous acid treatment. Similarily, the percentage of intact hexosaminyl bonds *remaining* after the hydrolysis period is represented by the deamination products in excess of those formed from the free hexosamine present before deamination (this gives exact values when the final hydrolysate contains only monosaccharides and the two possible disaccharides; when there are significant amounts of higher oligosaccharides present before deamination, as for heparin and heparan sulfate, only a minimum value for per cent intact hexosaminyl bonds remaining can be calculated). These calculations show that after hydrolysis in 1 N sulfuric acid at 100° for 24 hr, the percentages of total bonds *hydrolyzed* in chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, hyaluronic acid, heparin, and heparan sulfate, respectively, are, for hexosamine: 77, 93, 82, 100, <21, and <45, and, for hexuronic acid: 49, 40, >90, 49, 85, and 63. These values reflect the relative stabilities of these glycosidic bonds in the different polymers and are consistent with the observations on these polymers in the literature and with the data obtained here with model compounds.

The final question raised by these data is that of the nature of the acidic R_{Gl} 1.3 component so prominent in the profiles of heparin and heparan sulfate. This component is present even in the early stages of hydrolysis and, as hydrolysis progresses through the later stages, it does not appear to be formed at the expense of any of the end products or intermediates. Although the analytical data indicate that it is a part of the acid fraction of these polymers, it appears not to be a degradation product of D-glucuronic acid as indicated by its relative prominence in heparin and heparan sulfate, in contrast to the other polymers; however, this point requires further examination since the effects of various linkage types on the mode of degradation are poorly understood. On the other hand, its virtual absence in dermatan sulfate indicates that it is not a degradation product from L-iduronic acid. Furthermore, it is present in small amounts before nitrous acid treatment and not an artifact arising in the deamination reaction. It is an unknown which may be a *bona fide* constituent of these polymers.

Acknowledgments

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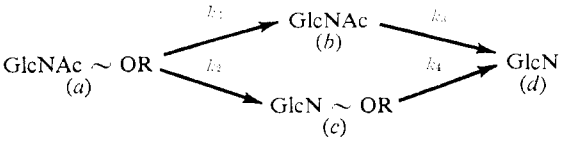
Appendix

Calculation of Rate Constants for Hydrolysis of 2-Acetamido-2-deoxy-D-glucosides.

I. Derivation of Equations.



$$-\frac{d(a)}{dt} = (k_1 + k_2)(a) \quad (1)$$

$$\frac{\ln (a)}{(a_0)} = -(k_1 + k_2)(t) \quad (2)$$

$$\frac{(a)}{(a_0)} = e^{-(k_1 + k_2)t}; \quad (a) = (a_0)e^{-(k_1 + k_2)t} \quad (3)$$

$$\frac{d(c)}{dt} = k_2(a) - k_4(c) \simeq k_2(a) \quad (4)$$

substituting eq 3 into eq 4

$$\frac{d(c)}{dt} = k_2(a_0)e^{-(k_1 + k_2)t} \quad (5)$$

$$(c) = \frac{k_2(a_0)}{k_1 + k_2}[1 - e^{-(k_1 + k_2)t}] \quad (6)$$

$$(c) = \frac{k_2}{k_1 + k_2}[(a_0) + (a)] \quad (7)$$

II. Calculation of Rate Constants k_1 and k_2.

A. A plot of ln (a)/(a_0) *vs.* t (eq 2) gives a line with slope $= -(k_1 + k_2)$, where a_0 and a are the concentrations of GlcNAc~OR at times 0 and t.

B. A plot of (c) *vs.* $[(a_0) - (a)]$ for each t (eq 7) gives a line with slope $= k_2/(k_1 + k_2)$, where (c) is the concentration of GlcN~OR and (a_0) and (a) are as defined above.

C. Solution of the simultaneous equations from A and B gives k_1 and k_2.

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