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THE DETERMINATION OF ALDONIC ACIDS AND 2-*C*-METHYLALDONIC ACIDS

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ABSTRACT

Specific, spectrophotometric methods are described for the determination of glyoxylic acid from aldonic acids and pyruvic acid from 2-*C*-methylaldonic acids, which allow their determination in admixture. Confirmation of the classification of these aldonic acids is obtained by ion-exchange chromatography of the products of periodate oxidation.

INTRODUCTION

2-*C*-Methylaldonic acids are formed in the alkaline degradation of carbohydrates. D-Fructose gave, *inter alia*, 2-*C*-methyl-D-ribonic acid on treatment with calcium hydroxide[1], and this compound has also been obtained from the degradation of 1-*O*-methyl-D-fructose[2]. 2-*C*-Methyl-D-erythronic and 2-*C*-methyl-D-threonic acids have been isolated from the reaction of D-xylose with calcium hydroxide[3]. Aldonic acids may also arise from the action of alkali on carbohydrates in the presence of oxygen[4,5].

Oxidation of aldonic acids with periodate gives glyoxylic acid, whereas 2-*C*-methylaldonic acids yield pyruvic acid. Pyruvic acid in periodate-oxidation mixtures may be determined by reaction with lactate dehydrogenase[6]. However, this method does not allow the distinction between pyruvic acid and glyoxylic acid where the latter could arise from the periodate oxidation of aldonic acids.

The satisfactory determination and identification of aldonic acids and their 2-*C*-methyl derivatives is of importance. This paper evaluates the methods available, and describes the determination and resolution of these acids by characterisation of the specific products of oxidation with periodate.

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EXPERIMENTAL

Standardised procedure for glyoxylic acid determination (Method A). — Reagents: (*1*) Periodic acid (25mM) in sulphuric acid (62.5mM); (*2*) M sodium metabisulphite; (*3*) 2,3,4-trihydroxybenzoic acid (THBA, 10 mg/ml) in ethanol; (*4*) conc. sulphuric acid.

Method: Sample and standard solutions (0.1 ml), containing 0–0.3 μmole of aldonic acid, were pipetted into stoppered test-tubes, and periodic acid reagent (0.1 ml) was added. The oxidation was terminated after 30 min by the addition of reagent *2* (0.05 ml). After a further 5 min, reagent *3* (0.1 ml) was added followed by reagent *4* (3.0 ml). Heating of the solutions at 50° for 30 min resulted in the production of the characteristic blue chromophore (λ_{max} 590 nm); after cooling to room temperature, the absorbance was determined at 590 nm.

Pyruvic acid, oxalic acid, glycolic acid, β-formylpyruvic acid, malonaldehyde, formaldehyde, acetaldehyde, formic acid, and glycolaldehyde gave no reaction when present in ten-fold excess. Tartronic acid, dihydroxytartaric acid, dihydroxymaleic acid, and D-*arabino*-hexulosonic acid gave the characteristic chromophore after oxidation with periodate.

Variation in colour development with concentration of THBA. — Solutions of tartaric acid (20.5 μg, 0.1 ml) were analysed by the standard procedure, but employing differing concentrations of THBA in reagent *3*. After termination of the oxidation with bisulphite, various volumes (0 to 0.2 ml) of ethanolic THBA (10 or 100 mg/ml) were added. After development of the chromophore, the absorbances were determined at 590 nm. The results are shown in Table I.

TABLE I

VARIATION OF COLOUR DEVELOPMENT WITH CONCENTRATION OF THBA IN THE REACTION WITH GLYOXYLIC ACID

Volume of THBA solution (ml)	0.01	0.02	0.05	0.10	0.20
Absorbance (590 nm; THBA, 10 mg/ml)	0.281	0.605	1.130	1.450	0.295
Absorbance (590 nm; THBA, 100 mg/ml)	1.430	1.770	1.820	1.540	0.795

Variation in colour development with time of heating. — Solutions of tartaric acid (8.0 μg, 0.1 ml) were analysed by the standard procedure, using heating times from 0–60 min at 50°. The rate of colour development is shown in Table II.

TABLE II

VARIATION OF COLOUR DEVELOPMENT WITH TIME OF HEATING IN THE REACTION OF THBA WITH GLYOXYLIC ACID

Heating time (min)	2	4	6	8	10	12
Absorbance (590 nm)	0.484	0.590	0.660	0.700	0.738	0.757
Heating time (min)	15	20	30	40	50	60
Absorbance (590 nm)	0.780	0.818	0.842	0.855	0.870	0.880

Determination of optimal conditions for oxidation with periodate. — Solutions of tartaric acid (27 μg, 1.0 ml) and glyoxylic acid monohydrate (173 μg, 1.0 ml) were placed in duplicate, stoppered test-tubes containing reagent *1* or 25mM periodic acid (1.0 ml). Aliquots were withdrawn at various time-intervals, and the glyoxylic acid content was determined by the standard analytical procedure. The results are shown in Table III.

TABLE III

RELEASE OF GLYOXYLIC ACID ON OXIDATION OF TARTARIC ACID WITH PERIODATE, AND THE STABILITY OF GLYOXYLIC ACID, AS DETERMINED BY THE THBA METHOD

Substrate	*Oxidant* HIO_4 *(25mM)*	*Oxidation time (min)*						
		2	*5*	*10*	*20*	*30*	*60*	*120*
		Absorbance (590 nm)						
Tartaric acid	Aqueous	—	0.950	0.930	0.900	0.820	0.640	0.525
Glyoxylic acid	Aqueous	—	0.760	0.750	0.682	0.655	0.590	0.502
Tartaric acid	In H_2SO_4 (62.5mM)	0.382	0.815	1.190	1.590	1.660	1.550	1.600
Glyoxylic acid	In H_2SO_4 (62.5mM)	1.700	1.690	1.730	1.720	1.540	1.580	1.600

Calibration of the standard method. — Aliquots (0.1 ml) of standard solutions containing tartaric acid (0–50 μg) were analysed for glyoxylic acid content by the standard procedure, a typical calibration and chromophore absorption spectrum being shown in Fig. 1(*a*).

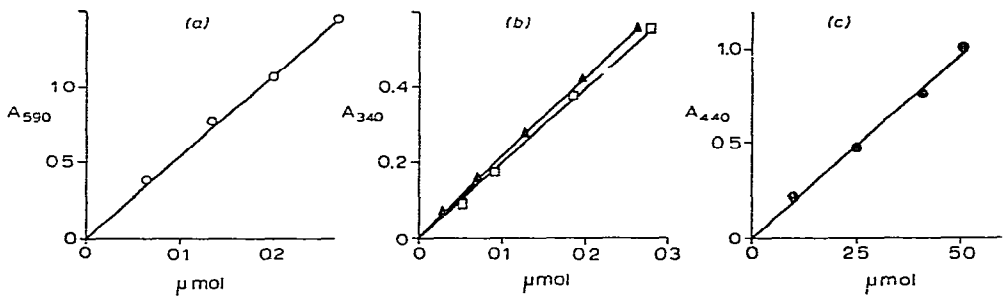

Fig. 1. (*a*) Calibration of the THBA method for glyoxylic acid (method *A*), —○—○—. (*b*) Calibration of the LDH method (method *B*) for pyruvic acid, —▲—▲—; and glyoxylic acid, —□—□—. (*c*) Calibration of the salicylaldehyde method for pyruvic acid (method *C*), —●—●—.

Determination of pyruvic acid and glyoxylic acid with lactate dehydrogenase (*Method* B). — *Reagents:* (*1*) Periodic acid (25mM) in sulphuric acid (62.5mM);

(*2*) aqueous ethylene glycol (1.1 mg/ml); (*3*) sodium dihydrogen phosphate (0.1M) adjusted to pH 7.4 with sodium hydroxide; (*4*) aqueous, reduced nicotinamide adenine dinucleotide (NADH) (3.9 mg/ml); (*5*) lactate dehydrogenase (LDH) suspension (ex beef heart, Koch–Light Ltd.); activity, 150 units/mg.

Method: Sample solutions of pyruvic acid (0–0.36 μmol), weighed as sodium pyruvate, and glyoxylic acid monohydrate (0–0.26 μmol) were diluted to 1.4 ml with water. Reagent *1* (0.1 ml) was added and the solutions were maintained at room temperature for 30 min. The oxidation was terminated by addition of reagent *2* (0.05 ml). After addition of reagents *3* (1.0 ml) and *4* (0.1 ml), the absorbances were determined at 340 nm. Reagent *5* (0.01 ml) was added, and the absorbances were redetermined after 15 min. The resulting calibration graph is shown in Fig. 1(*b*).

Determination of pyruvic acid with salicylaldehyde (*Method* C). — *Reagents:* (*1*) Periodic acid (15mM) in sulphuric acid (62.5mM); (*2*) aqueous ethylene glycol (5.5 mg/ml); (*3*) aqueous potassium hydroxide (1.67 g/ml); (*4*) 2% (v/v) ethanolic salicylaldehyde (redistilled *in vacuo*), freshly prepared before use.

Method: Sample solutions (0.1 ml) containing compounds giving pyruvic acid (0–1.0 μmol) were added to test-tubes containing reagent *1* (0.05 ml) and maintained at room temperature for 30 min. Excess of periodate was reduced by addition of reagent *2* (0.05 ml). After a further 5 min, reagents *3* (0.2 ml) and *4* (0.1 ml) were added and the solutions maintained at 38° for 10 min. After dilution with water (2.5 ml), the absorbance was determined at 440 nm. A typical calibration for sodium pyruvate is shown in Fig. 1(*c*).

Glyoxylic acid, glycolaldehyde, glycolic acid, β-formylpyruvic acid, malonaldehyde, formaldehyde, acetaldehyde, oxalic acid, formic acid, and acetic acid did not interfere in the determination of pyruvic acid when present in ten-fold excess.

Determination of aldonic acids and 2-C-methylaldonic acids by the analytical procedures for glyoxylic acid and pyruvic acid. — Sample solutions containing D-arabinonic acid (weighed as D-arabinono-1,4-lactone), calcium glycerate, and 2-*C*-methyl-D-ribonic acid (weighed as 2-*C*-methyl-D-ribono-1,4-lactone) were determined by the three analytical procedures described. The resulting molar yields of glyoxylic and pyruvic acids are shown in Table IV.

TABLE IV

OXIDATION OF ALDONIC AND 2-*C*-METHYLALDONIC ACIDS WITH PERIODATE

Substrate	*Formaldehyde*	*Products of periodate oxidation (mol/mol)*		
		Glyoxylic acid (THBA method)	*Glyoxylic/pyruvic acid (LDH method)*	*Pyruvic acid (salicylaldehyde method)*
D-Arabinonic acid	0.99	0.93	0.94	—
DL-Glyceric acid	0.97	0.95	0.97	—
2-*C*-Methyl-D-ribonic acid	1.01	—	1.00	1.00

Determination of L-*lactic acid with lactic acid dehydrogenase* (*Method* D). — *Reagents:* (*1*) Sodium carbonate (0.6M) and semicarbazide hydrochloride (0.45M), adjusted to pH 10.5 with sodium hydroxide; (*2*) aqueous nicotinamide adenine dinucleotide (NAD) (20 mg/ml); (*3*) lactate dehydrogenase (LDH) suspension (ex beef heart, Koch–Light Ltd.); activity, 150 units/mg.

Method: Sample solutions (0.4 ml) containing L-lactic acid were added to test-tubes containing reagent *1* (2.0 ml). Reagents *2* (0.2 ml) and *3* (0.025 ml) were added and, after thorough mixing, the solutions were maintained at 30°. After 1 h, the absorbance of the solutions was determined at 340 nm. Calcium D-lactate and pyruvic acid did not depress chromophore development when present in ten-fold excess.

Fractionation of glyoxylic, pyruvic, and lactic acids by anion-exchange chromatography. — Solutions containing glyoxylic acid (400 μg), sodium pyruvate (500 μg), and lactic acid (1 mg) were together loaded onto a column (26 × 0.55 cm) of Dowex-1 x8 (formate form) resin. The column was eluted with a linear gradient of formic acid (0–6.0M; eluant volume, 100 ml) at a rate of 0.6 ml/min. Fractions (20/h) of the eluate were collected, and analysed by method *A* for glyoxylic acid, method *C* for pyruvic acid, and method *D* for lactic acid [Fig. 2(*a*)].

Solutions containing D-arabinonic acid (1 mg) or 2-*C*-methyl-D-ribonic acid (1.2 mg) in distilled water (0.5 ml) were oxidised for 20 min with a solution of periodic

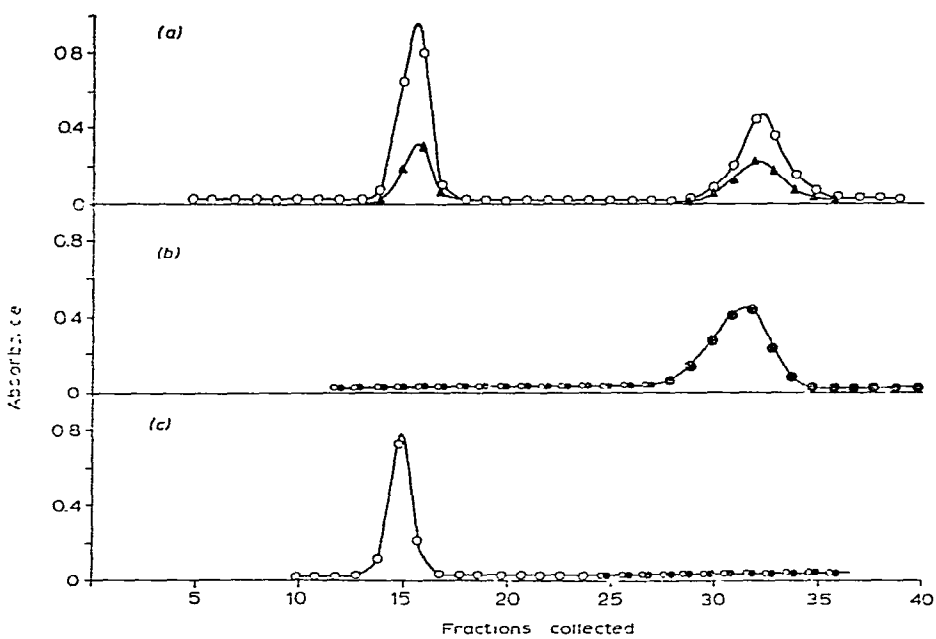

Fig. 2. Anion-exchange chromatography of (*a*) reference glyoxylic acid and pyruvic acid; (*b*) periodate-oxidised 2-*C*-methyl-D-ribonic acid; and (*c*) periodate-oxidised D-arabinonic acid: —○—○—, THBA analysis (method *A*); —▲—▲—, LDH analysis (method *B*); —●—●—, salicylaldehyde analysis (method *C*).

acid (25mM) in 62.5mM sulphuric acid (1.0 ml). Excess of periodate was removed with barium carbonate, the precipitates were removed by centrifugation, and the supernatant solutions were loaded separately onto columns of Dowex-1 x8 (formate form) resin and eluted with formic acid as described above. The resulting fractionations are shown in Figs. 2(*b*) and 2(*c*).

DISCUSSION

Determination of the products of periodate oxidation of carbohydrates provides a method for their quantitative analysis. Furthermore, by use of specific methods of analysis, structural information may also be obtained. Thus, 2-deoxyaldoses, 3-deoxyaldonates, and 3-deoxy-2-*C*-(hydroxymethyl)aldonates may be determined and differentiated by condensation of their respective periodate-oxidation products with 2-thiobarbituric acid[6,7]. Similarly, aldonic acids and 2-*C*-methylaldonic acids may be determined and resolved by oxidation with periodate to glyoxylic and pyruvic acid, respectively.

α-Hydroxy acids and α-keto acids are normally oxidised slowly by periodate[8,9]. In order to obtain quantitative data, conditions under which glyoxylic acid was stable towards oxidation with periodate were required. Glyoxylic acid is readily oxidised by periodate to formic acid and carbon dioxide[8,10–12], complete oxidation being observed, for example, with sodium periodate in acetate buffer (pH 3.6). However, glyoxylic acid (as its *p*-nitrophenylhydrazone) has been isolated in good yield from the oxidation of triose reductone with dilute, aqueous periodic acid[13].

2,3,4-Trihydroxybenzoic acid (THBA) has been employed[14] for the spectrophotometric determination of glyoxylic acid, a characteristic blue chromophore being formed with THBA and glyoxylic acid in conc. sulphuric acid[15]. This procedure has been modified to allow direct determination of glyoxylic acid in periodate-oxidation mixtures. Use of bisulphite proved to be the most satisfactory method for the reduction of excess of periodate, as it is rapid, avoids precipitation of an insoluble periodate or iodate salt, and gives no products interfering in the subsequent determination. Reproducible results were obtained by addition of THBA as an ethanolic solution.

Investigation of the factors that influence the extent of chromophore development in the analysis of glyoxylic acid, under the optimal conditions for periodate oxidation, revealed the conditions for maximal sensitivity. Although use of more-concentrated THBA solution (100 mg/ml) allowed a greater sensitivity (25%), the dilute solution (10 mg/ml) was more convenient, in that precipitation did not occur in the stored reagent, thereby avoiding the necessity for freshly prepared reagent. Examination of possible interfering compounds revealed the specificity of this reagent system for glyoxylic acid.

Accordingly, the stability of glyoxylic acid in periodic acid and periodic acid in dilute sulphuric acid was investigated (Table III). After a reaction time of 2 h in aqueous periodic acid, 66% of the glyoxylic acid remained, whereas in the presence

of sulphuric acid (62.5mM), 94% was intact, as evidenced by the THBA method. Maximal production of glyoxylic acid from tartaric acid occurred after oxidation for 30 min; at this time, less than 4% of the glyoxylic acid was destroyed.

Glyoxylic acid is unsuitable as a reference standard because of the difficulty of obtaining a suitable form for weighing, and therefore tartaric acid was adopted for reagent standardisations. Analysis of D-glyceric and D-arabinonic acid for glyoxylic acid and formaldehyde, produced on oxidation with periodate, with reference to tartaric acid and mannitol standards, respectively, indicated good molar recoveries of glyoxylic acid and formaldehyde (Table IV).

Pyruvic acid may be determined by reduction with lactate dehydrogenase (LDH) to lactic acid, the concomitant oxidation of NADH to NAD providing a change in absorbance at 340 nm which is observable spectrophotometrically. Investigation of the specificity of this enzyme system revealed that glyoxylic acid gave an equimolar response to pyruvic acid in the analytical method. This behaviour renders the LDH method unsuitable for the characterisation of 2-*C*-methylaldonic acids or their determination in the presence of aldonic acids. This led to an erroneous conclusion by Barker *et al.*[6] that 2-*C*-methylaldonic acids were amongst the products of aerobic alkaline-degradation of carbohydrates.

Pyruvic acid may also be determined by reaction with salicylaldehyde. Examination of the specificity of this analysis method revealed that glyoxylic acid neither interfered with, nor depressed, the chromophore formation from pyruvic acid. The original method was adapted for use in periodate-oxidation mixtures, ethylene glycol being a suitable agent for reduction of excess of periodate. The high reagent-blank obtained with this method necessitates the determination of absorptions with reference to a complete reagent-blank. Analysis of D-arabinonic acid, 2-*C*-methyl-D-ribonic acid, and calcium glycerate by the three procedures (Table IV) confirmed the specificity. Glyoxylic acid, produced on oxidation with periodate, from D-arabinonic acid and calcium D-glycerate resulted in equimolar responses in both the THBA and LDH methods. In contrast, 2-*C*-methyl-D-ribonic acid gave an equimolar response in the analysis by the LDH and salicylaldehyde methods, but no response with the THBA procedure.

Confirmatory evidence of the nature of the product from the oxidation of aldonic acids and 2-*C*-methylaldonic acids may be obtained by ion-exchange separation. Fractionation of authentic pyruvic, glyoxylic, and lactic acids by chromatography on Dowex-1 x8 (formate form) resin with a linear concentration-gradient of formic acid gave a convenient separation of these acids. Analysis of fractions by the three methods described above enabled classification by specific determination and elution position. Application of this technique revealed its value in the periodate oxidation of D-arabionic acid and 2-*C*-methyl-D-ribonic acids (Fig. 2). D-Arabinonic acid gave glyoxylic acid, whereas 2-*C*-methyl-D-ribonic acid gave pyruvic acid, as evidenced by both elution position and analysis response.

Combination of the two specific, spectrophotometric methods (the THBA method for glyoxylic acid and the salicylaldehyde procedure for pyruvic acid) for

the products of oxidation with periodate allows the distinction of aldonic from 2-*C*-methylaldonic acids. Confirmation may be obtained by fractionation of the periodate-oxidation products by anion-exchange chromatography, thus enabling satisfactory resolution and determination of these structurally related acids.

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