STRUCTURAL ANALYSIS BY HYPOIODITE OXIDATION OF PERIODATE-OXIDISED CARBOHYDRATES

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ABSTRACT

A method has been established for the structural analysis of polysaccharides, involving the application in sequence of periodate oxidation, hypoiodite oxidation, and acid hydrolysis. The resulting products have been separated by anion-exchange chromatography. L-Lactic acid was determined by reaction with lactic acid dehydrogenase and total lactic acid by conversion into acetaldehyde. Oxidation of the "polyaldehyde" to the "polyacid" with hypoiodite gave higher yields than use of bromine in the presence of strontium carbonate. Methyl α-L-rhamnopyranoside, on degradation, gave L-lactic acid and glyoxylic acid, whereas methyl 6-deoxy-α-D-allopyranoside yielded D-lactic acid and glyoxylic acid. Methyl α-D-glucopyranoside, on degradation, gave glyceric acid, the reactions of which were consistent with its being the D-isomer. Degradation of the polysaccharide nigeran gave equimolar amounts of erythronic acid and glucose.

INTRODUCTION

When determinations of polysaccharide structure are made by the Smith[1] method, difficulties may be encountered in isolating the polyalcohols and often very low yields of glycolaldehyde are obtained.

Fragments released on hydrolysis of the polyacid have previously been separated by paper chromatography[2], or by use of ion-exchange resins[3]. Column chromatography is the most desirable form of analysis since it is amenable to quantitation and automation.

The method of degradation involving further oxidation of the polyaldehyde to the polyacid was examined in detail, since it was considered that the absolute configuration of suitably linked sugar units in the polysaccharide would be revealed, and the separation of characteristic fragments would be easily achieved because of their acidic nature.

METHODS

Analytical procedures. — Uptake of periodate by carbohydrates was determined by using u.v. absorbance[4] or by titration[5]. 6-Deoxyhexoses and hexoses were deter-

mined by the L-cysteine–sulphuric acid reaction[6]. Glyceric acid and erythronic acid were analysed by release of formaldehyde on periodate oxidation[7,8]. Glycolic acid was determined by reaction with the 2,7-dihydroxynaphthalene–sulphuric acid reagent[9], glyoxylic acid with 2,3,4-trihydroxybenzoic acid–sulphuric acid[10], tartaric acid by release of glyoxylic acid on periodate oxidation[10], and L-lactic acid by reaction with lactic acid dehydrogenase[11]. Total lactic acid was determined by conversion into acetaldehyde by the following procedure. Sample solutions containing lactic acid (0–0.03 μmole, 0.020 ml) were treated with conc. sulphuric acid (M.A.R., 0.20 ml). After mixing thoroughly, the solutions were heated at 85° for 3 min, and then cooled to room temperature. The solutions were diluted by the addition of distilled water (0.10 ml), followed by the addition of aqueous D-fructose (0.4 mM, 0.25 ml) and

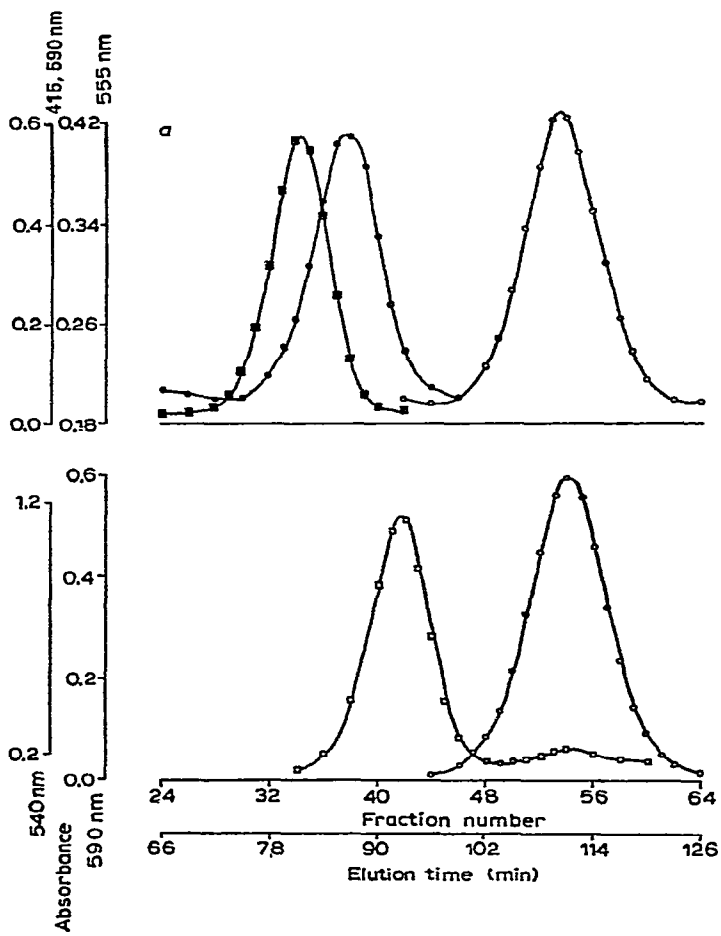

Fig. 1. (*a*) Fractionations on Dowex-AGI (x8, acetate form, 200–400 mesh, column 18×0.6 cm) resin using a linear gradient of ammonium acetate (0→0.5 M, during 3 h). Glyceric acid (–■——■–), lactic acid (–●——●–), glycolic acid (–□——□–), and glyoxylic acid (–○——○–).

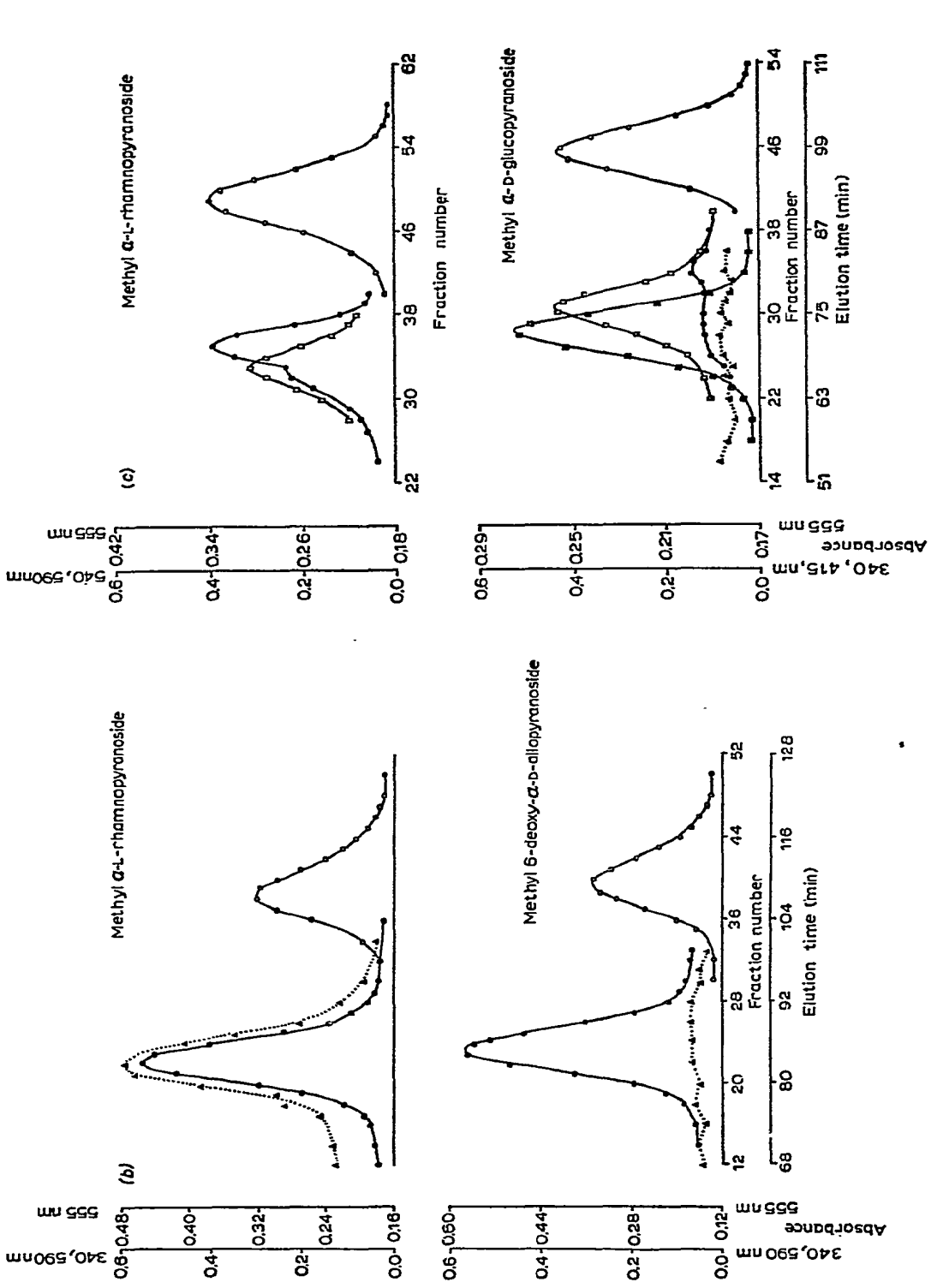

Fig. 1. (*b*) Degradation products of methyl 6-deoxy-α-hexopyranosides; —●—●—, total lactate assay; —○—○—, glyoxylate assay; ...▲.....▲..., LDH assay. (*c*) Degradation products of methyl α-L-rhamnopyranoside and methyl α-D-glucopyranoside; —■—■—, glycerate assay; —□—□—, 2,7-dihydroxynaphthalene–H_2SO_4 assay; —●—●—, total lactate assay; —○—○—, glyoxylate assay; ...▲.....▲..., LDH assay.

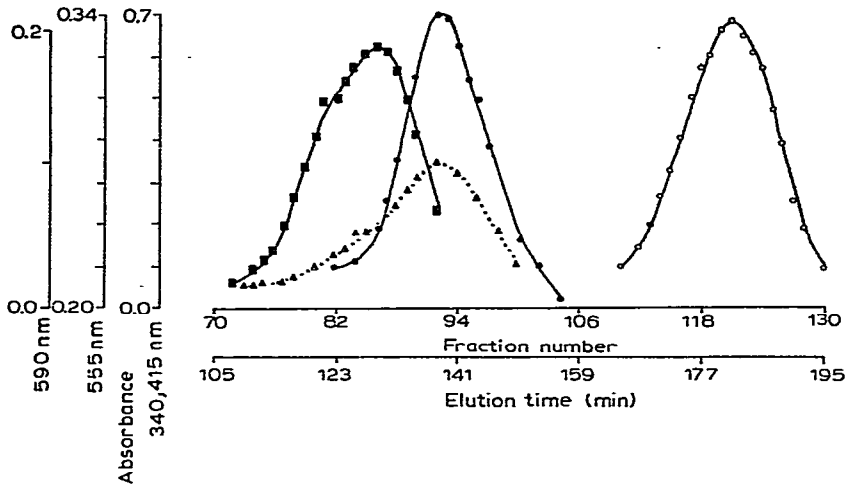

Fig. 2. Fractionation of D-erythronic acid, DL-glyceric acid, L-lactic acid, and glyoxylic acid on Dowex-AG1 (x8, acetate form, 200–400 mesh, column 18 × 0.6 cm) resin using a linear gradient of ammonium acetate (0→0.4M, during 5 h); —■——■—, glyceric acid, erythronic acid assay; —●——●—, total lactate assay; —○——○—, glyoxylate assay; .. ▲ .... ▲ .., LDH assay.

resorcinol (1.1 mM) in hydrochloric acid (10.5M, 2.5 ml). After mixing, the solutions were heated at 80° for 5 min and cooled to room temperature, and the absorbance was measured at 555 nm. A linear calibration was obtained.

Fractionation of degradation products by chromatography on an anion-exchange resin (acetate form). — Columns of Dowex-AG 1-x8 (200–400 mesh, acetate form, 18 × 0.6 cm) were equilibrated by pumping with distilled water at 0.60 ml/min for at least 30 min before use. Products from the degradation of carbohydrates, or standard solutions of D-erythronic acid, DL-glyceric acid, D- and L-lactic acid, glycolic acid, glyoxylic acid, and *meso*-tartaric acid, were brought to neutral pH by the addition of sodium hydroxide prior to application to the column. The column was eluted with a linear gradient of ammonium acetate (0 → 0.5M during 3 h, or 0 → 0.4M during 5 h). Samples of the eluate were analysed manually by using selected assays (Figs. 1*a*, 2). *meso*-Tartaric acid was eluted from the column by washing with hydrochloric acid (0.2M).

Preparation and g.l.c. analysis of the O-*(trimethylsilyl) derivatives of the degradation products.* — Solutions of the products from the degradation of carbohydrates, and standard solutions of the characteristic hydroxy acids, in ammonium acetate (0–0.5M) or hydrochloric acid (0.2M) as appropriate, were treated with Dowex-50W x8 (H^+) resin. After 1 h at room temperature, the resin was removed by filtration and the solutions were evaporated to dryness *in vacuo* (at less than 40°) with repeated co-evaporations with water. The residue was then trimethylsilylated by the hexamethyldisilazane–trimethylsilyl chloride method[12]. The resulting derivatives were separated on SE-30 (10%) with nitrogen carrier gas (40 ml/min), using either a constant oven temperature (150°) or a linear temperature programme (80–195° at 4°/min), in

a Pye 104 chromatograph.

Degradation of methyl α-L-rhamnopyranoside and methyl 6-deoxy-α-D-allopyranoside. — Methyl 6-deoxyhexopyranoside (20.5 μmoles) dissolved in water (5.0 ml) was treated with periodic acid (0.1M, 2.0 ml), and the solution was diluted to a total volume of 10 ml with distilled water. After 23 h at room temperature, the uptake of periodate was found to be constant (2.06 mol.).

An aliquot (5.0 ml) of the reaction mixture was neutralised by the addition of barium carbonate, and, after centrifugation, the solution was treated with Dowex-50W x8 (H^+) resin (5.0 g) at room temperature for 1 h. The resin was removed by filtration and the combined filtrate and washings were concentrated to 5.0 ml. The solution was treated with sodium carbonate(0.2M)–sodium hydrogen carbonate(0.2M) buffer (pH 10.5, 2.0 ml) and a standardised solution of iodine (0.05M, 1.0 ml). After thorough mixing, the solution was left at room temperature in the dark for 3 h. Unconsumed iodine was removed by the addition of silver carbonate, and, after centrifugation, the solution was deionised by the addition of Dowex-50W x8 (H^+) resin (5.0 g). After 1 h, the resin was removed by filtration and the solution concentrated nearly to dryness *in vacuo*.

The residue was hydrolysed in sulphuric acid (0.5M, 5.0 ml) at 100° for 3 h in a sealed tube. The hydrolysate was neutralised by the addition of barium carbonate, and, after centrifugation, the solution was passed through a column of Dowex-50W x8 (H^+) resin (20–50 mesh, 25 × 0.6 cm). The solution was concentrated *in vacuo* to 10 ml, and an aliquot was fractionated on Dowex-AG 1-x8 (acetate form) resin by using a linear gradient of ammonium acetate (0 → 0.5M), total eluate, 100 ml.

Possible degradation of the oxidised methyl 6-deoxyhexopyranosides in the alkaline solution employed for the hypoiodite oxidation was tested by omitting the iodine from the sodium carbonate–sodium hydrogen carbonate buffer. The resulting solution was hydrolysed and neutralised, and the products were fractionated as previously described.

Losses of lactic acid and glyoxylic acid in hydrolysates, which occurred on neutralisation of sulphuric acid with barium carbonate, were determined by subjecting standard solutions to the neutralisation procedure. The losses determined were as follows.

	D-*Lactic acid* (μg)	*Glyoxylic acid* (μg)
Before neutralisation of sulphuric acid	1380	2140
After neutralisation of sulphuric acid	1005	530
Loss on neutralisation (%)	27	75

Optimal conditions of hydrolysis of the diacid obtained from the degradation of methyl α-L-rhamnopyranoside were determined by analysis of the L-lactic acid released on hydrolysis using the lactate dehydrogenase method. The results were as follows.

Time (h)	0.0	0.5	1.0	1.5	2.0	2.5	3.0	4.0	5.0
Absorbance (340 nm, 10 mm)	0.105	0.760	0.770	0.770	0.775	0.785	0.790	0.780	0.750

The stability of lactic acid in sulphuric acid (0.5M) at 100° was checked by removing aliquots at intervals and assessing the lactic acid and acetaldehyde which may have been formed. Similarly, the stability of glyoxylic acid to 0.5M sulphuric acid at 100° was determined by removing aliquots and analysing by reaction with lactate dehydrogenase in the presence of sodium dihydrogen phosphate buffer (pH 7.4) and NADH.

The stability of lactic acid and glyoxylic acid to the conditions of hydrolysis are shown in Table I.

TABLE I

STABILITY OF GLYOXYLIC ACID IN SULPHURIC ACID (0.5M) AT 100° USING LDH/NADH ASSAY

Time (h)	0	0.5	1.0	1.5	2.0	2.5	3.0
Absorbance (340 nm, 10 mm)	0.403	0.413	0.408	0.415	0.410	0.401	0.403

STABILITY OF LACTIC ACID IN SULPHURIC ACID (0.5M) AT 100° USING TOTAL LACTATE ASSAY

Time (h)	0	0.5	1.0	1.5	2.0	2.5	3.0
Absorbance (555 nm, 10 mm)	0.090	0.081	0.085	0.090	0.087	0.081	0.083

RELEASE OF ACETALDEHYDE FROM LACTIC ACID IN SULPHURIC ACID (0.5M) AT 100°

Time (h)	0	0.5	1.0	1.5	2.0	2.5	3.0
Absorbance (555 nm, 10 mm)	0.000	0.003	0.005	0.007	0.011	0.006	0.004

The degradation of methyl α-L-rhamnopyranoside was repeated, but, instead of neutralizing the hydrolysate with barium carbonate, an aliquot of the hydrolysate in 0.25M sulphuric acid was brought to pH 7 by dropwise addition of sodium hydroxide prior to fractionation by anion-exchange chromatography. The yields of lactic acid and glyoxylic acid were 79%. In a parallel experiment with methyl α-D-glucopyranoside, the yields of glyceric acid and glyoxylic acid were 82 and 84%, respectively.

The use of bromine instead of iodine to effect the conversion of periodate-oxidised methyl α-L-rhamnopyranoside into the diacid was examined. A solution of periodate-oxidised methyl rhamnoside (derived from 40 μmoles of methyl rhamnoside in 20 ml of water) was treated with barium carbonate. After centrifugation, the solution was deionised with Dowex-50W x8 (H^+) resin and concentrated *in vacuo* to 10 ml. An aliquot (8.0 ml) was treated with distilled water (7.0 ml) and strontium carbonate (90 mg), followed by bromine (0.1M; 3.0 ml containing 2% of potassium bromide). The bromine was added at intervals of 1 h during a period of 3 h at room temperature in the dark. Excess bromine was removed by the addition of silver

carbonate, and, after centrifugation, the solution was deionised with Dowex-50W x8 (H^+) resin. The solution was concentrated *in vacuo* to 2.0 ml, and an aliquot (1.0 ml) was hydrolysed in sulphuric acid (0.5M, 1.0 ml) at 100° for 2 h. The hydrolysate was neutralised with sodium hydroxide and analysed by fractionation on the anion-exchange resin. A trace of lactic acid was found.

This method of oxidation with bromine was checked using glyceraldehyde. DL-Glyceraldehyde (20 μmoles) dissolved in water (5.0 ml) was treated with strontium carbonate (30 mg), and bromine (0.1M, 1.0 ml) was added at intervals of 1 h during 4 h. Silver carbonate was added to remove unconsumed bromine. Analysis of the deionised solution by anion-exchange chromatography revealed the presence of glyceric acid (in a yield of 31%).

Degradation of methyl α-D-glucopyranoside. — The title compound (50 μmoles) dissolved in water (12.5 ml) was treated with 0.1M periodic acid (5 ml), and the solution was diluted with distilled water to a total volume of 25 ml.

The uptake of periodate was followed and found to be constant (2.03 mol.) after 21 h at room temperature.

An aliquot of the reaction mixture was neutralised with barium carbonate, centrifuged, and treated with Dowex-50W x8 (H^+) resin. The solution was concentrated *in vacuo* to 10 ml.

The oxidation by hypoiodite reagent was carried out by treating the solution of dialdehyde (8 ml) with distilled water (7 ml), sodium carbonate–sodium hydrogen carbonate buffer (pH 10.5, 6 ml), and standardised iodine solution (0.05M, 3 ml). After 3 h at room temperature in the dark, the solution was treated with silver carbonate, centrifuged, deionized with Dowex-50W x8 (H^+) resin, and concentrated *in vacuo* to 2 ml.

An aliquot (0.5 ml) of the solution of diacid was treated with distilled water (1.0 ml) and M sulphuric acid (0.5 ml). After 2 h at 100°, the hydrolysate was neutralised (sodium hydroxide), and an aliquot (1.0 ml) was analysed by fractionation by anion-exchange chromatography (acetate form).

Degradation of nigeran. — Nigeran (10 mg) was dissolved in hot, distilled water (50 ml). On cooling, the "anhydro-glucose" content was determined by the cysteine–sulphuric acid method, and an aliquot (20 ml) was treated with 0.1M periodic acid (2.5 ml) and diluted to a total volume of 25 ml with distilled water. The oxidation mixture was left in the dark at 4°, and the periodate uptake and periodate-resistant sugar units were determined[13] at intervals. After 18 h, the uptake of periodate was constant (0.5 mole/mole of "anhydro-glucose"). An aliquot (10 ml) of the reaction mixture was neutralised by the addition of sodium hydroxide (0.1M), and unconsumed periodate was destroyed with 0.5M sodium arsenite (pH 7, 2 ml). The solution was dialysed against running tap water for 18 h and then changes of distilled water for 24 h. Loss of material on dialysis, assessed by use of the cysteine–sulphuric acid method, was ~0%.

An aliquot (10 ml) of dialysed polyaldehyde solution was treated with sodium carbonate–sodium hydrogen carbonate buffer (0.2M, pH 10.5, 4.0 ml) and standardised

iodine solution (0.05M, 2.0 ml) at room temperature for 3 h, at which time iodine consumption was constant. The solution was neutralised with sulphuric acid, and the excess iodine was removed by addition of 0.1M sodium thiosulphate (5.0 ml) and dialysis; loss of polyacid on dialysis was 33%. The dialysed polyacid was concentrated to *ca.* 1 ml and hydrolysed with M sulphuric acid (0.25 ml) at 100° for 4 h.

The hydrolysate was neutralised with sodium hydroxide prior to fractionation on a column of anion-exchange resin (acetate form) using a linear gradient of ammonium acetate (0→0.4M; total volume, 200 ml). Optimal conditions for hydrolysis of the polyacid were established by withdrawal of aliquots during hydrolysis and determination of reducing power by the alkaline ferricyanide method[14]. The results were as follows.

Time (h)	0	1	2	3	4	5
Absorbance (650 nm, 10 mm)	0.050	0.202	0.290	0.309	0.326	0.295

DISCUSSION

Alkaline hypoiodite has been applied to the analysis of aldoses, methylated sugars, and oligosaccharides by Hirst *et al.*[15], who found that the reaction was specific and stoichiometric. However, no application of the method has been made to fragments of smaller molecular weight, such as glyceraldehyde or erythrose, or other typical periodate-oxidation products of polysaccharides.

It was necessary to ascertain whether an internal Cannizzaro reaction[16] or degradation of the oxidised carbohydrate occurred in the alkaline conditions employed for oxidation. Oxycellulose is labile to alkali[17], and easily undergoes degradation to give acidic fragments. The possibility of racemisation of the asymmetric centres in the oxidised carbohydrate has also to be considered.

The hydroxy acids formed by the hydrolysis of the polyacid were separated by chromatography on Dowex-AG1 (x8, acetate form) resin using a gradient of ammonium acetate. The column eluate was analysed by using selected manual assays for hexose and 6-deoxyhexose, glyceric acid, erythronic acid, L-lactic acid, total lactic acid, glycolic acid, and glyoxylic acid. Total lactic acid was determined by conversion into acetaldehyde in sulphuric acid at 85°, followed by application of the fructose–resorcinol–hydrochloric acid reagent[18]. This method is one of the simplest for measuring lactic acid on a micro-scale. A small interference was found from 6-deoxyhexose (10% of the molar response of lactic acid), but this caused no difficulty since the method was only employed in the analysis of acidic products after fractionation. No interference was observed from pyruvic, glyceric, glycolic, or glyoxylic acids.

In the analysis of L-lactic acid by reaction with lactate dehydrogenase, interference was obtained from DL-glyceric acid and glyoxylic acid. In the converse analysis, used mainly for the analysis of pyruvic acid, glyoxylic acid interfered, and the method was in fact employed for the determination of this acid.

These interferences showed the necessity for a separation of the hydroxy acids under investigation before quantitative determination by spectrophotometric methods.

The separation of glyceric, lactic, glycolic, and glyoxylic acids using a gradient elution of ammonium acetate is shown in Fig. 1(*a*). Fractionation of erythronic acid, glyceric acid, lactic acid, and glyoxylic acid is shown in Fig. 2.

One disadvantage of the proposed method of degradation is that it is difficult to determine accurately threonic, erythronic, and glyceric acids in admixture in the ion-exchange separation systems, because of considerable overlap of the components. In these cases, confirmation of identity was obtained by gas–liquid chromatography of the *O*-trimethylsilyl derivatives[19].

The method of degradation was first applied to methyl α-L-rhamnopyranoside and methyl 6-deoxy-α-D-allopyranoside. Oxidation of the methyl 6-deoxyhexopyranoside in sequence with periodic acid and alkaline hypoiodite, followed by acid hydrolysis of the resulting diacid and anion-exchange chromatography of the products, gave lactic acid and glyoxylic acid (Fig. 1*b*) in yields of 61 and 33%, respectively, from the degradation of methyl α-L-rhamnopyranoside, and 64 and 29% from methyl 6-deoxy-α-D-allopyranoside.

Racemisation in alkaline solution did not occur since degradation of methyl α-L-rhamnopyranoside gave only L-lactic acid, and methyl 6-deoxy-α-D-allopyranoside gave only D-lactic acid.

Alkaline degradation of the oxidised methyl 6-deoxyhexopyranoside did not occur to a large extent since only 2% of glycolic acid was formed, although on examining the products arising from the degradation omitting the addition of iodine more glycolic acid was found (12% of possible yield). The low yields of lactic acid (5%) and glyoxylic acid (less than 2%) showed that an internal Cannizzaro reaction did not take place appreciably.

In the hydrolysis of the diacid, the low recoveries of lactic acid (73%) and glyoxylic acid (25%) were caused by adsorption when sulphuric acid was neutralised with barium carbonate. These losses could be decreased by using neutralisation with sodium hydroxide followed by anion-exchange chromatography. In the degradation of methyl α-L-rhamnopyranoside, the recoveries of lactic acid (which was entirely the L-isomer) and glyoxylic acid were both 79% by this procedure. The fractionation is shown in Fig. 1*c*. In a parallel experiment with methyl α-D-glucopyranoside, the yields of glyceric acid and glyoxylic acid were 82 and 84%, respectively (Fig. 1*c*).

The product formed from the hypoiodite oxidation of D-glyceraldehyde was shown not to respond in the lactate dehydrogenase (LDH) assay. A solution of calcium DL-glycerate was found to have a molar response of 50% of that of calcium L-lactate tetrahydrate.

Fractions of column eluate containing glyceric acid formed on degradation of methyl α-D-glucopyranoside gave no response in the LDH assay, suggesting that the glyceric acid formed was the D-isomer.

While examining the degradation products of methyl α-L-rhamnopyranoside and methyl α-D-glucopyranoside by anion-exchange chromatography, the presence

of an unidentified compound(s) was found. This component gave an intense blue colour (λ_{max} 630 nm) with the 2,7-dihydroxynaphthalene–sulphuric acid reagent. No interference in the assay of sugar acids (by release of formaldehyde on periodate oxidation) or of glyoxylic acid was found, although a small interference was noted in the assay of total lactic acid.

The interfering compound, found in all the degradations of methyl glycosides, was apparently non-carbohydrate in origin since it was formed on the addition of iodine solution to sodium hydrogen carbonate–sodium carbonate buffer. Its presence did not affect the analysis of characteristic acid fragments in the degradation mixtures. The identity of the interfering compound has not yet been established.

The above method of degradation was also applied to nigeran which is a linear D-glucan in which the glycosidic linkages are alternately α-(1 → 3) and α-(1 → 4). When the polysaccharide was oxidised with periodic acid, 49% of the "anhydroglucose" units were resistant to oxidation. The polyaldehyde solution was subjected to alkaline hypoiodite oxidation, and the resulting polyacid was hydrolysed; losses of polyaldehyde and polyacid which occurred on dialysis were 0 and 33%, respectively. Anion-exchange chromatography of the products showed glucose, erythronic acid, and glyoxylic acid to be present. The ratio of glucose and erythronic acid (0.96:1.00), together with the periodate consumption of nigeran, are consistent with the known structure of the polysaccharide and the postulated mode of fragmentation.

The possible use of oxidation of aldehydes by bromine[21] in the presence of strontium carbonate was investigated, but the oxidation of DL-glyceraldehyde gave a low yield of glyceric acid (31%). Although bromine oxidations were performed at a more favourable pH, the reaction was of less value than alkaline hypoiodite oxidation because of the low yields.

In conclusion, it has been shown that structural determinations of carbohydrates should be achieved readily by the method reported herein; high recoveries of characteristic fragments are obtained and the degradation is stereospecific. As in all degradations based on periodate oxidation, the fragments obtained are not completely specific for a given linkage (for example, glyceric acid would be obtained from a non-reducing, terminal hexopyranose or (1 → 6)-hexopyranose unit; erythronic acid from a (1 → 4)-hexopyranose or (1 → 6)-heptopyranose unit), thus necessitating care in the interpretation of the data obtained.

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