# Reduction of the Products of Periodate Oxidation of Carbohydrates. XIII. Determination of Sugars in Polysaccharides Oxidized by Periodate[1]

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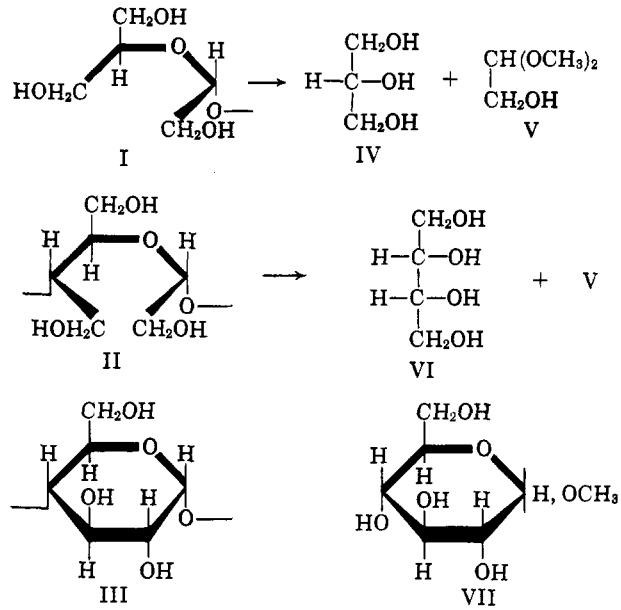
A method is described for the quantitative determination of sugar in periodate-oxidized polysaccharides. This is accomplished by reducing the polyaldehyde with sodium borohydride and subjecting the polyalcohol so formed to the action of methanolic hydrogen chloride which forms a mixture of alditols, methyl glycoside, and glycolic aldehyde dimethylacetal. Distillation under reduced pressure removes the glycolic aldehyde dimethylacetal, after which the residue is analyzed for sugar by the phenol–sulfuric acid method.

Periodate oxidation studies on polysaccharides[2] have necessitated the development of a simple method for determining the proportion of sugar units which survive oxidation. In a number of instances, the proportion of sugar residues in periodate-oxidized polysaccharides has been deduced indirectly from the periodate consumption[3] and from the results of the action of sodium hydroxide,[4] sodium borohydride,[5] or *p*-nitrophenylhydrazine[6] on the derived polyaldehyde.

Another approach in the case of glucans is to determine glucose in the hydrolysate of the polyaldehyde by means of glucose oxidase.[7] In other procedures, the hydrolysate of a polyalcohol obtained by reduction[8] of the corresponding polyaldehyde may be separated by paper chromatography and the reducing sugar determined by the Nelson–Somogyi[9,10] or by the phenol–sulfuric acid method.[11] The anthrone and orcinol reagents have been used for the determination of sugar in periodate-oxidized and reduced oligosaccharides[12] and glycopeptides,[13] respectively.

This article describes a simple and general method for the determination of sugar residues in polysaccharides which have been subjected to periodate oxidation. The procedure involves reducing the polyaldehyde to the polyalcohol with sodium borohydride, after which the polyalcohol is boiled with methanolic hydrogen chloride. This results in total cleavage giving glycerol and/or erythritol, the methyl glycosides of the sugar residues which survive periodate oxidation, and glycolic aldehyde dimethylacetal. Thus, in the case of a terminal, nonreducing glucose residue, periodate oxidation and reduction gives the residue I which upon methanolysis yields glycerol (IV) and glycolic aldehyde dimethylacetal (V). By the same procedure, a (1→4)-linked glucose unit will provide the residue II which in turn will give rise to erythritol (VI) and V, whereas any glucose unit surviving periodate oxidation, such as that depicted in III, will furnish methyl D-glucoside (VII). Evaporation of the neutralized solution removes the glycolic aldehyde dimethylacetal which interferes with the phenol–sulfuric acid method and leaves a mixture of methyl glycosides and sugar alcohol(s). This residual mixture is dissolved in water and the sugar is determined by the phenol–sulfuric acid method in the usual way.[11]




Although the present study deals only with the glucans, glycogen, and starch, the method is applicable to polysaccharides in general and, since protein does not interfere[14] with the phenol–sulfuric acid method, the procedure proposed herein is also applicable to glycoproteins and glycopeptides.

## Experimental

The apparatus used was a spectrophotomer (Coleman Junior or its equivalent) and a set of matched test tubes (18 × 150 mm.). The reagents used were phenol reagent,[11] prepared by dissolving freshly distilled phenol (5 g.) in distilled water (95 ml.), and sulfuric acid, reagent grade 95.5%, Spectro Grade 1.84.

**I. Periodate Oxidation of the Polysaccharide.**—A known weight of glycogen from calf liver was treated with an amount of 0.1 *N* sodium periodate calculated to bring about the desired level of oxidation, the reaction mixture being kept in the dark at 5°. A blank experiment was carried out at the same time. After about 3 weeks, the periodate consumption was determined volumetrically.[3] From the amount of periodate consumed, the degree of oxidation of the polysaccharide was calculated, assuming that 1 mole of periodate is consumed per glucose unit. The periodate-oxidized glycogen was isolated by freezing the reaction mixture and then allowing it to thaw,[15] after which the precipi-

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tated glycogen polyaldehyde was washed repeatedly with water until the washings were free from iodate (tested with acidified potassium iodide–starch solution). The periodate-oxidized glycogen was washed successively with ethanol (to remove water), ether, and petroleum ether (b.p. 30–60°) and dried *in vacuo*. Three samples of partially ozidized glycogen of 50, 75, and 90% oxidation were prepared in this manner.

A modified procedure can be used for polysaccharides which are soluble in the periodate oxidation reaction mixture. An aqueous solution of barium acetate is added until no more precipitate of barium iodate or periodate is formed. The solution is then filtered, the residue is washed with water, and the filtrate is treated as in section II below.

**II. Determination of Glucose in Periodate-Oxidized Polysaccharides.**—It is recommended that for 99% oxidized polysaccharides a 100-mg. sample should be used, and for 1% oxidized polysaccharides 10 mg. is a suitable quantity.

**A. By the Phenol–Sulfuric Acid Method.**[11] **Example 1.**—Periodate-oxidized glycogen (100.6 mg. oxidized to 90% of theory required for complete cleavage) was treated with 0.5% sodium borohydride solution (10 ml.), and the reaction mixture was allowed to stand overnight. A test portion of the solution, after acidification with dilute acetic acid, gave a negative Fehling's test. The solution was neutralized with dilute hydrochloric acid and concentrated to dryness under reduced pressure (35–40°), the last traces of water being removed by repeated addition and distillation of absolute ethanol. The dry residue was refluxed with 2.5% methanolic hydrogen chloride (5 ml.) for 2 hr. (or until the mixture gave a negative Fehling's test). The solution was neutralized by the addition of lead carbonate and filtered, and the filtrate was concentrated to dryness. The residue was dissolved in dry methanol (20 ml.) and the solution concentrated to dryness. This was repeated twice, and the product was heated finally for 2 hr. at 80–90° under reduced pressure to ensure complete removal of glycolic aldehyde dimethylacetal and trimethyl borate to give a residue R. This residue (R) was dissolved in water, the solution was filtered, and the volume was adjusted to 500 ml. by adding water.

Aliquots (1 ml.) of the solution in 18 × 150 mm. tubes were treated with the phenol reagent (1 ml.), followed by concentrated sulfuric acid (5 ml.), the latter being added quickly against the liquid surface to ensure rapid mixing.[11] The tubes were allowed to cool to room temperature, and the absorbancy of the characteristic orange-yellow color was determined at λ 490 mμ. Analyses were carried out in triplicate. The amount of glucose in each tube was computed by reference to a standard curve for glucose (10–50 μg.).[11]

**Example 2.**—Periodate-oxidized starch (30.4 mg. of 95% oxidation) was suspended in water (15 ml.) and treated with sodium borohydride (50 mg. in 10 ml. of water), and the mixture was kept for 24 hr. at room temperature. The procedure described above in example 1 was then followed except that methanolysis was continued for 7 hr. The solution was filtered, and the volume was adjusted to 50 ml. by the addition of water. The glucose content of 1-ml. aliquots was determined by the phenol–sulfuric acid method as described above.

**B. By the Glucose Oxidase Method.**[16,17]—The enzyme solution was prepared by combining a solution of glucose oxidase[18] (10 ml., 0.01% in 0.05 *M* acetate buffer of pH 4.8), peroxidase[19] (10 ml., 0.025% in the buffer), *o*-dianisidine (1 ml., 0.5% in 95% ethanol), and 0.05 *M* acetate buffer, pH 4.8 (20 ml.).

The residue (such as R, see example 1) was hydrolyzed by heating for 5 hr. at 100° with 1 *N* sulfuric acid (5 ml.). The solution was neutralized with barium carbonate and filtered, and the residue was washed well with water. The volume of the filtrate was adjusted to 100 ml. with 0.05 *M* acetate buffer, pH 4.8. Duplicate aliquots (1 ml., containing 20–80 μg. of glucose) were pipeted into 18 × 150 mm. tubes and allowed to equilibrate at 37° in a constant temperature bath. The enzyme solution (4 ml.), preheated to 37°, was pipeted into each tube, and the digest was incubated at 37°. The reaction was stopped after exactly 30 min. by rapid addition of 0.5 *N* hydrochloric acid (1 ml.), and the tubes were cooled in water for 20 min. The absorbancy of each tube was measured at λ 420 mμ. Glucose standards (20–80 μg./ml.) were analyzed concurrently in duplicate and a standard curve was constructed. The amount of glucose present in a 1-ml. aliquot was determined by reference to the standard curve.

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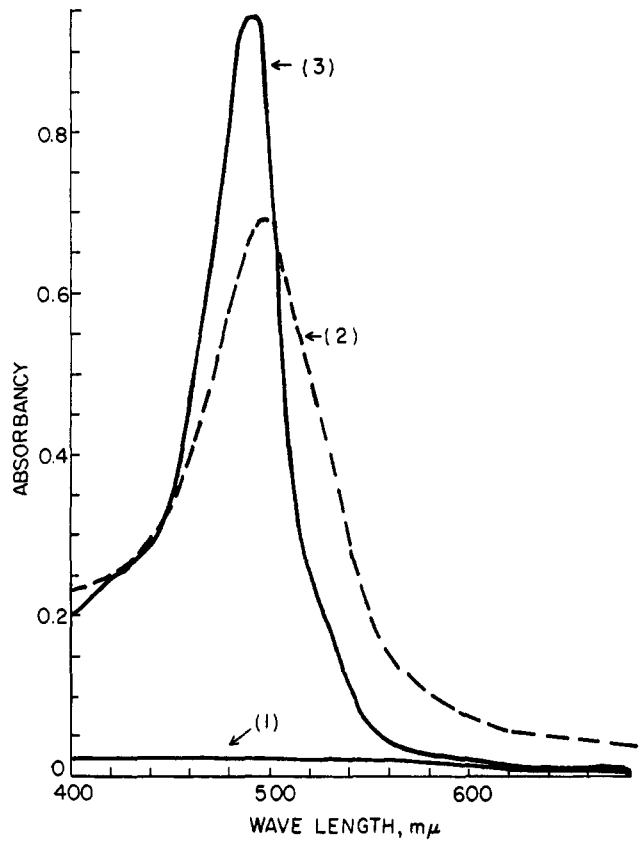


Fig. 1.—Test solutions contained an aqueous solution (1 ml.) of (1) glycerol and erythritol (45 μg., 730 μg.), (2) glycolic aldehyde (470 μg.), and (3) glucose (85 μg.), together with 5% aqueous phenol (1 ml.) and concentrated sulfuric acid (5 ml.).

## Results and Discussion

The analyses of three glycogen polyaldehydes and three starch polyaldehydes are summarized in Table I.

TABLE I
GLUCOSE CONTENT OF PERIODATE-OXIDIZED GLYCOGENS AND STARCHES

| | Weight of sample, mg. | Degree of oxidation, % | % Glucose, determined by— Periodate consumption | Phenol–sulfuric acid | Glucose oxidase |
|---|---|---|---|---|---|
| Glycogen | 57.3 | 50 | 50.0 | 46.3 | 44.2 |
| | 73.2 | 75 | 25.0 | 27.6 | 24.5 |
| | 110.6 | 90 | 10.0 | 14.2 | 13.8 |
| Starch | 28.0 | 3 | 97.0 | 95.0 | |
| | 27.4 | 60 | 40.0 | 41.0 | |
| | 30.4 | 95 | 5.0 | 5.3 | |

With specimens of periodate-oxidized glycogen, good agreement was obtained between the expected glucose content based on the periodate consumption data, and the glucose content as determined by the phenol–sulfuric acid method and by the glucose oxidase method.

The success of this simple method is dependent on complete removal of interfering compounds. Glycolic aldehyde, which is derived from C-1 and C-2 of the glucose units, produces a pink coloration with the phenol–sulfuric acid reagent ($\lambda_{max}$ 495 mμ, Fig. 1), and boric acid, generated in the sodium borohydride reduction,

decreases the color yield in the phenol–sulfuric acid reaction in an undefined manner.[20] Both of these compounds are converted to volatile derivatives (glycolic aldehyde dimethylacetal and trimethyl borate) by methanolysis and are eliminated by evaporation of the solvent. The complete removal of glycolic aldehyde dimethylacetal may be checked by testing the residue with the diphenylamine reagent.[21] In the event of incomplete removal of the aldehyde, the methanolysis and subsequent distillation is repeated. Glycerol, formed from terminal nonreducing hexopyranose and pentofuranose units, and erythritol, formed from nonterminal (1→4)-linked hexopyranose units, do not interfere with the glucose determination (Fig. 1).

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# Polynucleotides. II. Synthesis of (3′→5′)-Linked Diribonucleoside Phosphates Containing Uridine

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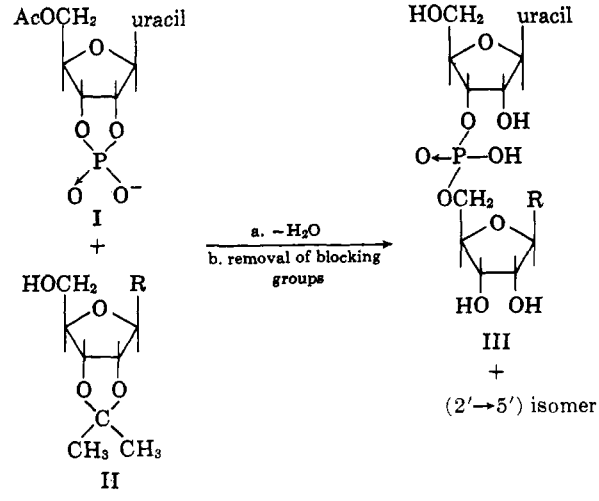
In the past few years, the syntheses of several (3′→5′)-linked diribonucleoside phosphates have been reported.[1-5] These syntheses fall into two groups, namely those which produce the (3′→5′) internucleotide bond and those which produce a mixture of (2′→5′)- and (3′→5′)-linked isomers. The second type of synthesis was utilized by us, and an ion-exchange procedure was developed which resolves the mixed isomers. The internucleotide bond was formed by treating 5′-*O*-acetyluridine-2′,3′ cyclic phosphate with a nucleoside blocked in the 2′- and 3′-positions in the presence of diphenyl phosphorochloridate. The following diribonucleoside phosphates have been prepared: uridylyl(3′→5′)-5-bromouridine, uridylyl(3′→5′)uridine, uridylyl(3′→5′)-6-thioinosine, and uridylyl(3′→5′)inosine.

One of the major problems in the synthesis of (3′→5′)-linked diribonucleoside phosphates stems from the requirement for an intermediate blocked in the 2′- and 5′-positions. The key intermediate can be prepared either by acetylation of the 2′-position of nucleoside-3′ phosphates[6] or by phosphorylation of ribonucleosides blocked in the 2′- and 5′-positions such as the easily prepared 2′,5′-di-*O*-trityluridine.[7] The first approach relies on the ability to obtain pure 3′-ribonucleotides and the second is limited to those nucleosides for which selective blocking of the 2′- and 5′-positions is practical. The difficulties in preparing diribonucleoside phosphates can be ameliorated to some extent if selective blocking of the 2′- or 3′-position is eliminated with the result that the final product becomes a mixture of (2′→5′)- and (3′→5′)-linked isomers. The usefulness of this method, therefore, rests on the ability to resolve the isomers. One of the first exponents of this approach to oligonucleotide syntheses was Michelson,[5] who prepared a series of [2′(3′)′→5′]-linked diribonucleoside phosphates and in some cases was able to separate the isomers. Such an approach to the synthesis of diribonucleoside phosphates results in a reduction of the number of steps and increased over-all yields. We have examined this approach with the intention of ascertaining its general usefulness for the synthesis of oligonucleotides and this paper describes the preparation of diribonucleoside phosphates in which uridylyl-3′ phosphate is linked to the 5′-position of 5-bromouridine, uridine, inosine, and 6-thioinosine.

The internucleotide bond was formed according to the method developed by Michelson[5] in which a ribonucleoside-2′,3′ cyclic phosphate (I) reacts with a 2′,3′-isopropylidene derivative of a ribonucleoside (II) under the influence of diphenyl phosphorochloridate and in the presence of tri-*n*-butylamine. Ready availability of ribonucleoside-2′,3′ cyclic phosphates is essential to




a, R = 5-bromouracil
b, R = uracil
c, R = hypoxanthine
d, R = 6-mercaptopurine

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