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# THE MANUAL AND AUTOMATED DETERMINATION OF ALDONIC ACIDS AND THEIR *O*-GLYCOSYL DERIVATIVES

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## ABSTRACT

Manual and automated spectrophotometric methods are described for the specific determination of aldonic acids by periodate oxidation and reaction with 2,3,4-trihydroxybenzoic acid. In combination with analyses for formaldehyde released on periodate oxidation, and for total aldose, the measurement of glyoxylic acid is employed for the determination of the substitution pattern of *O*-glycosylaldonic acids.

## INTRODUCTION

Aldoses are oxidised to the corresponding aldonic acid by iodine in weakly alkaline solution, under controlled conditions, and the stoichiometry of this reaction has allowed its use in the determination of reducing carbohydrates[1].

Aldonic acids are oxidised by periodate to yield, *inter alia*, glyoxylic acid if HO-2 and HO-3 are unsubstituted. The oxidation of aldobionic acids may be used as a method for distinguishing the mode of terminal substitution in disaccharides. 3-*O*-Substituted aldonic acids will not give glyoxylic acid on oxidation with periodate, in contrast to 4-*O*- and 6-*O*-substituted aldonic acids.

This paper describes manual and automated methods for the specific determination of aldonic acids, and their application to the determination of the substitution pattern of *O*-glycosylaldonic acids in eluates from anion-exchange columns.

## EXPERIMENTAL

*Manual spectrophotometric procedures.* — Glyoxylic acid, produced by oxidation of aldonic acids, was determined by the 2,3,4-trihydroxybenzoic acid (THBA) method[2,3]. Formaldehyde was determined by reaction with pentane-2,4-dione, using a manual version of the automated procedure of Samuelson and Stromberg[4], based on the original method of Nash[5]. Hexose and 6-deoxyhexose were determined

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by a manual version of the automated modification[6] of the original method of Dische *et al.*[7].

Solutions of the sodium salts of laminaribionic, isomaltobionic, and maltobionic acids were analysed by the above-mentioned methods for hexose, glyoxylic acid produced on oxidation with periodate, and formaldehyde produced on oxidation with periodate. The molar yields are shown in Table I.

TABLE I

DETERMINATION OF FORMALDEHYDE AND GLYOXYLIC ACID FROM OXIDATION OF *O*-GLYCOSYLALDONIC ACIDS WITH PERIODATE

| *Compound* | *Glucose determined (mol/mol)* | *Formaldehyde released (mol/mol)* | *Glyoxylic acid released (mol/mol)* |
|---|---|---|---|
| Laminaribionic acid | 0.98 | 0.96 | 0 |
| Maltobionic acid | 0.97 | 0.97 | 0.95 |
| Isomaltobionic acid | 1.02 | 0 | 1.01 |

*Automated analytical procedures.* — All operations were carried out by using the Technicon Autoanalyser system, employing a multipoint recorder when more than one assay method was combined. The automated procedures are shown in outline in Fig. 1, the determination of total hexose[6] being combined with the determination of formaldehyde and glyoxylic acid arising from oxidation with periodate. Reagent composition and flow rates are given in Tables II and III.

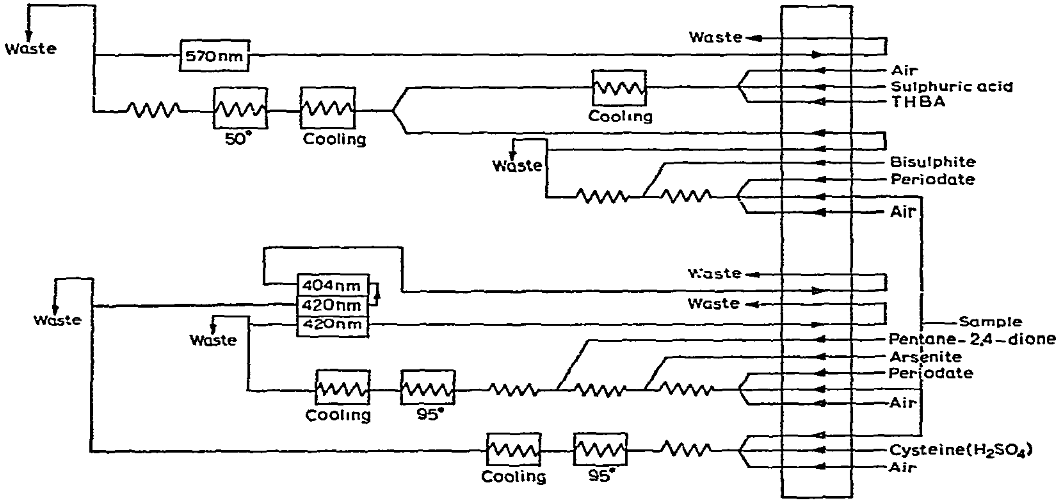

Fig. 1. Schematic diagram of the automated determination of glyoxylic acid and formaldehyde produced on periodate oxidation, and total hexose (reagent composition as in text).

TABLE II

REAGENTS AND FLOW RATES EMPLOYED IN AUTOMATED ANALYSIS OF FORMALDEHYDE

| *Reagent* | | *Flow rate* ($ml.min^{-1}$) |
|---|---|---|
| *A* | Periodic acid (15mM), neutralized with ammonia, in phosphate buffer (0.1M, pH 7.5) | 0.32 |
| *B* | Sodium arsenite (0.25M), adjusted to pH 7 with hydrochloric acid | 0 32 |
| *C* | Pentane-2,4-dione (0.02M) and acetic acid (0.05M) in ammonium acetate solution (M) | 0 60 |
| | Sample | 0.23 |

TABLE III

REAGENTS AND FLOW RATES EMPLOYED IN AUTOMATED ANALYSIS OF GLYOXYLIC ACID

| *Reagent* | | *Flow rate* ($ml.min^{-1}$) |
|---|---|---|
| *A* | Periodic acid (25mM) in sulphuric acid (62.5mM) | 0.05 |
| *B* | Sodium metabisulphite solution (M) | 0.05 |
| *C* | 2,3,4-Trihydroxybenzoic acid (10 mg/ml) in ethanol–water (1:2 v/v) | 0.05 |
| *D* | Conc. sulphuric acid, reagent grade | 1.19 |
| | Sample | 0.05 |
| | Sample on recycling | 0.10 |

*Determination of glyoxylic acid after periodate oxidation.* — The method was based on the manual procedure[3] employing THBA. Sample solution containing aldonic acids was oxidised with periodate for 10 min. Excess of periodate was reduced with sodium bisulphite reagent, and the solution was debubbled and recycled through the pump in order to give an even liquid-flow on mixing with reagents *C* and *D*. After initial cooling, the solution was passed through a bath controlled at 50 ±0.5°, for a total of 16 min, resulting in the formation of the characteristic blue chromophore ($\lambda_{max}$ 590 nm). The solution was cooled, debubbled, and drawn through the colorimeter flow-cell, and the absorption at 570 nm was recorded.

*Determination of formaldehyde after periodate oxidation.* — The basic method of Samuelson and Stromberg[4] was utilised, with modifications in reagent composition and flow rates as indicated in Table II.

*Determination of hexose and 6-deoxyhexose.* — This method was based on a modification[6] of the manual method of Dische *et al.*[7]. The absorptions of the characteristic chromophores (hexose, $\lambda_{max}$ 415 nm; 6-deoxyhexose, $\lambda_{max}$ 400 nm) were determined by using interference filters having absorption maxima of 404 and 420 nm. The concentrations of hexose and 6-deoxyhexose could be calculated by using the differential response at these wavelengths.

*Determination of pentose.* — The original manual method of Dische[8] was employed, with an alteration of reagent composition. In the automated procedure, a system similar to that employed for hexose determination was used, except that the heating coil was maintained at 25° to obtain greater sensitivity with respect to chromophore development ($\lambda_{max}$ 390 nm). In order to obtain good reproducibility, it is important that the junction of reagent and sample flow is cooled.

*Alkaline hypoiodite oxidation of oligosaccharides.* — The oligosaccharide (40 mg) was dissolved in distilled water (100 ml), and sodium hydrogen carbonate–carbonate buffer (0.2M, pH 10.6; 40 ml) and iodine solution (0.05M, 20 ml) were added. After 3 h at 25°, the solution was deionised by treatment with silver carbonate followed by Dowex-50W X8 ($H^+$) resin. After concentration of the solution to 10 ml, the acids were titrated to pH 7.5 with sodium hydroxide (0.5M). Laminaritetraose, isomaltotriose, laminaribiose, isomaltose, and maltose were oxidised by this method; xylobiose, xylotriose, and 6-*O*-β-L-rhamnopyranosyl-D-glucose were oxidised on a proportionately smaller scale (2 mg).

*Anion-exchange fractionation of* O*-glycosylaldonic acids.* — Anion-exchange separations were performed by using AG-1 X8 resin (200–400 mesh) in the acetate form. Aliquots of solutions of the *O*-glycosylaldonates (containing 1–5 mg of aldonate) were applied to a column (132 × 0.5 cm; bed volume, 26 $cm^3$) of the resin and eluted with a linear gradient of ammonium acetate (0→0.2M; total eluant, 200 ml). The column eluate (0.6 ml.$min^{-1}$) was analysed automatically for hexose, 6-deoxyhexose, or pentose (sample rate, 0.05 ml.$min^{-1}$), glyoxylic acid after oxidation with periodate (sample rate, 0.05 ml $min^{-1}$), and for formaldehyde after periodate oxidation (sample rate, 0.23 ml.$min^{-1}$). Typical elution profiles are shown in Fig. 2.

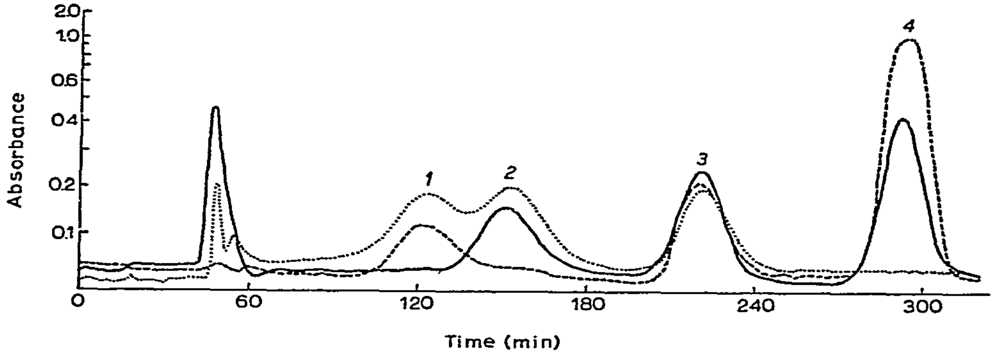

Fig. 2. Anion-exchange chromatography of laminaritetraonic acid (*1*), isomaltotrionic acid (*2*), maltobionic acid (*3*) and D-gluconic acid (*4*): ———, THBA analysis for glyoxylic acid produced on periodate oxidation; ......, cysteine–sulphuric acid analysis for total hexose; — — —, pentane-2,4-dione analysis for formaldehyde produced on periodate oxidation.

Alternatively, *O*-glycosylaldonates were fractionated on smaller columns (26 × 0.5 cm; bed volume, 5 $cm^3$), using a linear gradient of ammonium acetate

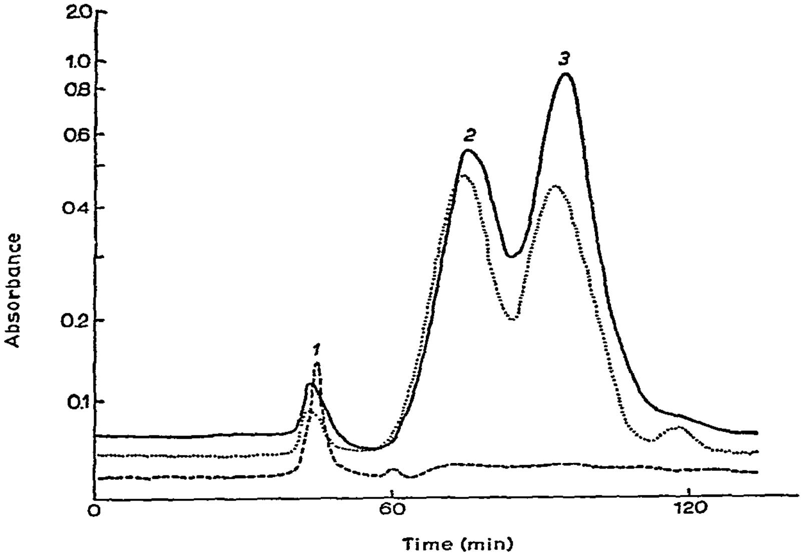

Fig. 3. Anion-exchange chromatography of xylotrionic acid (*2*) and xylobionic acid (*3*) produced from alkaline hypoiodite oxidation of xylotriose and xylobiose; *1* is unreacted oligosaccharide material: ———, THBA analysis for glyoxylic acid produced on periodate oxidation; ......, cysteine–sulphuric acid analysis for total pentose; — — —, pentane-2,4-dione analysis for formaldehyde produced on periodate oxidation.

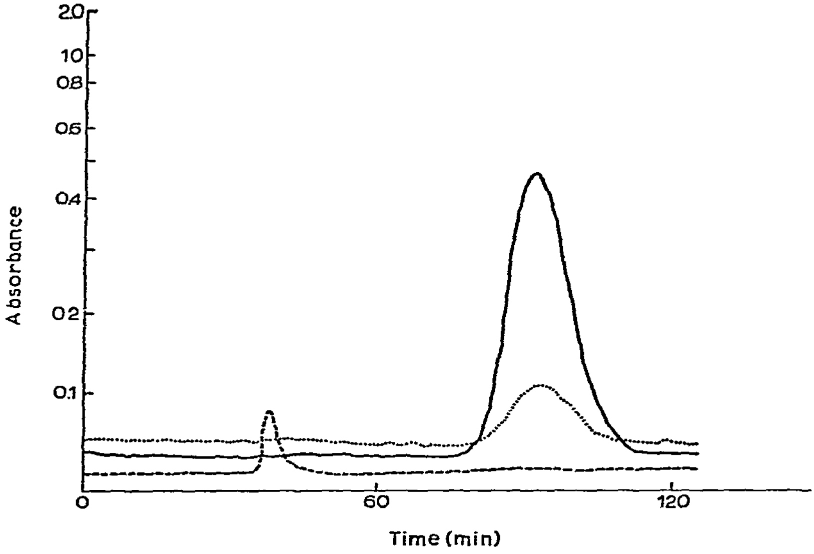

Fig. 4. Anion-exchange chromatography of 6-*O*-α-L-rhamnopyranosyl-D-gluconic acid: ———, THBA analysis for glyoxylic acid produced on periodate oxidation; ......, cysteine–sulphuric acid analysis for total 6-deoxyhexose; — — —, pentane-2,4-dione analysis for formaldehyde released on periodate oxidation.

(0→0.2M, total eluant, 100 ml) (Figs. 3 and 4). The comparative yields of neutral sugar, formaldehyde, and glyoxylic acid, determined by integration of peak areas, are shown in Table IV.

TABLE IV

DETERMINATION OF FORMALDEHYDE AND GLYOXYLIC ACID, RELEASED ON OXIDATION WITH PERIODATE, BY THE AUTOMATED METHODS IN THE ANION-EXCHANGE FRACTIONATION OF *O*-GLYCOSYLALDONIC ACIDS

| *Compound* | *Aldose determined (mol/mol)* | *Formaldehyde released (mol/mol)* | *Glyoxylic acid released (mol/mol)* |
|---|---|---|---|
| D-Gluconic acid | 0 | 1.01 | 1.04 |
| Maltobionic acid | 0.98 | 0.97 | 0.95 |
| Xylobionic acid | 1.03 | 0 | 1.00 |
| 6-*O*-β-L-Rhamnopyranosyl-D-gluconic acid | 1.01 | 0 | 1.01 |
| Isomaltotrionic acid | 1.96 | 0 | 1.03 |
| Xylotrionic acid | 1.95 | 0 | 1.04 |
| Laminaritetraonic acid | 3.00 | 1.07 | 0 |

## DISCUSSION

Oxidation of aldoses to aldonic acids provides a convenient method for introducing a charged functional-group into the molecule, thus facilitating separation by ion-exchange. With oligosaccharides, the *O*-glycosylaldonic acids produced are more readily separated on the basis of molecular size–charge ratio than are the original oligosaccharides by molecular size alone. Furthermore, the introduction of the terminal aldonic acid facilitates classification of the position of substitution in the reducing-sugar residue of the original oligosaccharide. Although various techniques of oxidation with periodate allow determination of the positions of intracatenary linkages, they do not permit distinction of the reducing terminal linkage, with the possible exception of 6-*O*-substitution. Periodate oxidation of aldonic acids gives, *inter alia*, glyoxylic acid from C-1 and C-2 (by oxidation of the C-2–C-3 bond), and formaldehyde from C-6 (by oxidation of the C-5–C-6 bond). By measurement of both glyoxylic acid and formaldehyde produced by oxidation with periodate, 3-*O*-, 4-*O*-, and 6-*O*-substituted hexonic acids may be distinguished.

Glyoxylic acid arising from periodate oxidation may be determined directly by a spectrophotometric method based on 2,3,4-trihydroxybenzoic acid (THBA). Quantitative yields of glyoxylic acid may be obtained from aldonic acids when the oxidation is performed in dilute mineral acid[3]. Direct determination of the formaldehyde and glyoxylic acid produced on oxidation of 3-*O*-, 4-*O*-, and 6-*O*-glycosylaldonic acids (Table I) with periodate showed the ready, quantitative distinction of the three types of substituted acid. Thus, 4-*O*-α-D-glucopyranosyl-D-gluconic acid gave equimolar yields of formaldehyde and glyoxylic acid, whereas 3-*O*-β-D-glucopyranosyl-D-gluconic acid gave formaldehyde but not glyoxylic acid, in contrast to 6-*O*-α-D-glucopyranosyl-D-gluconic acid, which gave glyoxylic acid but no formaldehyde.

The automation of the THBA method for the determination of glyoxylic acid necessitated minor variations in the reagent composition compared with the manual method[3]. An ethanolic solution of THBA, as employed in the manual method, was unsatisfactory because of the deleterious effect of ethanol on the peristaltic-pump tubing, and dissolution of the THBA in aqueous ethanol (1:2 v/v) was therefore required. The THBA reagent and sulphuric acid were mixed in a water-cooled coil before being added as a single reagent to the oxidised sample. Initial, erratic responses due to pulsing were overcome by debubbling the reaction stream after addition of the bisulphite reagent. The chromophore increased linearly with increasing concentration of glyoxylic acid generated from a standard solution of tartaric acid.

Hydroxy-carboxylic acids are generally eluted from anion-exchange resins in order of decreasing molecular weight when a salt gradient is applied. Thus, aldobionic acids are eluted before aldonic acids, and aldobiouronic acids before uronic acids. Introduction of the carboxyl group at the terminal residue of an oligosaccharide enables a separation to be made on the basis of chain length. Combination of the fractionation with automated analyses for glyoxylic acid and formaldehyde released on periodate oxidation and for neutral sugars enables determination of the chain length and terminal linkages of the *O*-glycosylaldonic acids in the column eluate. Fractionation of a series of *O*-glycosylaldonic acids (Fig. 2) illustrates this technique. Peak *1* [3-*O*-β-laminaritriosyl-D-gluconic acid] gave one mole of formaldehyde per three D-glucose residues and no glyoxylic acid (Table III). Peak *2* [6-*O*-α-isomaltosyl-D-gluconic acid] gave one mole of glyoxylic acid per two D-glucose residues and no formaldehyde, whilst Peak *3* [maltobionic acid] gave one mole of glyoxylic acid and one mole of formaldehyde per D-glucose residue. The values obtained demonstrate the feasibility of calculating chain length and terminal-substitution position from the autoanalytical output from the column eluate. Further examples with pentose and 6-deoxy sugars are presented in Figs 3 and 4 (see also Table III). In the pentose series, 4-*O*- and 5-*O*-substituted pentonic acids cannot be distinguished. When a 6-deoxyhexose occupies the terminal position, production of formaldehyde from oxidation of the 6-deoxyhexonic acid by periodate cannot occur, but confirmatory evidence of the substitution pattern may be obtained by determination of the acetaldehyde produced[9].

In the fractionation system described, *O*-glycosylaldonic acids of the same chain-length will not be separated. If the chain length is assumed from the elution position of a particular peak, then combination of the neutral sugar content with the yields of glyoxylic acid and formaldehyde enables the composition of the peak with respect to terminal linkage to be calculated for a peak containing three components each with a different terminal substitution (3-*O*-, 4-*O*-, or 6-*O*-substitution). This problem is obviated by use of a fractionation system which includes separation by sugar type as well as chain length of the *O*-glycosylaldonic acid, for example, by the inclusion of borate in the eluant.

It is hoped that the technique described, for separation and automated analysis of hypoiodite-oxidised oligosaccharides, will prove useful as an aid to the rapid classification of oligosaccharides produced in degradation studies on polysaccharides.

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