1 g.), was dissolved in 30 ml. of dry methanol, cooled to 0°, a small piece of sodium added, and the whole left in the ice-box overnight. A yellow amorphous precipitate formed which was separated by centrifugation, washed twice with dry methanol and dried; yield 0.7 g. This material was dissolved in 50 ml. of hot methanol and decolorized with carbon. The material in the filtrate was precipitated by the addition of acetone. The precipitate was redissolved in methanol and reprecipitated with acetone; yield 0.31 g. (49.5%) of a white powder, m.p. 148–150°, $[\alpha]^{27}D$ +52.1° (*c* 2, water).

Anal. Calcd. for $C_6H_{12}O_8NNaS$: C, 25.6; H, 4.3; N, 4.9; S, 11.4; Na, 8.2. Found: C, 25.9; H, 4.4; N, 4.8; S, 11.2; Na, 8.4.

Methyl 2-Deoxy-2-sulfoamino-α-D-glucopyranoside, Sodium Salt Monohydrate (XIII).—The crude reaction product (5 g.) from the above-described sulfation of VIII, was dissolved in 50 ml. of dry ethanol and deacetylated overnight in the cold (0–4°) in the presence of a small amount of sodium ethoxide. An amorphous precipitate formed which was separated by centrifugation, washed with ethanol until the washings were neutral, taken up in 50 ml. of dry methanol and an equal volume of dry ethanol added. A precipitate formed which was removed by centrifugation, and the supernatant was evaporated at low pressure and at room temperature to crystallization. The material was recrystallized from aqueous ethanol; yield 1.02 g. (30%), m.p. 159–161°, $[\alpha]^{27}D$ +103.1° (*c* 2.1, water). The material appears to be a monohydrate.

Anal. Calcd. for $C_7H_{14}O_8NNaS \cdot H_2O$: C, 26.8; H, 5.1; N, 4.5; S, 10.0; Na, 7.3. Found: C, 26.75; H, 4.9; N, 4.5; S, 9.8; Na, 7.6.

Methyl Mono-*O*-acetyl-2-amino-2-deoxy-α-D-glucopyranoside Hydrochloride.—Methyl tri-*O*-acetyl-2-amino-*N*-(benzoxycarbonyl)-2-deoxy-α-D-glucopyranoside (II, 8.3 g.) was mixed with 1 g. of a palladium-on-charcoal catalyst and added to 175 ml. of dry methanol containing 0.025 equivalent (1.2 molar ratio) of dry hydrogen chloride. Hydrogen was bubbled slowly through the solution for 12 hr. The solution was filtered and concentrated to dryness under reduced pressure to give a white solid (4.9 g.) which was dissolved in 20 ml. of ethanol and precipitated with light petroleum. The amorphous solid (4.3 g.) was filtered and was crystallized with difficulty from ethanol–acetone mixtures; m.p. 205–215° dec., $[\alpha]^{25}D$ +113.5° (*c* 2.6, water).

Anal. Calcd. for $C_7H_{15}O_5ClN(CH_3CO)$: C, 39.68; H, 6.68; Cl, 13.05; N, 5.16; CH_3CO, 15.8. Found: C, 39.75; H, 6.66; Cl, 13.17; N, 5.17; CH_3CO, 16.0.

COLUMBUS 10, OHIO

[CONTRIBUTION FROM THE RACKHAM ARTHRITIS RESEARCH UNIT, AND THE DEPARTMENTS OF BIOLOGICAL CHEMISTRY AND BACTERIOLOGY, UNIVERSITY OF MICHIGAN]

The Preparation of Glucosamine Oligosaccharides. I. Separation[1,2]

BY SYLVIA T. HOROWITZ, SAUL ROSEMAN[3] AND HAROLD J. BLUMENTHAL

RECEIVED MAY 6, 1957

Chitosan was partially degraded by the use of hydrochloric acid. The hydrolysate was fractionated by means of ion-exchange chromatography and yielded five discrete peaks. Analysis indicated that the materials in the peaks were glucosamine oligosaccharides which were eluted in order of increasing molecular weight. Procedures are presented for the analysis of glucosamine oligo- or polysaccharides and for large scale gradient chromatography.

In the course of studies in this Laboratory concerned with the biochemistry of chitin, it was desirable to prepare oligosaccharides of glucosamine (2-amino-2-deoxy-D-glucose) and N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose). The preparation of glucosamine oligosaccharides has not been reported previously. The preparation of N-acetylglucosamine oligosaccharides has been attempted by subjecting chitin to acetolysis, or to partial hydrolysis, followed by fractionation of the resulting mixtures by conventional techniques. Acetolysis of chitin[4] was reported to yield the fully acetylated disaccharide, octaacetylchitobiose (1,3,6-triacetyl-2-acetamido-2-deoxy-4-(3,4,6-triacetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-glucose) in 16.2% yield as the crude product. Subsequent partial hydrolysis of chitin[5] followed by fractionation of the chitodextrin mixture and acetylation gave low yields of the fully acetylated disaccharide and trisaccharide. It has been reported recently[6] that saponification of octaacetyl chitobiose with methanolic ammonia yields five products from which crystalline N,N¹-diacetylchitobiose was obtained by chromatography.

This report presents a method for the preparation of glucosamine oligosaccharides. Subsequent studies will deal with the isolation, properties and characterization of certain of these compounds. Although chitin is a polymer of N-acetylglucosamine, the amino groups can be liberated by deacetylation of chitin to chitosan. Partial hydrolysis of the resulting polyhexosamine yields the glucosamine oligosaccharides which are separated by ion-exchange chromatography. The separations achieved in this manner offer the following advantages over the older methods: (1) the free amino sugars can be isolated, (2) the separation appears to be much more efficient, (3) fractions of higher molecular weight than the trisaccharide can be obtained. For present purposes, effort was directed primarily toward the separation of the lower molecular weight materials.

The literature indicates the difficulty in obtaining purified chitin. The usual technique involves repeated treatment of the crude material with hot acid, hot alkali and alkaline permanganate at room temperature to remove colored material. It was found that preparations exhibiting satisfactory analyses could be obtained by treatment of

(1) The Rackham Arthritis Research Unit is supported by a grant from the Horace H. Rackham School of Graduate Studies of the University of Michigan. This investigation was supported in part by a grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health and a grant from the Michigan Chapter, Arthritis and Rheumatism Foundation.

(2) A preliminary report has appeared, *Federation Proc.*, **14**, 740 (1955).

(3) To whom requests for reprints and additional information should be addressed.

(4) M. Bergmann and E. Silberkweit, *Naturwiss.*, **19**, 20 (1931); *Ber.*, **64**, 2436 (1931).

(5) L. Zechmeister and G. Tóth, *ibid.*, **64**, 2028 (1931).

(6) F. Zilliken, G. A. Braun, C. S. Rose and P. György, THIS JOURNAL, **77**, 1296 (1955).

the crude material with concentrated formic acid which rapidly extracts colored impurities.

The chromatographic method used involved a gradient technique which employed 19 liter carboys for the reservoirs. The problem of maintaining a constant flow rate independent of hydrostatic pressure in the feed reservoir was solved by the use of a simple constant level siphon.

Finally, it was necessary to design a colorimetric method for the detection of the oligosaccharides and of chitosan. A modified anthrone procedure was developed which is sensitive, specific, and appears to offer many advantages over other procedures which have been used for the analysis of chitosan. It is possible to determine chitosan in the presence of polysaccharides which contain N-acetylhexosamine such as hyaluronic acid, chondroitin sulfate and chitin.

Experimental

Preparation of Chitin.—The polysaccharide was prepared from crab or lobster shell powder either by the standard method[7] (the acid and alkali treatments were repeated at least three times each) or by the procedure described below.

Decalcified powdered lobster shell (10 g.) was mechanically shaken for 18 hours with 100 ml. of concentrated formic acid solution (90%, Anal. Reag.). The supernatant solution turns brown within an hour. The mixture was filtered and the residue was washed with water. The precipitate was treated with 500 ml. of 10% sodium hydroxide solution on a steam-bath for 2.5 hours, filtered and washed with water. The white residue (60 to 70% yield) was dried *in vacuo* in a desiccator and gave the following analyses.

Anal.[8] Calcd. for $(C_8H_{13}O_5N)_n$: N, 6.90; $COCH_3$, 21.2. Found: N, 6.95; $COCH_3$, 19.2.

From the acetyl:nitrogen ratio it appears possible that some deacetylation may have occurred during the formic acid treatment. Time studies were not performed to determine whether the 24-hour extraction period could be shortened to avoid the possibility of deacetylation. The product was satisfactory for the preparation of chitosan.

Preparation of Chitosan.—Chitin (30 g.) was converted to chitosan by fusion with potassium hydroxide pellets (150 g.) in a nickel crucible under nitrogen.[9] The temperature of the stirred melt was maintained at 180° for 30 minutes. The melt was poured cautiously into ethanol and the gelatinous precipitate was washed with ethanol followed by water until the washings were neutral. Purification of the chitosan was effected by dissolving it in 5% acetic acid and reprecipitating the gel with dilute alkali. The purification step was repeated three times. The crude chitosan was converted to the hydrochloride salt[10] by dissolving the gel in 0.1 *N* hydrochloric acid at about 50°, adding concentrated hydrochloric acid until precipitation was complete, and cooling to room temperature. The salt was dissolved in the minimum quantity of boiling water and concentrated hydrochloric acid was added to the cooled solution until precipitation was complete. The mixture was heated until the solid dissolved, the solution was cooled in a Dewar flask and the salt was collected by filtration. After repeating the purification step twice, the precipitate was washed with ethanol, ether, and dried *in vacuo* over sulfuric acid (yield 15 g.).

Anal.[8] Calcd. for $(C_6H_{11}O_5N,HCl)_n$: N, 7.09; $COCH_3$, 0. Found: N, 6.63; $COCH_3$, 0.94.

After "recrystallizing" the hydrochloride 12 times by the described procedure,[10] it yielded the following analyses.

Anal.[8] Calcd. for $(C_8H_{11}O_5N, HCl)_n$: N, 7.09; $COCH_3$, 0. Found: N, 7.05, 7.00; $COCH_3$, 0.38.

In the purified sample, the acetyl value closely approaches the blank so that the acetyl content can be considered either a trace or insignificant. The change in analyses on purification indicates the heterogeneity of the crude chitosan. The chitosan prepared in this manner is almost free of N-acetyl group. It is usually held that approximately half of the amino groups of chitosan are acetylated.[11] The acetyl determination used here is the most reliable method available for this type of compound. The acetyl values reported for other preparations of chitosan may deserve re-examination by the chromic acid procedure.

Preparation and Chromatography of Oligosaccharide Mixture.—The purified chitosan hydrochloride (5.0 g.) was dissolved in 100 ml. of water, heated to 53°, and placed in a polyethylene bottle containing 900 ml. of concentrated hydrochloric acid at 53°. The sealed container was maintained at 53° for the desired time. Preliminary experiments demonstrated that a 72 hour hydrolysis under the specified conditions yielded extensive conversion to the monosaccharide while 24 hour hydrolysis gave considerable quantities of the higher oligosaccharides. The optimum time for the preparation of the lower molecular weight oligosaccharides appeared to be 48 hours.

After hydrolysis, the clear, amber colored solution was concentrated to dryness *in vacuo* with the bath temperature at 45°. The dried sample was dissolved in 50 ml. of water, treated once with carbon, and was carefully transferred to the top of a column of Dowex-50, hydrogen form ion-exchange resin,[12] 200–400 mesh, which had been prepared by the usual technique.[13] The glass column dimensions were 7.6 cm. × 61 cm. with a fritted glass disc sealed in the bottom and contained 1 l. of the ion-exchange resin. Water (100 ml.) was used to wash in the sample and this was followed by 10 l. of 0.1 *N* hydrochloric acid at 0.1 atm. air pressure. Gradient A (Fig. 1) was started at this point,

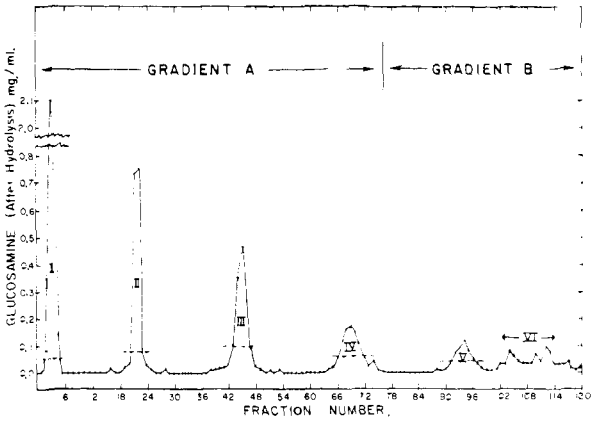

Fig. 1.—Chromatography of chitosan hydrolyzate by gradient ion-exchange technique. Each fraction contained 500 ± 50 ml. The ordinate represents mg. of glucosamine/ml. of fraction after acid hydrolysis with 4 *N* hydrochloric acid.

and was obtained by the continuous addition of 2.16 *N* hydrochloric acid into the mixing reservoir which initially contained 16 l. of 0.500 *N* hydrochloric acid. After Gradient A was complete, the mixing reservoir contained approximately 2.0 *N* hydrochloric acid. Gradient B was obtained by adding 4.15 *N* hydrochloric acid from the feed reservoir into the 2.0 *N* acid. After Gradient B, the column was washed with 6 *N* hydrochloric acid.

(7) H. Pringsheim and A. Steinbroever in (Houben-Weyl) "Die Methoden der Organischen Chemie," G. Thieme, Leipzig, Germany, 1930, 3rd edit., Vol. III, p. 314.

(8) N-Acetyl determinations were performed by a chromic acid procedure, E. Wiesenberger, *Mikrochemie ver Mikrochim. Acta*, **33**, 51 (1948); nitrogen determinations were performed by a Kjeldahl method.

(9) R. Jeanloz and E. Forchielli, *Helv. Chim. Acta*, **33**, 1690 (1950).

(10) O. von Fürth and M. Russo, *Beitr. Chem. Physiol. Path.*, **8**, 163 (1906).

(11) A. G. Richards, "The Integument of Arthropods," University of Minnesota Press, Minneapolis, Minn., 1951, pp. 26–28.

(12) Dowex-50 is a strong cationic exchange resin—a monofunctional, sulfonated, copolymer of styrene and divinylbenzene (Dow Chem. Co., Midland, Mich.).

(13) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192** 663 (1951).

The fraction collector was a moving arm type (Packard Instrument Co., LaGrange, Ill.) operating on a time-index principle. For this device, a constant flow rate through the column was highly desirable. The hydrostatic head in the feed reservoir constantly decreases with resultant decrease in the flow rate. This problem was solved as illustrated in Fig. 2. The reservoirs are 19 liter carboys. The important

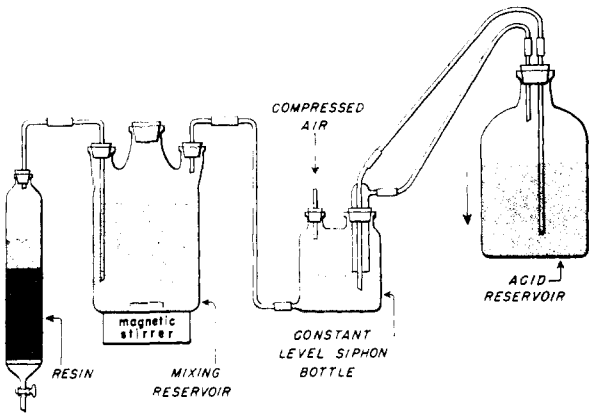

Fig. 2.—Apparatus for large scale gradient elution technique.

part of the apparatus is the "constant level siphon" bottle (usually a 2-l. aspirator bottle with a single neck) which contained a simple automatic siphon and the compressed air inlet. After the resin had packed in the column, and the stopcock was adjusted, the apparatus permitted the collection of 500 ± 50 ml. fractions/hour for extended periods of time.

Within 12 hours after collection, the fractions were chilled to −18° and stored in this manner. After analysis, the desired fractions were pooled to yield the various peaks,[14] excess lead carbonate was added to neutralize the acid, and the mixtures were again stored at −18°.

Detection of Peaks.—The peaks[14] were detected by use of either a modified anthrone procedure or by an acetylacetone method. In either case, the hydrochloric acid present in the samples was removed by drying *in vacuo* over soda lime and calcium chloride.[15] The residues were dissolved in water and aliquots were analyzed.

The anthrone procedure was convenient (requiring no hydrolysis of samples), sensitive and specific. Its disadvantages were (a) the occasional formation of brown colors rather than the desired blue-green and (b) the molar extinction coefficients of the oligosaccharides were not known so that the data could not be quantitated. The method is quite satisfactory for screening many samples. The aliquots (0.2 ml.) were placed in Pyrex colorimeter tubes followed by 1% sodium nitrite solution (0.1 ml.) and 20% acetic acid solution (0.1 ml.). After vigorous shaking, the tubes were allowed to stand for 10 minutes and were then treated with 6% ammonium sulfamate solution (0.1 ml.; Anal. Reag.). Intermittent shaking of the tubes for 5 minutes was followed by the addition of 1.2 ml. of a freshly prepared anthrone reagent (200 mg. of anthrone/100 ml. of concentrated sulfuric acid). Color development proceeded for 30 minutes and the absorbances were determined in the Coleman Colorimeter Model #9 using the 590 mμ filter. It will be noted that glucosamine and its polymers yield no color with the anthrone reagent *per se*. Glucosamine determination by deamination followed by treatment with indole has been used before,[16] a procedure which is subject to certain difficulties. The standard curves obtained with glucosamine and chitosan by the anthrone method are presented in Fig. 3. It is of interest that chitosan yields more color than does glucosamine.

The hexosamine technique is a variation of that described by Boas.[17] In the present studies, hydrolysis was performed in sealed Pyrex test-tubes with 4 *N* hydrochloric acid solution for 30 hours at 100°. The hydrochloric acid was removed as described above, the samples were dissolved in water and analyzed *directly* with the acetylacetone reagent. The necessity for prolonged hydrolysis is indicated by a time study with chitosan (Fig. 4).

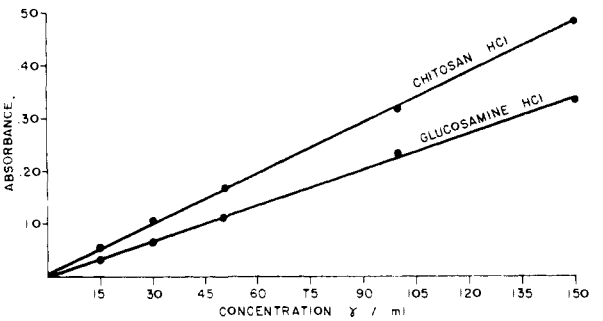

Fig. 3.—Absorbance *vs.* concentration of chitosan and glucosamine hydrochlorides by modified anthrone method.

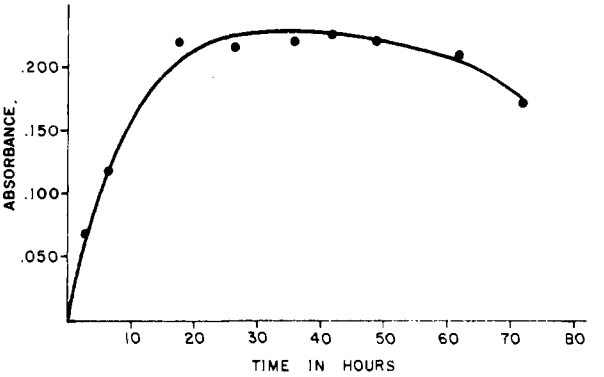

Fig. 4.—Effect of time on hydrolysis of chitosan by 4 *N* hydrochloric acid at 100°.

Results

A typical chromatogram is presented in Fig. 1 where the glucosamine content (after hydrolysis) per ml. of each fraction is plotted against fraction number. The separation of the lower molecular weight oligosaccharides is apparently quite satisfactory. The small peaks between the main fractions, and the multiple peaks after VI were not saved or characterized since they were obtained in such low yield.

Quantitative analysis of the fractions (Table I) demonstrates that 78.6% of the hydrolyzed chitosan placed on the column was recovered in this case. Occasionally, the recovery was as high as 90% which is within the experimental errors of the analytical procedures. When Fig. 1 and Table I are examined, it is clear that the band width of the various peaks increases with order of elution despite the fact that the total quantity of material per peak decreases. Preliminary experiments were conducted to characterize the materials in the various peaks. The pooled fractions were analyzed for nitrogen content and for reducing sugar by a ferricyanide method,[18] and the molar ratios are presented in Table I. Since the reducing equivalent values of the oligosaccharides are unknown, the data must be interpreted with caution. Never

(14) A "peak" is defined as the sequence of fractions where the hexosamine content proceeds from a value close to zero, through a maximum, and back again toward zero.

(15) S. Roseman and I. Daffner, *Anal. Chem.*, **28**, 1743 (1956).

(16) Z. Dische, "Methods of Biochemical Analysis," Vol. 2, Interscience Publishers, Inc., New York, N. Y., 1955, pp. 352-356.

(17) N. F. Boas, *J. Biol. Chem.*, **204**, 553 (1953).

(18) J. T. Park and M. J. Johnson, *ibid.*, **181**, 149 (1949).

theless, it is clear that the materials in the various peaks represent oligosaccharides of increasing molecular weight.

TABLE I

GLUCOSAMINE CONTENT AND END-GROUP ANALYSIS OF THE PEAKS

Peak	Glucosamine content,[a] g.	% of material placed on column	N/reducing value[b] mol./mol.
I	1.37	27.5	1.03
II	0.822	16.5	1.90
III	.614	12.2	2.90
IV	.434	8.7	4.30
V	.244	4.8	5.40
VI-end	.443	8.9	

[a] After acid hydrolysis (4 *N* HCl for 30 hours at 100°). [b] Reducing value by ferricyanide method[8] with glucosamine as standard.

The material in peak I was characterized as glucosamine hydrochloride by the following criteria: (a) isolation of the crystalline hydrochloride, (b) demonstration by paper chromatography and ionophoresis that the compound was homogeneous, (c) conversion to a crystalline derivative, the α-1,3,-4,6-tetraacetyl - N - (*p* - methoxybenzylidene) - D - glucosamine.[19] The derivative melted at 185–186° (uncor.) and did not depress the m.p. of an authentic sample.

An aliquot of the material in peak II was isolated and acetylated by methods to be described elsewhere. The octaacetate so obtained melted at 285–288° (uncor., dec.) and did not depress the dec. point of octaacetyl chitobiose obtained by the acetolysis of chitin.[4] The characterization of the material in peak II is preliminary, however, due in part to the high decomposition point of the acetylated derivative.

From the data offered above it would appear that it is now possible to separate glucosamine oligosaccharides. Although acidic oligosaccharides have been obtained[20] which contain N-acetylglucosamine and glucuronic acid, this is apparently the first report of the separation of some members of a series of positively charge hexosamine oligosaccharides.

Acknowledgment.—It is a pleasure to acknowledge the expert technical assistance provided by Mr. Roy M. Seppala.

(19) M. Bergmann and L. Zervas, *Ber.*, **64b**, 975 (1931).

(20) M. M. Rapport, K. Meyer and A. Linker, THIS JOURNAL, **73**, 2416 (1951); B. Weissmann, K. Meyer, P. Sampson and A. Linker, *J. Biol. Chem.*, **208**, 417 (1954).

ANN ARBOR, MICHIGAN

[CONTRIBUTION FROM THE RESEARCH AND DEVELOPMENT DEPARTMENT, U. S. NAVAL POWDER FACTORY]

Studies on the Decarboxylation of Oxidized Cellulose Acetate through the Formation of the Acid Chloride from the Carboxyl Group and its Reaction with Silver Oxide and Bromine[1,2]

BY F. A. H. RICE AND ARTHUR RUSSELL JOHNSON

RECEIVED APRIL 5, 1957

It has been found that the carboxylic acid groups in oxidized cellulose acetate, oxidized cellulose acetate after reduction with sodium borohydride and oxidized cellulose acetate after reduction and reacetylation can be replaced by acid chloride groups and the acid chloride group can in turn be replaced with bromine by reaction in tetrachloroethane with a mixture of silver oxide and bromine. Treatment of the decarboxylated polymer with sodium borohydride leads to the loss of the halide without any marked decrease in the intrinsic viscosity of the product. Viscosity studies on the products indicate that very little if any depolymerization takes place when reduced reacetylated oxidized cellulose is decarboxylated. The decrease in intrinsic viscosity shown on decarboxylation of oxidized cellulose acetate, however, suggests that chain scission takes place during the treatment of oxidized cellulose acetate containing acid chloride groups with silver oxide and bromine.

In previous communications it was shown that the silver salt of penta-*O*-acetyl-D-gluconic acid would react with bromine to form silver bromide, carbon dioxide and *aldehydo*-1-bromopenta-*O*-acetyl-D-arabinose.[3] Furthermore, it was demonstrated that the silver salt of alginic acid, a polymannuronic acid,[4] would, on treatment with bromine, quantitatively evolve carbon dioxide.[5] Reduction of the product of decarboxylation of alginic acid proved to be extremely difficult and hydrolysis of the product, although it yielded the expected compounds, did so only in very small amounts.[5]

In the course of studies on the decarboxylation of silver salts, it was discovered that an acid could be quantitatively decarboxylated *via* its acid chloride[6] by treating the acid chloride with silver oxide and bromine. It was apparent that this latter reaction would be of particular value for studying acetylated polysaccharides which contain carboxylic acid groups, insofar as it should be possible to transform the carboxylic acid group of the acetylated polysaccharide into the acid chloride and hence substitute by reaction with silver oxide and a halide the carboxylic acid groups in the polysaccharide with the halide. If the halide could be replaced by hydrogen, the over-all reaction would have substituted a pentose for the original uronic

(1) Published with permission of the Bureau of Ordnance, Navy Department. The opinions and conclusions are those of the authors.

(2) Presented in part at the 131st Meeting of the American Chemical Society, Miami, Florida, April, 1957.

(3) F. A. H. Rice and Arthur Russell Johnson, THIS JOURNAL, **78**, 428 (1956).

(4) S. Peat, *Ann. Repts. Progress Chem. (Chem. Soc. London)*, **38**, 150 (1941).

(5) F. A. H. Rice, Abstracts 127th Meeting, Am. Chem. Soc., Cincinnati, Ohio, 1955, p. 11E.

(6) F. A. H. Rice, THIS JOURNAL, **78**, 3173 (1956); F. A. H. Rice and W. Morganroth, *J. Org. Chem.*, **21**, 1388 (1956).