solvent present is believed to be quite constant. However, it may be that formaldehyde oxime, postulated above as a precursor of the cyanide, can arise from the interaction of formaldehyde with one of the reduction products of the nitrogen oxides such as hydroxylamine. The presence of such nitrogenous intermediates is supported by the identification of ammonia as an ignition product at pressures above atmospheric.[15]

The role of the nitrogen oxides in the above processes cannot be ignored; however, their participation in the formation of the initially produced carbon fragments may be negligible compared to their major role in the further oxidation, to carbon monoxide and dioxide, of the entities first formed. This is particularly true of nitric oxide since it is not appreciably reduced to nitrogen at initial pressures below 20 atmospheres.[15]

The above discussion suggests possible modes of formation for the compounds isolated after the ignition of cellulose nitrate. However, except for formaldehyde, the data herein reported do not define either the modes of formation or the position of origin, in the original anhydro-D-glucose units, of the carbon isolated as small organic molecules. Accordingly, we are currently seeking further insight into these matters through the use of tracer techniques.[63]

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, PURDUE UNIVERSITY]

Action of Alkaline Hypochlorite on Corn Starch Amylose and Methyl 4-O-Methyl-D-glucopyranosides[1,2]

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Hypochlorite oxidation of corn starch amylose at 25° and at *p*H 9 and 11 with oxidant levels of 0.5 to 2.0 equivalents per anhydro-D-glucose unit shows that 75 to 88% of the polysaccharide remains non-dialyzable and that up to 27% of the oxidant is used for cleavage of the anhydro-D-glucose residues between carbons C2 and C3. From the hydrolyzate of the oxidized amylose can be isolated D-glucose, D-erythronic acid and its γ-lactone and glyoxylic acid. Likewise, by oxidation of methyl 4-*O*-methyl-β-D-glucopyranoside with sodium hypochlorite at *p*H 9 and 25°, there is obtained 43–47% of glyoxylic acid, isolated as the 2,4-dinitrophenylhydrazone and 10% of glyoxal as the 2,4-dinitrophenylosazone, again showing evidence of preferential oxidation at the C2 and C3 positions of the substituted D-glucopyranosidic ring. The corresponding α-D-glucopyranoside is oxidized similarly but more slowly.

Oxidative reactions are of importance in dealing with all polysaccharides but assume greatest industrial significance with starch and cellulose. Oxidative modification of starch to introduce new properties which widen its industrial application has been long practiced in the starch industry. Bleaching of cellulose pulps to improve whiteness by the destruction of color bodies subjects the cellulose molecules to oxidative degradation. In both of these industrial processes hypochlorite is the principal oxidant. While much information exists as to how the properties of these polysaccharides are altered by oxidants, little information exists either as to the sites at which oxidation occurs or as to the nature of the molecular fragments that may be produced. The present preliminary report describes some of the products formed when hypochlorite reacts with corn starch amylose and with the β- and α-anomeric forms of methyl 4-*O*-methyl-D-glucopyranoside, which may be regarded as models for cellulose and starch.

It is well known that specific oxidants such as periodate,[3] lead tetraacetate and perhaps one or two others[4] bring about cleavage between C2 and C3 of anhydro-D-glucose units of starch and cellulose. Nitrogen dioxide oxidizes starch[5,6] and cellulose[7] preferentially at C6, although some other non-specific oxidation also occurs.

Most other oxidants have far less specific action as indicated by various analytical procedures designed to measure the amounts of carbonyl and carboxyl groups which may be produced. Bromine water in the presence of calcium carbonate[8] and alkaline hypochlorite[9] react with starch to produce degradation products, among which are acids with less than six carbons. Hypochlorous acid, on the other hand, seems to oxidize starch preferentially[10] at C6 with little oxidation[11] occurring at C2 and C3.

At *p*H 11 sodium hypochlorite reacts with cellulose to give an abrupt rise in carboxyl content

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(2) Paper presented before the Division of Carbohydrate Chemistry at the 129th Meeting of the American Chemical Society at Dallas, Texas, April, 1956.

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with little uronic acid formation and with the production of carbonyl but no aldehyde groups.[12] Under these alkaline conditions hypochlorite drastically reduces the apparent degree of polymerization of cellulose.[13]

The reaction of oxidants with simple glycosides also provides information helpful in understanding the nature of polysaccharide oxidations. Jackson and Hudson[14] find that barium hypobromite oxidizes methyl α-D-mannoside to produce 12% of the uronide and, by excision of C3 as formic acid, 25% of strontium α-(hydroxymethyl)-α′-methoxydiglycolate dihydrate, thus indicating attack at carbons 2, 3, 4 and 6. Oxidation of menthol α-D-glucoside[15] with sodium hypobromite in pyridine solution produces small amounts of uronide at low oxidant levels, but with methyl α-D-glucoside at high oxidant levels large amounts of glyoxylic acid are recoverable, again indicating chain cleavage. Bromine in sodium carbonate solution is reported[16] to yield 30% uronide from methyl α-D-glucoside, but alkaline solutions of iodine have little or no action on methyl α-D-glucoside.[17]

Bromine water at either low[18] or high[19] *p*H acts on free D-glucose to produce, initially, D-gluconic acid and subsequently,[20] at low *p*H, 5-keto-D-gluconic acid and D-glucaric acid. It is significant that methyl α- and β-D-glucosides[21] are likewise oxidized by aqueous chlorine to D-gluconic acid with subsequent formation of 5-keto-D-gluconic acid and D-glucaric acid.[22] Similar reactions occur with methyl α- and β-D-galactoside, mannoside, xyloside[23] and β-cellobioside.[22] In these reactions oxidation is not preceded by hydrolysis[21] and, just as with the free sugars, the β-forms react more rapidly than the α-forms.[21,23]

In the first part of the present work the action of hypochlorite on amylose is investigated at *p*H 9 and *p*H 11. The *p*H values are selected to duplicate those commonly used in industrial practice. Amylose is used to reduce the number of possible oxidation products. The rate and extent to which hypochlorite is consumed at three oxidant levels for each of the two *p*H values is shown in Fig. 1. At *p*H 9 the reaction proceeds swiftly to completion, while at *p*H 11 the reaction is slow and not all of the oxidant is used in the period investigated. Incomplete reactions may, in part, be due to retrogradation effects which, because of the slowness of the oxidation, intervene and remove some of the amylose molecules from the reaction.

To determine in a more detailed way the nature

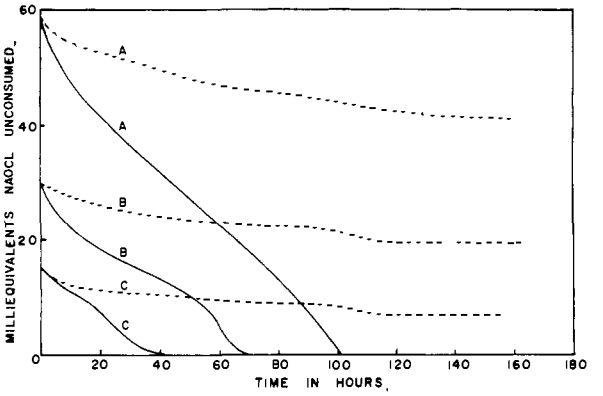

Fig. 1.—Oxidation of amylose with sodium hypochlorite at *p*H 9 (———) and *p*H 11 (– – – – –); equivalents of hypochlorite per anhydro-D-glucose unit used initially: A (2.0); B (1.0); C (0.5).

of the reaction a larger quantity of amylose is oxidized at *p*H 9 with a hypochlorite level of 2 equivalents per anhydro-D-glucose unit. This high oxidant level is selected to increase the yield of end products. Extensive depolymerization occurs, since 25% of the amylose becomes dialyzable. Hydrolysis of the oxidized amylose yields large amounts of D-glucose, indicating that many of the sugar units in the chain were unattacked by the oxidant. However, among the oxidation products are found, by paper chromatographic separation, D-erythrono-γ-lactone, D-erythronic acid and glyoxylic acid. The appearance of these complementary 2- and 4-carbon fragments suggests that extensive oxidation occurs at carbon atoms C2 and C3 of anhydro-D-glucose units.

If it is assumed that a portion of the hypochlorite is used in concerted oxidation of carbon atoms C2 and C3, to the extent that cleavage occurs and carboxyl groups are formed at positions C2 and C3, then the quantitative determination of glyoxylic acid produced on hydrolysis is a measure of the extent of this specific oxidation. Such quantitative measurement by spectrophotometry indicates that about 25% of the hypochlorite is consumed in oxidative cleavage between C2 and C3 bonds.

Chromatographic examination of the oxidized amylose hydrolyzate indicates only one unidentified component which appears in minor amounts and which is not gluconic, glucaric, tartaric or glucuronic acid. It must be presumed that at *p*H 9 the oxidative action of hypochlorite on amylose is by no means random but is extensively specific for positions C2 and C3. A large part of the hypochlorite seems to be consumed in oxidation of the fragments cleaved from the chains; these fragments appear as dialyzable material at the end of the reaction. Depolymerization might be expected if a carbonyl group is formed at C2 or C3 of sugar units, for its presence would weaken the bond at the C1 position.

In a second part of this work the α- and β-forms of methyl 4-*O*-methyl-D-glucopyranosides are oxidized with 10 equivalents of sodium hypochlorite per mole of sugar derivative at *p*H 9 and 25°. Again a high oxidant equivalent is used to increase the yield of end products. On oxidation of the

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β-anomer, what is apparently the disodium salt of 2-*O*-methyl-3-*O*-(glyoxylic acid methyl acetal)-D-erythronic acid is obtained in 30% yield and identified through isolation of glyoxylic acid in quantitative amounts. Isolation of the glyoxylic acid directly in yields of 43–47% from the oxidation reaction indicates a still greater degree of cleavage between atoms C2 and C3.

Interestingly, glyoxal can be isolated from the reaction products as the 2,4-dinitrophenylosazone. It is formed early in the reaction and the amount present does not increase during the remainder of the reaction.

From the reaction products about 10% of the starting material can be recovered, but a chromatographic search did not reveal any uronic or glyconic acids.

Methyl 4-*O*-methyl-α-D-glucopyranoside is obtained crystalline for the first time. Oxidation of this compound under the same conditions as used for the β-anomer, produce the same reaction products, but at a slower rate, thus again demonstrating the lower reactivity of the α-form.[21]

The recovery of an intermediate oxidation product and particularly the recovery of glyoxylic acid and glyoxal in large yields emphasizes that the oxidation is by no means random but that there is a distinct preference for glycol cleavage between C2 and C3. No information is obtained here concerning the mechanism of this oxidation, but it may be presumed to follow one or more of those previously presented in which either C2 or C3 is oxidized to carbonyl[9,12,24] with transformation to a 2,3-enediol[25–27] before further oxidative attack[28,29] or more likely it may proceed by direct oxidation to a 2,3-diketo derivative[9,12] which is further cleaved.[30,31] A possible explanation for isolation of a glyoxal derivative is the development of a 3,4-diketone from an intermediate 3-keto component, with cleavage to 2-carboxyglycolic aldehyde, which in reaction under acidic conditions with 2,4-dinitrophenylhydrazine, decarboxylates and forms the glyoxal osazone. This type of reaction is well known.[32–34] A similar reaction could explain the high yield of glyoxal osazone from the alkaline hypochlorite oxidation of methyl 2-*O*-methyl-α-D-glucopyranoside.[35]

Experimental

Oxidation of Amylose.—Approximately 1% dispersions of amylose were prepared by addition of 3 l. of boiling water to 30 g. of amylose,[36] well mixed with 720 ml. of 1-butanol. The mixture was heated with rapid stirring on a steam-bath to remove 1-butanol. The dispersion was quickly cooled, divided into 6 equal parts, 3 portions adjusted to *p*H 9 and 3 portions to *p*H 11. Sodium carbonate and sodium hydrogen carbonate were the buffers used.[37] To each *p*H group a stock solution of 2.2 *N* sodium hypochlorite[38] was added in such amount as to introduce into the dispersions 0.6, 1 and 2 equivalents of oxidant per anhydro-D-glucose unit, and the final volumes were adjusted to 500 ml. The reaction mixtures were maintained at 25° in the dark. Hypochlorite consumption was followed by titration of acidified aliquots containing excess potassium iodide with sodium thiosulfate. Hypochlorite solutions serving as blanks showed very little loss of oxidant. Oxidations at *p*H 9 were more rapid and attended with less retrogradation than at *p*H 11. Dispersions at the two higher oxidant levels at *p*H 9 remained clear. The dispersions at the lowest oxidant level at *p*H 9 and all dispersions at *p*H 11 became somewhat cloudy and at the lower two oxidant levels at *p*H 11 a precipitate formed.

Isolation of D-Erythrono-γ-lactone.—Following oxidation, the reaction mixtures were dialyzed against distilled water to remove inorganic salts and small organic molecules. Concentration of the dialyzate, followed by paper chromatography, showed that one organic component was present in very high proportion. This compound remains to be identified.

To determine the nature of oxidation in the remaining polymer, a portion of the non-dialyzable product from amylose oxidized with 2 equivalents of hypochlorite per anhydro-D-glucose unit at *p*H 9 was hydrolyzed in *N* sulfuric acid at 98° for 5 hours. Sulfate ions were removed as barium sulfate. The residual solution was stirred with IR-120 (H) resin[39] to remove cations and was decolorized with a small amount of charcoal (Darco G-60).[40] Concentration to near dryness, followed by distillation with benzene, yielded a colorless sirup. In 50-mg. portions the sirup was chromatographed on standard sheets of Whatman No. 1 filter paper using ethyl acetate–acetic acid–formic acid–water [18:3:1:4 v./v. (irrigant I)] at 25° as the irrigating solvent. After 9 hr. the papers were removed and guide strips sprayed with ammoniacal silver nitrate solution.[41] Five locations were revealed with $R_{glucose}$ values of 1.00, 2.27, 2.65, 3.41 and 4.07. Where indicated by the guide strips, sections of filter paper were excised and eluted with water and the eluents evaporated to sirups. The fastest moving component, $R_{glucose}$ 4.07, on treatment of its aqueous solution with IR-120 (H) resin,[39] decolorization with charcoal (Darco G-60)[40] and concentration yielded a colorless sirup. After azeotropic distillation with benzene it spontaneously crystallized and was recrystallized from ethyl acetate as long glistening needles, m.p. 104–105°, $[\alpha]^{25}D$ −72.4 (*c* 2.36, water). A mixed melting point with authentic D-erythrono-γ-lactone[42] was undepressed. Identical $R_{glucose}$ values were given by the two samples in several chromatographic solvents and their X-ray patterns were identical.

The component with $R_{glucose}$ value 2.27 had the same chromatographic flow rate as D-erythronic acid. It was more easily separated on filter paper sheets irrigated with 1-butanol–acetic acid–water [4:1:1 v./v. (irrigant II)] where its $R_{glucose}$ value was 1.52. For purification it was again chromatographed on paper with irrigant I under the conditions listed in the paragraph above. Guide strips sprayed with ammoniacal silver nitrate solution developed a yellow zone which gradually turned black, a color reaction indicative of aldonic acids. In irrigant I the substance showed the same $R_{glucose}$ value as D-erythronic acid, namely, 2.27. After treatment with IR-120 (H) resin,[39] evaporation to dryness and azeotropic distillation with benzene, the material crystallized. Recrystallization from ethyl acetate produced D-erythro-γ-lactone identical with that obtained above.

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Isolation of Glyoxylic Acid.—Hydrolysis of the non-dialyzable material with sulfuric acid followed by removal of sulfate ions with barium hydroxide precipitated much of the glyoxylic acid ($R_{glucose}$, 2.65 in irrigant I) as its insoluble barium salt. Consequently, for the isolation of this acid, hydrolysis of the non-dialyzable material from amylose oxidized with 2 equivalents of hypochlorite per anhydro-D-glucose unit at *p*H 9 was performed by treating 100 ml. of a 2% carbohydrate dispersion with 40 g. of IR-120 (H) resin[39] at 80° for 48 hr. After removal of the resin the light yellow solution was concentrated to 0.5 volume and mixed with an equal volume of 2 *N* hydrochloric acid containing 0.4% of 2,4-dinitrophenylhydrazine. The bright yellow crystalline precipitate was purified by dissolution in methyl ethyl ketone–water azeotrope and chromatography on a magnesium sulfate[42a] column with the same solvent. The eluent containing the yellow band was evaporated to dryness and the residue crystallized from hot ethanol by addition of water; m.p. 190–191°. On admixture with authentic glyoxylic acid 2,4-dinitrophenylhydrazone the melting point was undepressed. Chromatographic comparison and comparison of X-ray patterns confirmed the identity of the hydrazone. The same derivative was also obtained by proper treatment of the purified glyoxylic acid hydrate isolated from the resin hydrolyzate by paper sheet chromatography with irrigant II.

Isolation of D-Glucose.—D-Glucose from the hydrolyzate crystallized readily from the aqueous extract of appropriate sections of paper chromatographs developed with irrigant I. Its $R_{glucose}$ value was, of course, 1.0. Its phenylosazone melted at 210–211° and did not depress the melting point of an authentic sample. The X-ray pattern of the phenylosazone was identical to that from an authentic sample.

Quantitative Analysis.—To obtain some knowledge of the specificity of the oxidation, hypochlorite oxidations were performed on separate 5 g. portions of amylose under the conditions outlined in the first section of the Experimental. Oxidized amyloses were hydrolyzed in 1% dispersions with 0.5 *N* hydrochloric acid at 98° for 5 hr. Glyoxylic acid was determined spectrophotometrically by modification of the method of Snell and Snell.[43] The method used to determine glyoxylic acid in the standard solution was as follows. To 5 ml. of 0.1 *N* iodine solution was added 10 ml. of glyoxylic acid reagent. To this was added a mixture of 25 ml. of *N* disodium hydrogen phosphate and 8.67 ml. of *N* sodium hydroxide over a period of 20 minutes. After standing an additional 70 min. the solution was treated with 10 ml. of concentrated hydrochloric acid and the liberated iodine titrated with sodium thiosulfate. The difference between this and a blank gave the equivalents of glyoxylic acid in the standard. Results are shown in Table I. Three commercially oxidized starches[44] were also analyzed as shown in the table.

TABLE I

SODIUM HYPOCHLORITE OXIDATION OF AMYLOSE AND STARCH

NaOCl consumed per anhydro-glucose residue, equiv.	Non-dialyzable product, %	Glyoxylic acid from hydrolysis of 10 g. of non-dialyzable product, meq.	NaOCl used to effect 2,3-cleavage, %	Residues in non-dialyzable product which contain glyoxylic acid, %
		*p*H 9		
0.48	76	0.92	14	1.8
0.96	79	2.9	23	5.5
1.9	75	6.7	25	13
		*p*H 11		
0.25	88	0.18	5.9	0.34
.34	88	0.87	21	1.7
.60	77	2.1	27	4.0
		Commercially oxidized starch		
Light[a]	..	0.045	..	0.09
Medium[a]	..	.11	..	.21
Heavy[a]	..	.054	..	.12

[a] Designates relative degree of commercial oxidation.

Methyl 4-*O*-Methyl-D-glucopyranosides.—Methyl 4-*O*-methyl-β-D-glucopyranoside was prepared by the method of McGilvray[45] and had m.p. 102–103° and $[\alpha]^{20}D$ −17° (*c* 1, water); reported m.p. 102–103° and $[\alpha]^{20}D$ −17.6° (*c* 1, water). The triacetate derivatives of this compound, prepared in the usual way,[46] had m.p. 107–108° and $[\alpha]^{25}D$ −33.9° (*c* 1, chloroform); reported values, m.p. 107–108° and $[\alpha]^{20}D$ −33.5° (*c* 1, chloroform).[47]

Methyl 4-*O*-methyl-α-D-glucopyranoside was prepared by a similar method. Eleven grams of methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside was treated with 8.0 g. of triphenylchloromethane in 200 ml. of dry pyridine at 100–105° for 5.5 hr. The trityl derivative was obtained[45] as a sirup (19 g.) with $[\alpha]^{25}D$ + 16° (*c* 2, chloroform). Two methylations of this compound with Purdie's reagents[48] gave 19 g. of a glass with $[\alpha]^{25}D$ +36° (*c* 2, chloroform). The trityl derivative was treated with 350 ml. of 80% acetic acid for 1 hr. at 95–100°. Fifty milliliters of water was then added to precipitate more of the insoluble triphenylcarbinol which was filtered off. The filtrate was extracted with chloroform and washed with water until free of acid. After drying with anhydrous sodium sulfate and evaporation of the chloroform, a sirup with $[\alpha]^{25}D$ + 43° (*c* 1, chloroform) was obtained. Debenzylation with sodium and ethyl alcohol following the McGilvray procedure gave a sirup which failed to crystallize. This sirup was chromatographed on a cellulose column using water saturated methyl ethyl ketone as irrigant. From 12 g. of the above sirup, 0.99 g. of crystalline methyl 4-*O*-methyl-α-D-glucopyranoside were recovered. Recrystallization from methyl ethyl ketone followed by a second recrystallization from ethyl acetate gave needles, m.p. 94–95° and $[\alpha]^{25}D$ + 167° (*c* 1.17, water).

Anal. Calcd. for $C_8H_{16}O_6$: C, 46.2; H, 7.7. Found: C, 45.7; H, 7.7.

The rotational shift of the methyl 4-*O*-methyl-α-D-glucopyranoside in cuprammonium[49] was determined by Dr. R. E. Reeves and found to be −1021°, in good agreement with the calculated value: $[\alpha]^{25}_{436}$ −709° ± 5 (*c* 0.6, cuprammonium); $[\alpha]^{25}_{436}$ +312° ± 2 (*c* 1.17, in water).

The 4-*O*-methyl-D-glucophenylosazone of this compound was prepared[50] and had m.p. 158°; reported value, 158–159°. This derivative showed no change in melting point on admixture with 4-*O*-methyl-D-mannophenylosazone.

Oxidation of Methyl 4-*O*-Methyl-β-D-glucopyranoside.—The glycoside derivative was dissolved in 1% concentration in sodium hypochlorite solutions buffered with sodium bicarbonate to *p*H 9. Approximately 10 equivalents of oxidant per mole of sugar were used. The course of the reaction at 25° was followed by optical rotation and chlorine consumption.

Recovery of Glyoxal.—Methyl 4-*O*-methyl-β-D-glucopyranoside (0.4 g.) was oxidized with 40 ml. of 0.46 *N* sodium hypochlorite. The rotation changed from −0.20° to +0.52° after 96 hr. At this point a sample of the mixture was diluted with water to 50 ml., acidified to *p*H 6.5 with 0.5 *N* hydrochloric acid and aerated until free of chlorine as indicated by the starch–potassium iodide test. Treatment of this solution with 2,4-dinitrophenylhydrazine reagent[51] on a steam-bath for 2 hr. gave glyoxal 2,4-dinitrophenylosazone, m.p. 328°; reported m.p. 326–328°.[52] No change in melting point was obtained on admixture with authentic compound prepared from glyoxal bisulfite.

Anal. Calcd. for $C_{14}H_{10}O_8N_8$: N, 26.8. Found: N, 26.7.

Separation on Cellulose Column.—A second sample of methyl 4-*O*-methyl-β-D-glucopyranoside (1.000 g.) was oxidized with 100 ml. of 0.51 *N* sodium hypochlorite. In 48

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hours the change in $[\alpha]^{25}D$ was $-22°$ to $+46°$. During this time the oxidant decreased from 10.6 equivalents per mole to 0.03 equivalent per mole. Eighty-five milliliters of solution containing 0.85 g. of β-glucoside was adjusted to *p*H 6.5, aerated to remove chlorine and evaporated to dryness. The residue was then extracted with absolute alcohol. The alcohol insoluble residue, consisting mostly of inorganic salt and a small amount of sodium glyoxylate, was discarded. The alcohol soluble fraction was evaporated to dryness. The residue (0.95 g.) was then chromatographed on a cellulose column with butanol–pyridine–water [6:4:3 v./v. (irrigant III)]. Effluent portions were grouped into four fractions after examination with paper chromatograms.

From Fraction I, 75 mg. of unreacted methyl 4-*O*-methyl-β-D-glucopyranoside was recovered. Other components were present in insufficient amounts to identify.

Fraction II (60 mg.) was not identified. The crystalline compound melted at 258–260° with considerable evolution of gas. It had no optical activity.

Fraction III (350 mg.) was largely inorganic salt. Treatment with 2,4-dinitrophenylhydrazine gave glyoxal 2,4-dinitrophenylosazone and glyoxylic acid 2,4-dinitrophenylhydrazone in small amounts.

Fraction IV gave 350 mg. of an amorphous powder with $[\alpha]^{25}D$ +43° (*c* 1, water) and appeared quite pure by paper chromatographic analysis. It is assumed that this compound is the disodium salt of 2-*O*-methyl-3-*O*-(glyoxylic acid methyl acetal)-D-erythronic acid.

Anal. Calcd. for $C_8H_{12}O_8Na_2$: Na, 16.3. Found: Na, 15.6.

One hundred milligrams of this compound was refluxed with Amberlite resin IR 120 (H)[39] for 24 hr. The solution was filtered and concentrated. Extraction with ether followed by evaporation of the ether gave 30 mg. of a brown sirup. This sirup gave a strong lactone test[53] and showed chromatographic evidence of small amounts of glyoxylic acid. Attempts at crystallization were not successful. For comparative purposes 2-*O*-methyl-D-erythrono-γ-lactone was prepared. Half a gram of methyl 4-*O*-methyl-β-D-glucopyranoside was reacted with 20 ml. of 0.27 *M* sodium metaperiodate to constant rotation. The dialdehyde was isolated in the usual way[54] as a sirup. This sirup (0.4 g.) was oxidized with bromine water and 1.0 g. of barium carbonate for 48 hr. After filtration of the insoluble material and removal of the bromine by aeration, the solution was refluxed with Amberlite resin IR 210 (H)[39] for 24 hr. The resin was filtered off and the filtrate concentrated. From the ether extract of this solution, 0.35 g. of a brown sirup was recovered. This sirup was chromatographically separated on paper using irrigant III and recovered from the paper by elution with water. About 50 mg. of a sirup was obtained which appeared pure by paper chromatography. The sirup gave the same color with the lactone test and with silver nitrate as the compound isolated from the hydrolysis of fraction IV. Both compounds had the same chromatographic flow rate which compared to 4-*O*-methyl-D-glucose was 1.58, in irrigant III.

The amount of glyoxylic acid formed upon acid hydrolysis of fraction IV was determined gravimetrically as the 2,4-dinitrophenylhydrazone. A sample of 10–20 mg. in 5 ml. of water was refluxed on a steam-bath for 3 hr. with 5 ml. of 2,4-dinitrophenylhydrazine. After cooling, the solution was diluted with water to 50 ml. and cooled to 0°. Dilution and cooling of the solution are necessary to minimize the solubility of the derivative. The precipitate was then filtered, washed with cold water, dried and weighed. Quadruplicate determinations showed 85% of theoretical isolation of glyoxylic acid from fraction IV, assuming that it is the disodium salt of 2-*O*-methyl-3-*O*-(glyoxylic acid methyl acetal)-D-erythronic acid. The precipitates of each of the hydrazones were compared with authentic glyoxylic acid 2,4-dinitrophenylhydrazone as to melting point, mixed melting point and paper chromatographic flow rate and found to be the same.

The amount of glyoxylic acid formed upon acid hydrolysis of fraction IV was determined by a second method which was a slight modification of the colorimetric method of Cavallini.[55] A sample of fraction IV was dissolved in 10 ml. of water and refluxed on a steam-bath for 3 hr. with 10 ml. of 2,4-dinitrophenylhydrazine reagent. The solution was cooled and made up to 100 ml. with water. A one milliliter aliquot was then treated with 5 ml. of 5% sodium hydroxide and the transmittancy measured on a spectrophotometer at wave length 460 mμ. The amount of glyoxylic acid was then obtained from a standard curve. The average of six determinations showed 95% of the theoretical isolation of glyoxylic acid from fraction IV assuming it to be the disodium salt of 2-*O*-methyl-3-*O*-(glyoxylic acid methyl acetal)-D-erythronic acid.

Glyoxylic Acid Recovery and Oxidant Consumption for the β-Anomer.—At various stages of the oxidation of methyl 4-*O*-methyl-β-D-glucopyranoside, determination was made for oxidant consumed[56] and for glyoxylic acid and glyoxal produced. Glyoxal determination by the above method indicated that throughout the reaction the amount present remained constant at about 0.1 mole per mole of the D-glucopyranoside. Glyoxylic acid was determined by the above gravimetric procedure and results are shown in Table II.

TABLE II

GLYOXYLIC ACID RECOVERY FROM OXIDIZED METHYL 4-*O*-β-D-GLUCOPYRANOSIDE (GRAVIMETRY)

Oxidation period, hr.	Moles glyoxylic acid/mole sugar	Equiv. of oxidant consumed/mole sugar
5	0.10	1.3
24	.31	5.6
72	.41	9.3
120	.47	9.5

Transmittancy curves indicated that the colorimetric method of Cavallini[55] also could be used to determine glyoxylic acid obtained upon hydrolysis of the oxidized mixture. A one-milliliter aliquot equivalent to 0.01 g. of methyl 4-*O*-methyl-D-glucopyranoside was diluted with 9 ml. of water, acidified with *N* hydrochloric acid to *p*H 6.5 and aerated until free of chlorine. The mixture was then refluxed on a steam-bath for 3 hr. with 10 ml. of 2,4-dinitrophenylhydrazine reagent. The solution was filtered to remove the glyoxal 2,4-dinitrophenylosazone and made up to 100 ml. with water. To a 1-ml. aliquot of this diluted solution was added 9 ml. of water and 5 ml. of 5% sodium hydroxide. After mixing, the transmittancy was measured at 460 mμ with a spectrophotometer. A sample of methyl 4-*O*-methyl-D-glucopyranoside (0.01 g.) was refluxed with the 2,4-dinitrophenylhydrazine made up to same dilutions and used as the blank. Results are shown in Table III.

TABLE III

GLYOXYLIC ACID RECOVERY FROM OXIDIZED METHYL 4-*O*-METHYL-D-GLUCOPYRANOSIDES (COLORIMETRY)

	β-Anomer		α-Anomer	
Oxidation period, hr.	Moles of glyoxylic acid/mole sugar	Equiv. of oxidant consumed/mole sugar	Moles of glyoxylic acid/mole sugar	Equiv. of oxidant consumed/mole sugar
5	0.21	2.7	0.08	1.4
12	.34	5.2	.15	2.7
24	.43	7.2	.24	5.2
48	.43	8.9	.29	7.9
72	.43	9.5	.31	9.2
96	.43	9.7	.32	10.0
144	.43	10.0	.32	10.4

Uronic and Glucuronic Acids.—Samples of the oxidized glucoside products were examined by paper chromatography for uronic acids with the following indicators: naphthoresorcinol,[57] *o*-phenetidine,[58] aniline phthalate[59] and *p*-anisidine,[59] and for gluconic acids with the indicators aniline phthalate,[59]

(53) M. Abdel-Akher and F. Smith, THIS JOURNAL, **73**, 5859 (1951).

(54) R. Adams, "Organic Reactions," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 302.

(55) D. Cavallini, *Ricerca Sci.*, **20**, 803 (1950).

(56) R. B. Bradstreet, "The Standardization of Volumetric Solutions," 2nd Ed., Chemical Publishing Co., Inc., New York, N. Y., 1944, p. 106.

(57) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(58) P. C. Arni and E. G. V. Percival, *J. Chem. Soc.*, 1822 (1951).

(59) S. M. Partridge, *Nature*, **164**, 443 (1949).

o-phenylenediamine[60] and silver nitrate,[61] but none were detected.

Oxidation of Methyl 4-*O*-Methyl-α-D-glucopyranoside.—The α-anomer was oxidized under the same conditions as the β-glucoside. Identification of glyoxylic acid, 2-*O*-methyl-D-erythrono-γ-lactone[33] and glyoxal was made in the same manner. Results are shown in Table III.

(60) M. C. Lanning and S. S. Cohan, *J. Biol. Chem.*, **189**, 109 (1949).

(61) E. L. Hirst, L. Hough and J. K. N. Jones, *J. Chem. Soc.*, 928 (1949).

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LAFAYETTE, INDIANA

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH]

Stevioside. IV. Evidence that Stevioside is a Sophoroside

BY ERIK VIS[1] AND HEWITT G. FLETCHER, JR.

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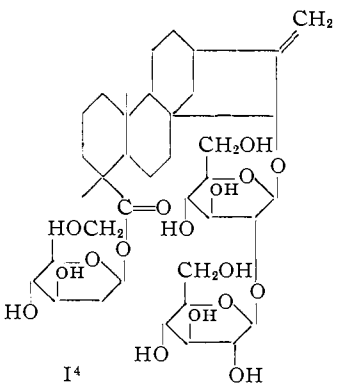
Treatment of steviolbioside heptaacetate with hydrogen bromide in glacial acetic acid has been found to give α-acetobromosophorose (3,4,6-tri-*O*-acetyl-2-*O*-(tetra-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucosyl bromide). The disaccharide residue in stevioside is, therefore, a sophorosyl group.

Structural studies of stevioside,[2,3] the intensely sweet principle of *Stevia Rebaudiana* Bertoni, have shown that the substance contains one glucopyranose unit attached to a carboxyl group and two glucopyranose units linked to $C_1 \rightarrow C_2$ and thence to an alcoholic hydroxyl group. These facts may be represented by I, it being understood that precise details of the structure of the aglucon remain to be elucidated. Since stevioside is not attacked by the enzymes usually employed in settling questions of anomerism and attempts to cleave the disaccharide fragment as a whole failed, the configurations of the three anomeric carbons were left open in the original work. Subsequently a study of a simple



I[4]

analog of a portion of the stevioside molecule[5] indicated with some certainty that the ester-linked sugar residue is a β-D-glucopyranosyl group. We now wish to report evidence bearing on the configuration of the linkage joining the glucose residues of the disaccharide portion.

As described earlier,[2] treatment of stevioside with alkali results in loss of the ester-linked sugar residue and formation of a glucoside which was given the trivial designation of "steviolbioside." Acetylation of this substance has now provided an amorphous heptaacetate which, in glacial acetic acid, is readily cleaved by hydrogen bromide to give a relatively insoluble acetobromobiose. The physical constants of this substance agreed closely with those of α-acetobromosophorose; treatment with silver acetate converted the product to β-sophorose octaacetate, identification being confirmed through comparison with authentic material. Since sophorose has been unequivocally demonstrated to be 2-*O*-(β-D-glucopyranosyl)-D-glucose,[6] the configuration of the linkage joining the two glucose units in stevioside may now be considered established. While the configuration of the sophorose–steviol linkage remains unproved, it is very probably β also.

To the authors' knowledge stevioside is but the second sophoroside to be found, the first being kaempferol sophoroside which occurs in the fruit of the widespread ornamental tree *Sophora japonica* L.[6a,7]

While the present work was not directly concerned with the fate of the aglucon in the cleavage, ketoisostevic acid (isosteviol) was isolated after one rather prolonged run. That an unrearranged product such as hydroxydehydroisostevic acid (steviol)[3] or its acetate might be isolable under milder conditions is not excluded.

Experimental[8]

Steviolbioside Heptaacetate.—Anhydrous steviolbioside (2.5 g.) was dissolved in 10 ml. of dry pyridine and the solution cooled to −70°. Acetic anhydride (3.5 ml.) was then added and the solution slowly warmed to 20°. After 20 hr. at 20° and 4 hr. at 60°, the reaction mixture was poured into 200 ml. of 1% aqueous acetic acid at 0°. The white powder thus precipitated was removed after 1 hr., dissolved in ether and the solution washed successively with dilute

(1) Chemical Foundation Fellow, 1956.

(2) H. B. Wood, Jr., R. Allerton, H. W. Diehl and H. G. Fletcher, Jr., *J. Org. Chem.*, **20**, 875 (1955).

(3) E. Mosettig and W. R. Nes, *J. Org. Chem.*, **20**, 884 (1955).

(4) The hydrogen atoms attached to the rings of the glucose moieties have been omitted for clarity.

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(6) (a) K. Freudenberg, H. Knauber and F. Cramer, *Chem. Ber.*, **84**, 144 (1951); (b) J. Rabaté, *Bull. soc. chim.*, **7**, 565 (1940); (c) K. Freudenberg and K. Soff, *Ber.*, **69**, 1245 (1936); (d) K. Freudenberg, H. Toepffer and C. C. Andersen, *ibid.*, **61**, 1750 (1928).

(7) J. Rabaté and J. Dussy, *Bull. soc. chim. biol.*, **20**, 459, 467 (1938).

(8) Melting points are corrected.