g. (43%) of a crystalline product (m.p. 110–111°) was obtained from absolute ethanol. After two recrystallizations from absolute ethanol (initially dissolved in dichloromethane, which was removed by boiling), the pure substance melted at 111–112° and showed no appreciable rotation ($[\alpha]^{20}D$ + 0.2°) in chloroform (*c*, 1.8). The absence of the hydroxyl group was demonstrated by the lack of absorption in the region of 3600 cm.$^{-1}$ and the recovery of 86% of the starting material when it was treated with *p*-nitrobenzoyl chloride–pyridine for 65 min. at room temperature.

Anal. Calcd. for $C_{18}H_{13}NO_8S$ (403.4): C, 53.60; H, 3.25; N, 3.47; S, 7.95. Found: C, 53.74, 53.78; H, 3.47; 3.47; N, 3.35; S, 7.88, 7.95.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, PURDUE UNIVERSITY]

Alkaline Degradation of Amino Sugars[1]

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2-Acetamido-2-deoxy-D-glucose with a glycosidic linkage at C-4 is transformed by the action of saturated lime water into calcium D-isosaccharinate which is the same product obtained from other 4-*O*-substituted D-hexoses. The D-isosaccharinate is isolated and identified as "α"-D-isosaccharino-1,4-lactone. Sodium hyaluronate is also degraded with the production of acids in saturated lime water.

Recently, there has been great interest in animal and bacterial polysaccharides, some of which may come in contact with alkaline solutions during their isolation or purification. Many of these substances contain amino sugars (frequently *N*-acetylated). There is increasing interest in the commercial use of deacetylated chitin, prepared by treating chitin with hot, concentrated alkaline solutions. It is, therefore, of interest to ascertain the effect of alkaline solutions on *O*-substituted 2-acetamido-2-deoxyaldoses and 2-amino-2-deoxyaldoses. As used herein, "*O*-substituted" will indicate that the substituent group is alkyl or glycosyl but not acyl.

2-Amino-2-deoxy-D-glucose readily undergoes autoxidative degradation in aqueous solution by reactions which are not fully understood but which are accelerated in alkaline solutions.[2] The kinetics of this reaction have been determined.[3] The presence of an *N*-acetyl group stabilizes the molecule so that 2-acetamido-2-deoxy-D-glucose undergoes little, if any, autoxidative degradation in water solutions.[2] In dilute aqueous base at room temperature, 2-acetamido-2-deoxy-D-glucose rapidly undergoes epimerization and becomes equilibrated with 2-acetamido-2-deoxy-D-mannose (and vice versa) in proportions of 2–4:1, respectively.[4–7] Likewise, 2-acetamido-2-deoxy-D-ribose equilibrates with 2-acetamido-2-deoxy-D-arabinose under the same conditions.[8] The facility of these equilibria had been attributed to the inductive effect of the acetamido group,[9] and their existence is an indication of the presence of enol structures such as III.

4-O-Substituted 2-acetamido-2-deoxy-D-glucose. Little is known of the action of alkaline solutions on *O*-substituted amino sugars. It has been reported that 3-*O*-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-glucose is easily degraded to D-galactose and an unidentified product in dilute sodium carbonate solution and that, under the same conditions, 4-*O*-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-glucose is stable.[10] If these disaccharides are degraded by a mechanism similar to that advanced for other *O*-substituted aldoses,[11,12] it would be expected that the former disaccharides would degrade more rapidly than the latter, but both should be labile.

As demonstrated by this work, *N*-acetylated chitotriose, which is a 4-*O*-substituted 2-acetamido-2-deoxy-D-glucose (I), is degraded in 0.04 *N* calcium hydroxide solution with the formation of

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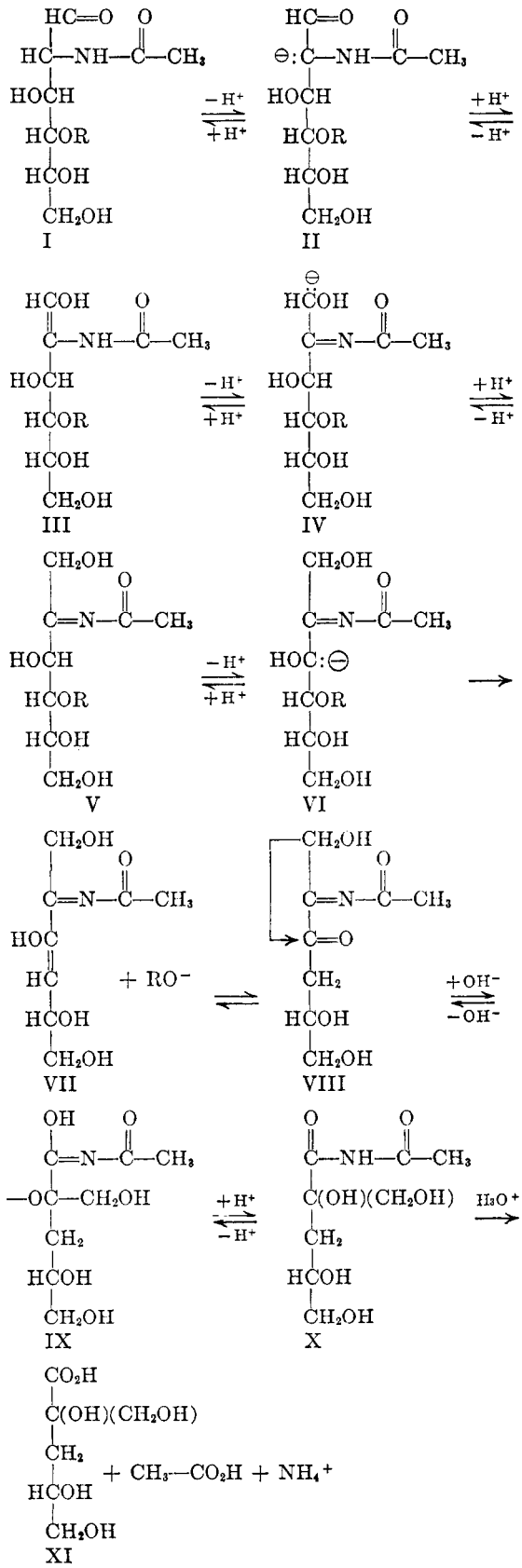
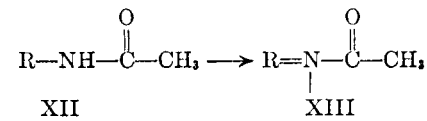


D-isosaccharinate, a product characteristic of 4-*O*-substituted D-hexoses. Its rate of degradation (Table I) approximates closely those of other β-D-(1→4)-linked oligosaccharides containing D-glucose or D-fructose reducing endunites, such as lactose,[13] lactulose,[14] cellobiose,[15] cellobiulose,[15] and cellotetraose.[15] A mechanism has been proposed for the formation of D-isosaccharinates (XI) from 4-*O*-substituted 2-acetamido-2-deoxy-D-glucose in which the penultimate compound (X) is an imide. Imides are easily hydrolyzed to an acid and an amide so that the products of this reaction would be D-isosaccharinic acid and acetamide or D-isosaccharinamide and acetic acid. "α"-D-Isosaccharinamide is known to be unstable in warm water,[16] and either amide should be hydrolyzed when the solution is acidified. The reaction VIII→X as written is analogous to a benzilic acid rearrangement. It is unlikely that the acetyl group is removed during the early steps of this reaction since 2-acetamido-2-deoxy-D-glucose is one of the final reaction products. However, it is possible that the nitrogen atom will loose a proton (XII→XIII) as occurs during the alkaline hydrolysis of proteins[17]; such a reaction would undoubtedly lead to a splitting of the carbon chain in the case of 4-*O*-substituted 2-acetamido-2-deoxy-D-glucose.



That 2-acetamido-2-deoxy-D-glucose is an early reaction product is not surprising since it would be expected that the rates of degradation of tri-*N*-acetylchitotriose and di-*N*-acetylchitobiose would be almost equal. D-Isosaccharinate is also formed from deacetylated chitotriose, but considerably more coloration occurs.

3-O-Substituted 2-acetamido-2-deoxy-D-glucose. In hot, dilute sodium carbonate solution, 2-acetamido-2-deoxy-D-glucose is transformed into a substance which gives a direct positive test with the Ehrlich reagent (*N*,*N*-dimethyl-*p*-aminobenzaldehyde) used in the Morgan-Elson test.[18] The nature of this substance has been investigated by several workers. White[19] gave evidence to support his contention that this substance is "glucoxazoline," although there has been some question of the purity of his material. Kuhn and Krüger,[20] using modern chromatographic and spectrophoto-

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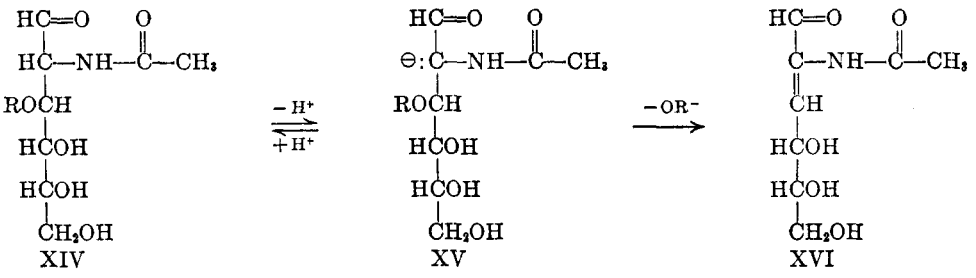

metric methods, separated the "chromogen" into three components and proved the structure of chromogen III to be that of D(+)-3-acetamido-5-(1,2-dihydroxyethyl)furan. Treatment of 2-acetamido-2-deoxy-D-glucose with dilute sodium carbonate solution at room temperature does not, however, produce a chromogen which will react with the Ehrlich reagent.[21]

Kuhn and co-workers[10,21] reported that the treatment of 2-acetamido-2-deoxy-3-*O*-methyl-D-glucose and 3-*O*-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-glucose with dilute sodium carbonate solution at room temperature yields D-galactose and "anhydro-*N*-acetyl-D-glucosamine." It was inferred that this "anhydro-*N*-acetyl-D-glucosamine" is the chromogen obtained from the treatment of 2-acetamido-2-deoxy-D-glucose with hot alkaline solutions. Wolfrom and Juliano[22] treated 3-*O*-β-D-glucopyranosyl-2-acetamido-2-deoxy-D-galactose with 0.04 *N* sodium carbonate at room temperature and obtained D-glucose and a Morgan-Elson reactive sugar, "anhydro-*N*-acetyl-D-galactosamine."

Since it has already been shown here that 2-acetamido-2-deoxy sugars behave in alkaline solutions quite similarly to other aldoses and ketoses, the cleavage of the glycosidic linkages observed in the case of 3-*O*-substituted 2-acetamido-2-deoxyaldoses probably occurs by the reaction sequence XIV→XVI. 2-Acetamido-2-deoxy-D-glucose (XIV. R = H) or its 3-*O*-substituted derivative (XIV. R = CH_3 or glycosyl) would form the compound XVI which apparently is sufficiently stable in dilute bases to allow it to be cyclized to the furanose form and dehydrated to form chromogen III.

Hyaluronic acid is composed of repeating units of 3-*O*-(β-D-glucopyranosyluronic acid)-D-glucopyranose in (1→3) linkages.[23] *O*-Substituted uronic acids are degraded by alkaline solutions in the normal manner.[24] Likewise, 3-*O*-substituted 2-acetamido-2-deoxyaldose units in alkaline solutions eliminate the glycosyloxy group at C-3 and expose a new reducing end group. Therefore, hyaluronic acid should undergo alkaline degradation by a peeling process which begins at the reducing end. The rate of the alkaline degradation of hyaluronic acid is given in Table II. No products were identified in this work; it is evident, however, that hyaluronic acid is degraded in alkaline solutions and that exposure to alkaline solutions should be avoided during its isolation.

EXPERIMENTAL

Chitin. Chitin was prepared from lobster shells. Clean, dried lobster shells (500 g.) were ground and soaked in 10% oxygen-free sodium hydroxide solution for 3 days at room temperature, the alkaline solution being changed every day. The deproteinized particles were then washed with water until free of alkali. The particles were triturated with 95% ethanol and filtered until the filtrate was colorless, a total of 6 l. of 95% ethanol being used in this operation. The particles were then triturated with the following organic solvents in small portions and filtered: acetone (1 l.), absolute ethanol (2.5 l.), ether (500 ml.). The nearly colorless particles were dried under reduced pressure. They were then put into 37% hydrochloric acid at −20°, and the mixture was kept 4 hr. at this temperature. The swelled particles were separated by centrifugation at 0° and washed with water at 0° until the washings were acid-free. After washing with several changes of 95% ethanol, absolute ethanol, and ether in that order and drying under reduced pressure, the acid treatment was repeated; yield of chitin about 100 g., sulfated ash 0.15%, N 7.1% (theoretical 6.9%).

Tri-N-acetylchitotriose. Tri-*N*-acetylchitotriose was prepared by the method of Stacey and co-workers.[25] Final purification was by paper chromatography using the upper phase of 1-butanol–ethanol–water–ammonium hydroxide (40:49:10:1 v./v.) as the irrigant; decomp. point 304–306°, $[\alpha]_D^{25}$ +2° (at equilibrium; *c* 1.0, water); the physical constants are in agreement with those reported.[25]

Alkaline degradation of tri-N-acetylchitotriose. Tri-*N*-acetylchitotriose (197 mg.) was dissolved in 100 ml. of oxygen-free, 0.0435 *N* calcium hydroxide solution at 25°. One milliliter aliquots were removed at intervals and passed through a column containing 2.3 ml. of Amberlite IR-120-(H^+) cation-exchange resin. The resin was washed with 10 ml. of water, and the acids were determined by back titration with 0.0103 *N* sodium hydroxide to the first semipermanent end point with phenolphthalein according to the method of Corbett and Kenner.[15] The results are found in Table I.

The resin was then washed with 2 *N* hydrochloric acid, and the combined washings were concentrated under reduced pressure and investigated by paper chromatography using ethyl acetate–pyridine–water (10:4:3 v./v.) as the

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TABLE I

Production of Acids from *N*-Acetylated Chitotriose in Saturated Lime Water at 25°

Time, Hr.	Meq. of Acid	Meq. of Acid/ Mmoles of Sugar
2	0.0027	0.69
3	0.0035	0.90
13	0.0043	1.10
24	0.0045	1.15
48	0.0049	1.26
102	0.0065	1.67
144	0.0072	1.85
240	0.0087	2.24
288	Temperature increased to 50°	
312	0.0119	3.06
336	0.0160	4.10

irrigant. "α"-D-Isosaccharino-1,4-lactone, di-*N*-acetylchitobiose, and 2-acetamido-2-deoxy-D-glucose were detected early in the reaction. In the later stages, the products were "α"-D-isosaccharino-1,4-lactone and 2-acetamido-2-deoxy-D-glucose; only traces of tri-*N*-acetylchitotriose and di-*N*-acetylchitobiose remained after 312 hr. No other reaction products were indicated with the silver nitrate–sodium hydroxide reagents of Trevelyan and co-workers.[26]

The solution remaining after 336 hr. was treated with Amberlite IR-120(H^+) cation-exchange resin and concentrated under reduced pressure to a sirup which was extracted with hot ethyl acetate. Upon evaporation of the extract, crystals of "α"-D-isosaccharino-1,4-lactone were deposited; yield 15 mg., m.p. 96° (reported,[27] m.p. 95–96°).

Alkaline degradation of chitotriose. Chitotriose was prepared by the method of Stacey and co-workers.[25] Following paper chromatography using 2-pentanol–pyridine–water (1:1:1 v./v.) as the irrigant, the fraction corresponding to chitotriose was lyophilized. Alkaline degradation of this material and subsequent investigations by the procedures described above showed "α"-D-isosaccharinic acid to be the main acid present; some colored substances were also formed.

Sodium hyaluronate. Sodium hyaluronate was prepared by a modification of the procedure of Meyer and co-workers.[28] Human umbilical cords which had been stored in acetone were rinsed with distilled water and cut into 2-cm. segments. The segments were blended in a mechanical food blender with as little water as necessary to make a thin slurry (about 500 ml.). The slurry was transferred to a 1-l. Erlenmeyer flask and acidified to *p*H 2.0 with 6 *N* hydrochloric acid. One gram of crystalline pepsin was added with stirring, and the mixture was covered with toluene and placed in a constant temperature bath at 37° with slow agitation. The *p*H was occasionally adjusted to 2.0. After 24 hr., the *p*H was brought to 7.9–8.0 with 30% sodium hydroxide solution. One gram of "ichtozyme" (a product of Enzyco, Inc., 486 California Street, San Francisco 4, Calif.) was added with stirring, and the mixture was dialyzed in a constant temperature bath at 45° saturated with chloroform and toluene. The water was changed every day, and, after 3 days, the contents of the dialysis tubing were nearly clear. The dialyzed solutions were filtered through folded filter paper (J. Green No. 788 1/2, available from Arthur H. Thomas Co., Philadelphia, Pa.), and the residue was washed with a small amount of water. The filtrate was then treated by a procedure similar to that used by Sevag;[29] a mixture of 3.3 l. of chloroform, 1.7 l. of 1-pentanol, and 1 l. of an aqueous solution containing 160 g. of glacial acetic acid and 300 g. of anhydrous sodium acetate was mixed with the filtrate in a ratio of 5:6 (v./v.). The resulting mixture was shaken about 20 hr. and then centrifuged in 250 ml. bottles at 3500 r.p.m. for 30 min. The top layer was siphoned off and immediately placed in a refrigerator. The middle and lower layers were poured into a separatory funnel. After a few minutes, the bottom layer was discarded, and the middle layer was recentrifuged at 35 r.p.m. for 30 min. The top layer was again siphoned off and cooled. Two volumes of a saturated solution of anhydrous sodium acetate in 95% ethanol were added, and the mixture was mixed thoroughly and kept 24 hr. at 0°. When the polysaccharide was well precipitated, it was collected by centrifugation and dissolved in water. The solution was dialyzed for 2 days against frequent changes of distilled water. The solution was then filtered through a Supercel[30] mat in a medium fritted-glass funnel and lyophilized.

TABLE II

Production of Acids from Sodium Hyaluronate in Saturated Lime Water at 25°

Time, Hr.	Meq. of Acid	Meq. of Acid/Mmoles of Sodium Hyaluronate
0	0.0064	0.32
20	0.0083	0.42
116	0.0096	0.48
164	0.0119	0.60
212	0.0121	0.61

Anal. Calcd. for $C_{14}H_{21}NO_{11}$ Na(sodium hyaluronate): N, 3.5; sulfated ash, 18. Found: N, 3.5; sulfated ash, 19.

Alkaline degradation of sodium hyaluronate. Sodium hyaluronate (400 mg.) was dissolved in 100 ml. of oxygen-free 0.0435 *N* calcium hydroxide at 25°. Aliquots were taken, and the total acid production was determined as previously described. The results are found in Table II.

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