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AUTOMATED HYPOIODITE OXIDATION OF CARBOHYDRATES

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ABSTRACT

An automated system is described for the hypoiodite oxidation of aldoses and substituted aldoses to the corresponding aldonic acids. Automated determination of the glyoxylic acid and formaldehyde obtained on oxidation with periodate enables the 3-*O*-, 4-*O*-, and 6-*O*-substituted aldonic acids to be distinguished. The method is applied to the analysis of oligosaccharides in column eluates.

INTRODUCTION

Oxidation to the corresponding aldonic acid provides a method for the determination of the reducing terminal linkage of oligosaccharides. Subsequent oxidation of the derived aldonic acid with periodate enables distinction of 3-*O*-, 4-*O*-, and 6-*O*-substituted aldoses. Thus, a 4-*O*-substituted aldonic acid gives equimolar proportions of glyoxylic acid and formaldehyde, 6-*O*-substituted acids give glyoxylic acid and no formaldehyde, and 3-*O*-substituted acids give formaldehyde and no glyoxylic acid. Automation of such a reaction scheme would provide a useful parameter for the analysis of oligosaccharide structure. This paper describes the automation of the oxidation by hypoiodite, the analysis by periodate oxidation of the resulting aldonic acids, and the application of the method to mono- and oligosaccharides.

EXPERIMENTAL

Glyoxylic acid in periodate-oxidation mixtures was determined by the 2,3,4-trihydroxybenzoic acid (THBA) procedure[1,2]. Formaldehyde was determined by the pentane-2,4-dione method[3], and neutral sugars by the cysteine–sulphuric acid method[2,4].

Oxidation of D-*glucose by alkaline solutions of iodine.* — Aliquots of solutions of D-glucose (10 mg/ml) were mixed with a solution of iodine (0.05M) in potassium iodide (0.24M, 0.5 ml) and carbonate buffer (0.2M, pH 10.5; 1.0 ml). Solutions of D-glucono-1,5-lactone (10 mg/ml) were similarly treated. Control solutions were prepared by addition of water (0.5 ml) and carbonate buffer (1.0 ml) to aliquots (0.1 ml) of a

solution of D-glucose (10 mg/ml). After 3 h at 25°, the reaction mixtures were diluted with water (9.0 ml), and aliquots (0.1 ml) were removed for analysis by the THBA method for glyoxylic acid produced on oxidation with periodate. D-Gluconic acid (0.99 mole) was obtained from D-glucose on the basis of the analysis for glyoxylic acid.

Solutions of D-glucono-1,5-lactone (1 mg/ml) in carbonate buffer (0.2M, pH 10.5; 2.0 ml), when mixed with either water (1.0 ml) or a solution of iodine (0.05M) in potassium iodide (0.24M, 1.0 ml), gave identical colour yields in the analysis for glyoxylic acid, produced on oxidation with periodate, by the THBA method.

Effect of iodine concentration. — Solutions of D-glucose (1 mg/ml, 0.1 ml) were mixed with carbonate buffer (0.2M, pH 10.5; 1.0 ml) and iodine (0.05M) in potassium iodide (0.24M; 0.5 ml, 0.25 ml, or 0.1 ml). After dilution with water (to 6.6 ml), the solutions were maintained at 25° for 3 h, and aliquots (0.1 ml) were removed for analysis by the THBA method for glyoxylic acid produced on periodate oxidation. Quantitative conversion into the aldonic acid was only effected at the most concentrated level of hypoiodite employed. A similar experiment, in which aliquots were removed for analysis at time intervals up to 3 h, showed that complete conversion was obtained after 5 min with the concentrations of aldoses employed.

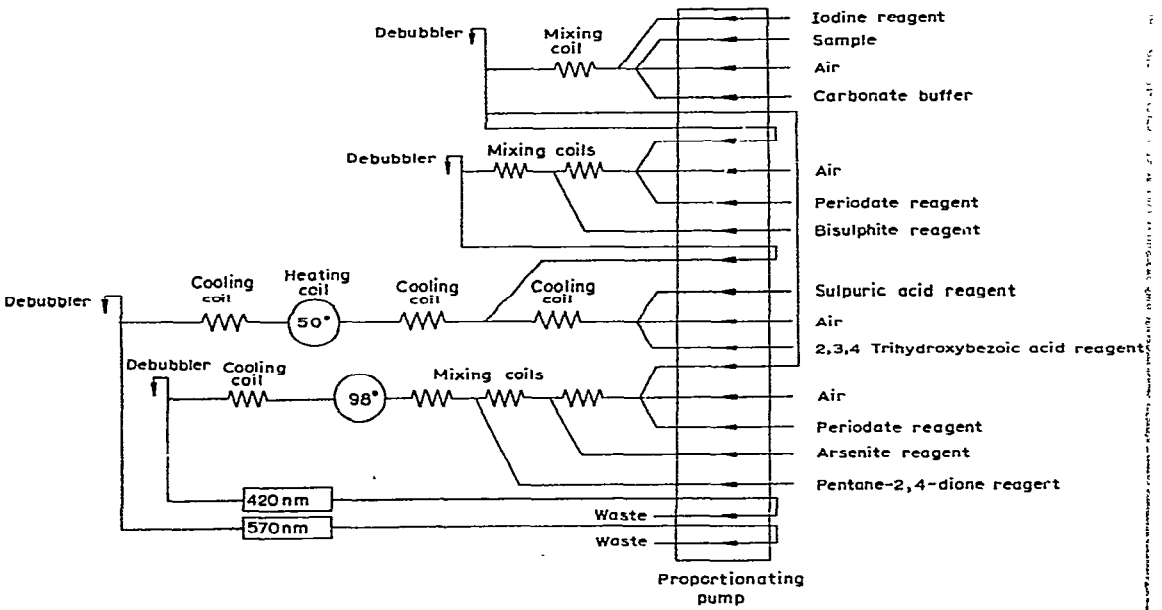

Fig. 1. Schematic representation of the automated system for the hypoiodite oxidation. Reagent flow-rates: iodine reagent (0.05 ml/min), sample (0.10 ml/min), air (0.10 ml/min), carbonate buffer (0.05 ml/min), sample from debubbler to THBA assay (0.05 ml/min), air (0.10 ml/min), periodate reagent (0.05 ml/min), bisulphite reagent (0.05 ml/min), sample from debubbler (0.10 ml/min), sulphuric acid reagent (0.68 ml/min), air (0.32 ml/min), 2,3,4-trihydroxybenzoic acid reagent (0.03 ml/min), waste-return line (0.43 ml/min), sample from debubbler to pentane-2,4-dione assay (0.05 ml/min), air (0.16 ml/min), periodate reagent (0.05 ml/min), arsenite reagent (0.10 ml/min), pentane-2,4-dione reagent (0.10 ml/min), waste-return line (0.16 ml/min).

Automation of the hypoiodite oxidation. — Technicon Auto Analyzer modular equipment was employed throughout, and a schematic representation is shown in Fig. 1. The solution of carbohydrate was sampled continuously and mixed with sodium carbonate buffer (0.1M, pH 10.5), air, and iodine solution (12.5mM in potassium iodide, 10 g/l) during 14 min. After debubbling, the reaction stream was split into two portions, and each was recycled through the pump. Stream (a) was mixed with air and periodic acid (25mM in 62.5mM sulphuric acid) during 15 min. Oxidation was terminated with M sodium metabisulphite, and the stream was debubbled and recycled through the pump. 2,3,4-Trihydroxybenzoic acid (10 mg/ml in ethanol–water, 1:2) and conc. sulphuric acid (reagent grade) were mixed, segmented by air bubbles, and immediately cooled in a jacketed coil. The resulting solution was mixed with the sample from periodate oxidation and again cooled. The reaction stream was heated for 10 min at 50° to develop the characteristic chromophore (λ_{max} 570 nm), cooled, and debubbled, and the absorption was determined at 570 nm. Stream (b) was mixed with air and periodic acid (25mM in 62.5mM sulphuric acid), and oxidised for 4 min. Oxidation was terminated with arsenite reagent and, after further mixing, pentane-2,4-dione was added, The reaction stream was heated at 95° for 2 min, cooled, and debubbled, and the absorption at 420 nm was determined.

Hypoiodite oxidation of some reference compounds. — Aliquots (0.1 ml) of solutions of D-glucose, D-glucono-1,5-lactone, maltose, maltobionic acid (0.5 calcium salt), lactose, lactobionic acid (0.5 calcium salt), lactitol, and D-galactose (all 10 mg/ml) were diluted with water (10 ml). To each solution, sodium carbonate buffer (0.2M, pH 10.5; 1.0 ml) was added, and aliquots were withdrawn and analysed for total sugar content by the cysteine–sulphuric acid method. To the remaining solutions, 0.05M iodine solution in 0.24M potassium iodide (0.5 ml) was added, and the mixture was maintained at room temperature for 30 min. Aliquots (0.1 ml) were withdrawn and analysed for aldonic acids by using the THBA method. The results are shown in Table I.

Solutions (~200 μg/ml) of D-glucose, D-glucono-1,5-lactone, maltose, maltobionic acid (0.5 calcium salt), cellobiose, lactose, lactobionic acid (0.5 calcium salt),

TABLE I

HYPOIODITE OXIDATION OF SOME REFERENCE COMPOUNDS (MANUAL)

Compound	*Oxidation sample (mole)*	*Glyoxylic acid (mole)*	*Molar yield of aldonic acid*
D-Glucose	1.00	0.98	0.98
D-Glucono-1,5-lactone		1.00	
Maltose	0.51	0.51	1.00
Maltobionic acid	0.45	0.43	0.96
Lactose	0.51	0.57	1.10
Lactobionic acid	0.49	0.50	1.02
Lactitol	0.50	0.00	0.00
D-Galactose	1.00	1.00	1.00

and D-galactose were oxidised by hypoiodite, using the automated system. Glyoxylic acid and formaldehyde, produced on oxidation with periodate, were determined by the automated THBA and pentane-2,4-dione procedures (Table II).

Solutions of various substituted aldoses were subjected to the automated procedure, and analysed for glyoxylic acid and formaldehyde produced on oxidation with periodate (Table III).

TABLE II

AUTOMATED HYPOIODITE OXIDATION OF SOME REFERENCE COMPOUNDS

Compound	*Yield of aldonic acid (mole)*
D-Glucose	0.88
Maltose	0.86
Maltobionic acid	0.86
Cellobiose	0.89
Lactose	1.00
Lactobionic acid	1.25
D-Galactose	0.90

TABLE III

AUTOMATED HYPOIODITE OXIDATION OF SOME OLIGOSACCHARIDES AND SUBSTITUTED HEXOSES FOLLOWED BY PERIODATE OXIDATION

Compound	*Glyoxylic acid (mole)*	*Formaldehyde (mole)*
Maltose	1.04	1.06
Lactose	1.12	1.00
Lactulose	0.09	0.89
Melibiose	1.12	0
Isomaltose	0.99	0
6-*O*-Methyl-D-galactose	1.12	0
Laminaribiose	0	1.02
Turanose	0	1.06
3-*O*-Methyl-D-glucose	0	0.99
6-*O*-β-L-Rhamnopyranosyl-D-glucose	1.12	0

Analysis of oligosaccharides in column eluates. — Fractionation of oligosaccharides was effected by chromatography on a column (90 × 5 cm) containing cation-exchange resin (AG 50 x2, 200–400 mesh, lithium form). The column was eluted with water at a rate of 2.0 ml/min. The eluate was monitored by the automated cysteine–sulphuric acid method for total hexose content, and the automated hypoiodite oxidation followed by the THBA and pentane-2,4-dione methods for glyoxylic acid and formaldehyde produced on oxidation with periodate, In a typical separation, a mixture of starch, laminaripentaose, isomaltotriose, maltose, and D-glucose (10 mg/ml, 1.0 ml) was fractionated (Fig. 2).

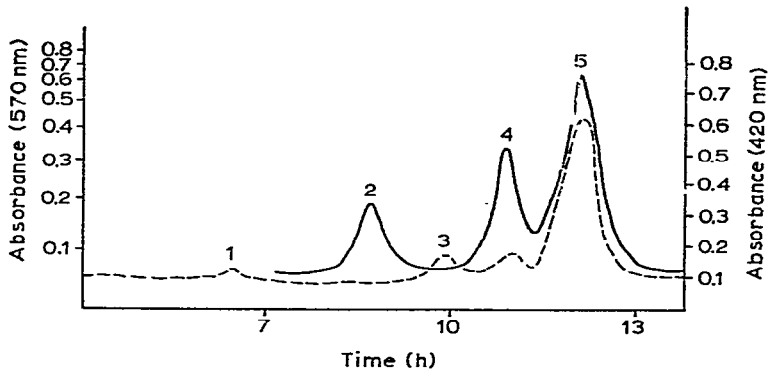

Fig. 2. Analysis of column eluates by the automated hypoiodite-oxidation procedure. Starch (1), laminaripentaose (2), isomaltotriose (3), maltose (4), and D-glucose (5); pentane-2,4-dione assay, ———; 2,3,4-trihydroxybenzoic acid assay, — — —.

DISCUSSION

Halogen or hypohalite oxidations are the most common methods for conversion of aldoses into aldonic acids. In the oxidation system, oxidant must be removed prior to cleavage of α-glycol with periodate, in order to avoid further oxidation of the glycol-cleavage products. This would not only remove glyoxylic acid by oxidation to oxalic acid but also result in the formation of a glyoxylic acid acetal by oxidation of the "dialdehyde" produced by glycol cleavage of the sugar ring. Similarly, if the "dialdehyde" is exposed to an alkaline environment, a glyoxylic acid acetal may be produced by a Cannizzaro reaction[5]. Alkaline solutions of iodine stoichiometrically oxidise aldoses, methylated aldoses, and oligosaccharides to the corresponding aldonic acids[6]. Change in the reaction pH to an acid value should effectively remove hypoiodite and thus terminate the oxidation. This could not be used, for example, to terminate the oxidation of aldoses by hypochlorite since, at pH 4.5–5.0, this reagent causes cleavage between C-1 and C-2 of the aldonic acid formed at alkaline pH[7]. The subsequent oxidation with periodate has to be performed at pH 1.0 in order to prevent loss of the glyoxylic acid by overoxidation[2]. Under drastic conditions, hypoiodite oxidation may yield keto acids, for example, a 5-hexulosonic acid from D-glucose[8]. Ketoses are essentially inert to the action of hypoiodite under the conditions for oxidation of aldoses, although oxalic acid may be produced[9] under more drastic conditions. In general, halogen oxidations are complicated by the change in the nature of the oxidation as the conditions of temperature, acidity, and concentration vary.

Excess hypoiodite is normally removed by precipitation with silver carbonate or acetate. Although this is satisfactory in a manual analysis, albeit time-consuming, precipitations are not amenable to automated, flow-through systems. Reducing agents cannot be used since this would prohibit subsequent oxidation with periodate.

Examination of the reaction of D-glucose with iodine showed that, as expected, no D-gluconic acid was formed (as determined by periodate oxidation to glyoxylic

acid) at an acid pH. A precipitate of iodine was observed on acidification, which, although not interfering with the periodate oxidation, was not satisfactory for the automated system. However, this precipitation was avoided by dilution of the hypoiodite reagent (two-fold) without affecting the quantitative nature of the reaction. Careful control of the proportions of reagents at this stage was necessary to avoid precipitation. The iodine colour was removed on addition of bisulphite at the termination of the periodate oxidation. The periodate reagent and sufficient acid were added simultaneously to avoid complications due to lactone formation.

A study of the oxidation of D-glucose to D-gluconic acid in comparison to standard D-gluconic acid showed that (*a*) hypoiodite reagent after acidification did not interfere in the subsequent determination of glyoxylic acid, and (*b*) a quantitative yield (0.99 mole) of D-gluconic acid could be obtained from D-glucose. Although the quantitative yield of glyoxylic acid indicated that no oxidation of aldehyde groups was occurring during or after glycol cleavage, this experiment did not show whether glyoxylic acid would be formed by oxidation during glycol cleavage. Consequently, the oxidation of various disaccharides and their derivatives was investigated prior to commencing the development of an automated procedure. Since no glyoxylic acid was obtained from oxidation and glycol cleavage of lactitol, no glyoxylic acid could have been formed from further reactions of the "dialdehyde" produced by periodate oxidation. Furthermore, maltose gave a quantitative yield of maltobionic acid as evidenced by the yield of glyoxylic acid (1.00 mole), and authentic maltobionic acid, on treatment with alkaline hypoiodite and subsequent periodate oxidation, gave 0.96 mole of glyoxylic acid.

In the automated procedure, the hypoiodite reagent (iodine solution and carbonate buffer) deteriorated rapidly on standing. However, these reagents were stable individually and were therefore added separately. Analysis of the aldonic acid formed was effected by the automated THBA method, and total sugar content was determined on a separate aliquot by the cysteine–sulphuric acid method. Quantitative yields of aldonic acids were obtained from D-glucose, D-galactose, maltose, cellobiose, and lactose, using the automated procedure (Table II). The ratio of glyoxylic acid obtained from D-glucose and maltose containing the same equivalent of D-glucose was 1:0.49 (theoretical 1:0.50).

The presence of the hypoiodite reagent did not affect the pentane-2,4-dione assay for formaldehyde, provided that a separate periodate-oxidation stage was used (bisulphite cannot be used to terminate the periodate oxidation in this assay). Maltose, laminaribiose, and isomaltose were oxidised to the corresponding aldobionic acids by the automated procedure, and the glyoxylic acid and formaldehyde produced on oxidation with periodate were automatically analysed. Maltose gave quantitative, equimolar yields of glyoxylic acid and formaldehyde. Laminaribiose gave only formaldehyde (1.02 mole), whereas isomaltose gave only glyoxylic acid (0.99 mole). A number of other disaccharides and substituted aldoses were analysed by this procedure (Table III). Pentoses and hexoses (Table IV) gave equimolar yields of glyoxylic acid and formaldehyde. 6-Deoxyhexoses gave only glyoxylic acid. Lactose

and 4-*O*-methyl-D-glucose gave equimolar yields of glyoxylic acid and formaldehyde. 6-*O*-substituted sugars, such as melibiose, 6-*O*-methyl-D-galactose, or 6-*O*-β-L-rhamnopyranosyl-D-glucose, gave only glyoxylic acid, whereas only formaldehyde was obtained from 3-*O*-methyl-D-glucose and sophorose (2-*O*-β-D-glucopyranosyl-D-glucose). Such non-reducing sugars as trehalose did not give either product.

TABLE IV

AUTOMATED HYPOIODITE OXIDATION OF SOME MONOSACCHARIDES FOLLOWED BY PERIODATE OXIDATION

Compound	*Glyoxylic acid to formaldehyde ratio*
D-Galactose	1.08
L-Arabinose	1.09
D-Ribose	1.02
D-Xylose	1.09
D-Lyxose	1.00
D-Mannose	0.90
D-Glucose	0.99
D-Glucono-1,5-lactone	1.00

Substituted ketoses, for example, lactulose and palatinose, did not give significant amounts of glyoxylic acid, the small amount formed being due, presumably, to alkaline epimerisation to the corresponding aldose during hypoiodite oxidation prior to glycol cleavage. Turanose did not give glyoxylic acid, since even if epimerisation did occur, no glyoxylic acid can be obtained from 3-*O*-substituted aldonic acids.

Application of the automatic technique to the analysis of oligosaccharides in a column eluate is illustrated by the fractionation of a mixture of starch, laminaripentaose, isomaltotriose, maltose, and D-glucose, using an ion-exchange resin as support[10] (Fig. 2). As expected, laminaripentaose gave only formaldehyde, isomaltotriose gave only glyoxylic acid, whilst maltose and D-glucose gave both glyoxylic acid and formaldehyde. Thus, the technique can be used to classify the terminal linkage in oligosaccharides in column eluates.

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