263. *Studies on Fructosans. Part I. Inulin from Dahlia Tubers.*

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Repeated purification of inulin isolated from the tubers of a known variety of Dahlia failed to remove combined glucose (*ca.* 6%).

Methylated inulin on hydrolysis and separation by solvent extraction and by chromatography on a cellulose column gave 3 : 4 : 6-trimethyl fructose (91%) and tetramethyl fructofuranose (3·2%), together with tetramethyl glucopyranose (2·2%) and a mixture of trimethyl glucoses (3·7%). No dimethyl hexoses were detected, so that a branched structure is excluded. The principal trimethyl glucose component appears to be the 2 : 4 : 6-isomer. The high proportion of tetra- to tri-methyl glucose suggests that the glucose does not arise from the hydrolysis of an associated polyglucosan but is attached directly to the fructofuranose chain. Possible structures are suggested with a chain of about 35 fructofuranose residues with the potentially reducing fructose residue linked to a glucopyranose unit by a sucrose-type linkage, with a second glucopyranose residue linked through $C_{(1)}$ and $C_{(3)}$ at some undetermined position in the chain. The alternative possibility of a trehalose-type linkage between the two glucose residues is considered to be unlikely.

In previous work on the constitution of inulin (Haworth, Hirst, and Percival, *J.*, 1932, 2384), it was deduced that the polysaccharide, which had been shown previously (Haworth and Learner, *J.*, 1928, 619) to be composed mainly of D-fructofuranose residues linked through the 1 : 2-positions was made up of a chain of *ca.* 30 such units. This conclusion was reached on the basis of the isolation of tetramethyl fructofuranose (3·7%) on the hydrolysis of methylated

inulin. No methylated glucose derivatives were recognised among the products of hydrolysis, but Irvine and Montgomery (*J. Amer. Chem. Soc.*, **1933**, **55**, 1988) claimed that 3 : 4 : 6-trimethyl glucose, in small and variable amounts, accompanied the 3 : 4 : 6-trimethyl fructose and tetramethyl fructofuranose of methylated inulin hydrolysates, and it was claimed that the first sugar was produced from the second in some way by the agency of the acid hydrolytic agent.

Many workers, however (Tanret, *Bull. Soc. chim.*, 1893, [iii], **9**, 233; Schlubach and Elsner, *Ber.*, 1929, **62**, 1493; Adams, Richtmyer, and Hudson, *J. Amer. Chem. Soc.*, 1943, **65**, 1369; Ohlmeyer and Pringsheim, *Ber.*, 1933, **66**, 1292), have reported the presence of glucose in the products of hydrolysis of inulin, but it has always been uncertain whether the aldose originated in an associated polyglucosan, was produced from fructose, or formed an essential part of the inulin molecule. With the improved methods of analysis now available it was hoped to throw light on this subject.

Seven recrystallisations from water of a specimen of inulin ($[\alpha]_D$ − 40°) isolated from " Blue Danube " dahlia tubers failed to remove glucose from the polysaccharide, the quantity amounting to 5·7% estimated by paper-strip chromatography (Flood, Hirst, and Jones, *J.*, 1948, 1697) and corresponding to one glucose residue in *ca.* 19 hexose units.

The dahlia inulin was methylated in an atmosphere of nitrogen with sodium hydroxide and methyl sulphate, followed by a treatment with methyl iodide and silver oxide. The methylated inulin was then hydrolysed by oxalic acid in methanolic-aqueous solution, and the products were converted into glycosides with methanolic hydrogen chloride. The mixture was then extracted in a liquid-extractor with light petroleum, as described by Brown and Jones (*J.*, 1947, 1344), to give a mixture of methylglycosides (A), and thereafter with chloroform to give another mixture (B).

Examination, by the paper chromatogram, of the sugars obtained on the hydrolysis of A and B indicated, apparently, the presence of two components of R_G 0·88 and R_G 1·00, respectively. Tetramethyl fructofuranose and tetramethyl glucopyranose have the same R_G value (1·00), but it was possible to distinguish between these sugars and to identify them on the chromatogram when both were present, by the use of aniline oxalate to detect the glucose derivatives, and of urea oxalate (private communication by Dr. J. K. N. Jones) for the methylated fructoses. In this way A was found to contain most of the tetramethyl methylfructofuranoside, together with trimethyl fructose and trimethyl glucoses, whilst B contained tetramethyl methylglucopyranoside with only a little tetramethyl methylfructofuranoside, together with trimethyl sugars as before. A further separation was then carried out on a column of powdered cellulose (Hough, Jones, and Wadman, *J.*, 1949, 2511) after conversion of the glucosides into the free sugars. Pure tetramethyl fructofuranose was isolated from A together with a mixture of trimethyl fructose and trimethyl glucoses. B gave a much smaller yield of tetramethyl fructofuranose, mixed with crystalline tetramethyl glucopyranose, the relative proportions being determined by hypoiodite oxidation; the same method was adopted for the evaluation of the mixtures of trimethyl sugars arising from both A and B.

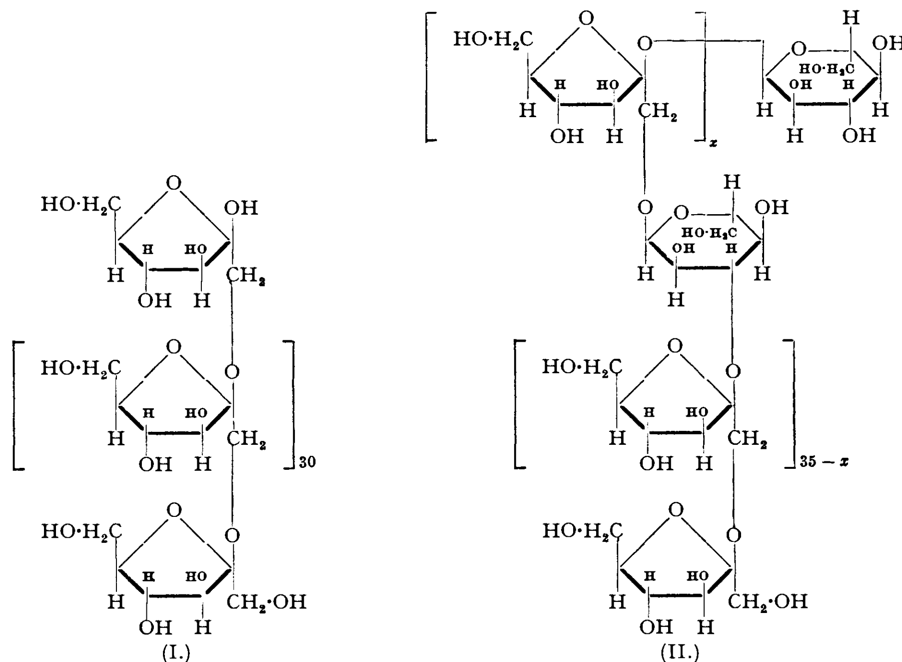
From these experiments it was computed that methylated dahlia inulin gave, on hydrolysis, tetramethyl fructofuranose 3·2%, tetramethyl glucopyranose 2·2%, 3 : 4 : 6-trimethyl fructofuranose 90·9%, and trimethyl glucoses 3·2%. It will be observed that the quantity of methylated glucoses estimated is in good agreement with the glucose content of the polysaccharide.

Owing to the identity of the R_G values of the trimethyl fructofuranose and the trimethyl glucoses, at any rate in the solvent mixtures employed, we were compelled to abandon direct chromatographic methods of separation and to seek others. Partial success was achieved by oxidation with bromine to give the corresponding aldonic acids, followed by the removal of the excess of trimethyl fructofuranose on the cellulose column. The trimethyl glucoses were eventually isolated as methyl esters, the derived amides from which gave a negative Weerman reaction and yielded no formaldehyde on oxidation with periodate. It was diagnosed, therefore, that methoxyl groups were present on $C_{(2)}$ and $C_{(6)}$. To decide between the possible alternatives, 2 : 4 : 6- and 2 : 3 : 6-trimethyl gluconamides, the possibility of the presence of glucofuranose units in the polysaccharide being neglected, the total consumption of periodate was measured in comparison with that of 2 : 3 : 6-trimethyl gluconamide. The result indicated that 71% of the unknown was not attacked by periodate. It is possible, therefore, that the principal component of the mixture was 2 : 4 : 6-trimethyl gluconamide, but we are well aware of the danger of relying exclusively on evidence in which the isolation of crystalline derivatives plays no part.

It is noteworthy that no dimethyl hexoses were encountered in this investigation, so that

the isolation of tetramethyl glucopyranose cannot be accounted for by the postulation of a branched structure. Furthermore, it appears most unlikely that the glucose is derived from an associated polysaccharide, for the high ratio of tetra- to tri-methyl glucose is inexplicable on this basis.

We appear to be left, therefore, with two main alternatives, both of which concern the potential reducing group of the main chain of fructofuranose units, the basic features of which were outlined by Haworth and Learner, and by Haworth, Hirst, and Percival (*locc. cit.*) (I).



(I.) (II.)

The amount of glucose found corresponds to 2 units in a total of 38 hexose units. A structure such as that depicted in (II), on methylation and hydrolysis, would give tetramethyl fructofuranose 3·0% (3·2), tetramethyl glucopyranose 3·0% (2·2), 2 : 4 : 6-trimethyl glucose 2·8% (2·6), and 3 : 4 : 6-trimethyl fructose 91·5% (90·9); the figures in parentheses represent the observed values. It is seen that the chain is terminated by the type of linkage present in sucrose, although no claim can be made to any knowledge of the stereochemical arrangements of the glycosidic linkages. So far as the present evidence goes, the glucose isolated as trimethyl glucose could occur at any intermediate point of the chain and could adjoin the terminal glucose unit. There is another formal possibility, with two glucose residues united as in the trehalose series terminating the chain, but this structure can be dismissed because of the stability of the trehalose linkage. Either formulation would give 1 mole of formic acid from 38 hexose units on oxidation with periodate, and the methylated derivative would have a molecular weight of 7800. The corresponding values found experimentally were 1 formic acid from 48 hexose units and 6210 (Carter and Record, *J.*, 1939, 624).

Although the constitution of inulin cannot be decided without further study, independent evidence exists which can be interpreted as supporting the views outlined above. Bacon and Edelman (*Biochem. J.*, 1949, **45,** xxviii) have reported that in extracts from the subterranean organs of several species of *Compositæ, e.g.*, Jerusalem artichoke tubers, at least six fructose-containing components are present, of which one is sucrose and the remainder have R_F values on the chromatogram ranging between that of sucrose and zero. Furthermore these authors have demonstrated that on hydrolysis under mild conditions the polysaccharide portion gives rise to both glucose and fructose. These studies by Bacon and Edelman (*loc. cit.*) and by Edelman and Bacon (*loc. cit.*, p. xxix) on the enzymic degradation of inulin are clearly highly important and may eventually throw light on the mechanism of its biosynthesis.

Experimental.

Isolation of Dahlia Inulin.—Dahlia tubers (3·3 kg.), variety "Blue Danube" purchased from Dobbie and Co., were minced (February, 1948), and the expressed juice was filtered through cloth. After an hour the filtrate solidified; it was then treated with hot water (1500 c.c.), milk of lime was added to pH 8, and the precipitate removed by filtration. Dilute aqueous oxalic acid was added to the solution at 60—70° to give pH 7, followed by decolorising charcoal; after filtration and chilling to *ca.* 3°, inulin separated. The separated solid (1*a*) was kept in acetone overnight and dried (50 g.).

The mother-liquor was concentrated at 40°/15 mm. to 300 c.c. and a further crop of inulin (1*b*; 35 g.) was isolated.

The minced pulp was extracted with water (5 l.) at 60° for 1½ hours. After treatment as above inulin (2*a*; 64 g.) was isolated and a further crop (2*b*; 108 g.) was obtained from the mother-liquor.

By a final extraction of the pulp with water (4 l.) at 60° for 1 hour a further quantity (3; 29 g.) of inulin, making a total yield of 286 g., was isolated. Examination by the filter-paper chromatogram showed glucose to be present in the hydrolysates of all five fractions, the specific rotations of which varied from −34° to −38°.

Eight "recrystallisations" of fraction 2*b* from hot water followed by chilling gave a product of $[\alpha]_D^{15}$ −40·0° (*c*, 1·0 in water). This specimen was used in the experiments described below.

Hydrolysis with aqueous oxalic acid (2·25%) and estimation of the sugars by the method of Flood, Hirst, and Jones (*loc. cit.*) gave fructose 94·0 and glucose 5·7% (mean of five determinations).

Methylation of Inulin.—To inulin (20 g.) dissolved in sodium hydroxide (280 c.c.; 35%) methyl sulphate (120 c.c.) was added dropwise in portions (25 c.c.) every 30 minutes with constant stirring at 35°, in an atmosphere of nitrogen. The stirring was continued overnight. More sodium hydroxide (200 c.c.; 35%) was then added, followed by methyl sulphate (120 c.c.) in portions as before, the addition extending over 8 hours. After neutralisation with sulphuric acid half the volume of ethanol was added. The precipitated sodium sulphate was filtered off and washed with chloroform and the washings were dried (Na_2SO_4). The aqueous-ethanolic filtrate was evaporated at 35°/15 mm. and the residue extracted with chloroform. The combined chloroform extracts were evaporated to give a pale yellow solid (17 g.) which was dissolved in boiling acetone (500 c.c.). Sodium hydroxide (250 c.c.; 30%) was added, followed by methyl sulphate (3 × 25 c.c.) at 55—60° with stirring under nitrogen as before. This was followed by sodium hydroxide (150 c.c.; 30%) and methyl sulphate (2 × 25 c.c.). Five hours after the addition of the methyl sulphate, acetone was removed by distillation, and the pellets of partly methylated inulin were filtered off and washed with boiling water. After dissolution of the product in boiling acetone (400 c.c.) sodium hydroxide (150 c.c.; 30%) was added, followed by methyl sulphate (50 c.c.) dropwise with stirring under nitrogen. Five hours after the termination of this process further similar quantities of the reagents were added, the product was recovered as before and dissolved in chloroform, the solution dried (Na_2SO_4), and the solvent removed (diminished pressure).

The white solid (18 g.) so obtained was dissolved in methyl iodide, and silver oxide (100 g.) added during 5 days at 45°. The product was recovered by extraction in chloroform, concentration of the extract, and precipitation by the addition of light petroleum (b. p. 40—60°), two fractions, (*a*) 4·2 g. (Found: OMe, 44·9%) and (*b*) 7·4 g. (Found: OMe, 45·5%), being obtained.

Hydrolysis of Methylated Inulin.—Fraction (*b*) (7·0 g.) in methanol (210 c.c.) and water (70 c.c.) was heated with crystallised oxalic acid (2·8 g.) at 80° for 18 hours. The solution was then neutralised with calcium carbonate and evaporated at 40°/15 mm., the syrup extracted with chloroform, and the extract dried (Na_2SO_4), evaporated to a syrup which was dissolved in methanol containing hydrogen chloride (0·25%), and kept at 20° for 70 hours. Barium carbonate was then added, the solvent removed in the presence of the solid, and the residue extracted with chloroform which on distillation at 40°/15 mm. gave a syrup (7·57 g.) which was kept in a vacuum over phosphoric oxide.

Partial Fractionation by Solvent Extraction.—The above syrup was dissolved in water (50 c.c.) and, in the presence of a little barium carbonate, was extracted with purified light petroleum (b. p. 40°) in a liquid-extractor (Brown and Jones, *loc. cit.*) for 3 periods of 3 hours each. The solvent was removed from these extracts, and samples were examined in duplicate on the paper chromatogram after hydrolysis with oxalic acid (0·4 c.c.; 2·25%) at 80° for 2 hours, using urea oxalate as a developing agent (for fructose derivatives) and aniline oxalate. All three samples contained tetramethyl fructofuranose, trimethyl fructose, and trimethyl glucose, and a trace of tetramethyl glucopyranose was present in the third extract. The fractions were then combined to give (A) (1·76 g.).

The original aqueous solution was exhaustively extracted with chloroform, and the extracts were evaporated to give (B) (5·80 g.), a sample of which on hydrolysis and examination on the paper chromatogram was shown to contain tetramethyl glucopyranose, trimethyl fructose, and trimethyl glucose.

Fractionation on the Cellulose Column.—A column of powdered cellulose was prepared in a tube 50 × 3·5 cm., according to Hough, Jones, and Wadman (*loc. cit.*), the column being *ca.* 40 cm. long. The solvent employed for elution was a mixture of purified light petroleum (b. p. 100—120°) (70%) with *n*-butanol saturated with water (30%). Fraction A was hydrolysed with aqueous oxalic acid (120 c.c.; 4%) at 80° for 3 hours. The acid was neutralised with calcium carbonate, and the mixture heated at 90° for 30 minutes. The filtered solution was evaporated to a syrup (1·43 g.) and dried with benzene–ethanol. This syrup was dissolved in the minimum of the solvent and added by pipette to the top of the column, which was then developed with solvent (200 c.c.), no sugars passing through at this stage. The column was then clamped above the rotating disc containing the receiving tubes, which changed position each six minutes, 400 c.c. of solvent being placed in the constant-head flask. When the disc had moved through a circumference the contents of each tenth tube were evaporated and the residue, if any, analysed on the paper chromatogram. Tetramethyl sugars were found in tubes 30—55, and tubes 65 onwards contained trimethyl sugars only. The contents of the first set of tubes were evaporated to give a syrup (*a*) (199 mg.). The contents of the remaining tubes were also concen-

trated and the concentrate (*b*) was set aside. The column was then washed with water to remove the remainder of the trimethyl fraction (*c*).

Fraction B was hydrolysed, and the reducing syrup (5·28) in the minimum of solvent added to the cellulose column. The tetramethyl hexose portion of this eluate (*d*) (198 mg.) was obtained by the evaporation of tubes 15—55; the trimethyl sugars were obtained as before and combined with (*b*) and (*c*) to give (*e*) (5·685 g.) (total recovery, 90·6%).

Fraction (*a*). Examination on the paper chromatogram showed this syrup, $[\alpha]_D^{14}$ +42·1° (*c*, 1·0 in water), to be a mixture of tetramethyl fructofuranose and tetramethyl glucopyranose.

The proportion of tetramethyl glucose was estimated as follows. A known quantity (*ca.* 60 mg.) was dissolved in water (5 c.c.), the opalescence due to a trace of grease removed with "Filter Cel," and the solution filtered. Portions of this solution (*ca.* 500 mg.) were weighed accurately into tubes fitted with ground-glass stoppers, and buffer (pH 11·4; 5·0 c.c.) was added, followed by iodine solution (N/10; 2·5 c.c.). The stoppers were sealed with 10% potassium iodide solution, and the tubes kept at room temperature for 4 hours. Sulphuric acid (4N.; 2·5 c.c.) was then added and the iodine liberated was titrated with sodium thiosulphate (0·02N.). Tubes containing water were treated in an identical manner. Pure tetramethyl fructofuranose having been tested [water blank 12·390 c.c.; solution (63 mg./5 c.c.) 12·385 c.c.] and found not to react with alkaline hypoiodite, the proportion of tetramethyl glucose was estimated to be 13%; it was concluded that (*a*) contained 173 mg. of tetramethyl fructofuranose and 26 mg. of tetramethyl glucopyranose.

Fraction (*d*). Examination on the chromatogram showed the tetramethyl glucopyranose to be contaminated with trimethyl fructose. This syrup was, therefore, separated again on the column, and tetramethyl glucose (135·4 mg.) crystallised completely after evaporation and keeping overnight (*d'*). The trimethyl sugar from this separation was added to (*e*) to give a total of 5·783 g.

Fraction *d'* was not pure tetramethyl glucopyranose; it showed $[\alpha]_D^{14}$ +68° (*c*, 1·0 in water), and hypoiodite oxidation gave a value of 80·7% aldose. It is estimated, therefore, that 109·3 mg. of tetramethyl glucopyranose and 26·1 mg. of tetramethyl fructofuranose were present.

Fraction *d'* was converted into tetramethyl glucopyranose anilide, m. p. 134—135° unchanged on admixture with an authentic specimen (Found: C, 61·7; H, 7·9; N, 4·6; OMe, 38·2. Calc. for $C_{16}H_{25}O_5N$: C, 61·6; H, 8·0; N, 4·5; OMe, 39·8%).

Fraction (*e*). Examination on the paper chromatogram showed (*e*) to contain trimethyl glucose and a trimethyl fructose of identical R_G value, $[\alpha]_D^{14}$ +38° (*c*, 0·9 in water) (Found: OMe, 38%). Hypoiodite oxidation gave an aldose equivalent of 3·9%, which corresponds to 225 mg. of trimethyl glucose and 5·558 mg. of trimethyl fructose.

To sum up, the total sugars estimated amount to tetramethyl fructofuranose 0·199 (3·2%), tetramethyl glucopyranose 0·135 (2·2%), trimethyl fructofuranose 5·558 (90·9%), and trimethyl glucose 0·225 g. (3·7%).

Oxidation of fraction (*e*). Fraction (*e*) in water (60 c.c.) was treated with bromine (2 c.c.). After 4 days at 15° examination on the paper chromatogram showed that trimethyl glucose was still present, and this was still the case after the addition of more bromine and storage for a further 4 days. Lead carbonate was then added to the aqueous solution, together with bromine (2·5 c.c.), and the oxidation was continued for a further 4 days; free bromine was removed by aeration, and the solution neutralised with silver carbonate. After filtration, treatment with hydrogen sulphide was followed by concentration to a thin syrup, which was dissolved in water, neutralised by silver carbonate, and again treated with hydrogen sulphide. Examination on the paper chromatogram showed no trimethyl glucose on spraying with aniline oxalate. When a paper was run in an acidic solvent [acetic acid (10%)–*n*-butanol (40%)–water (50%)] and the dried paper sprayed with bromophenol-blue (0·05%), two yellow spots were observed 4·5 cm. and 5·8 cm. respectively above the solvent boundary.

The syrup was then transferred to a cellulose column and the column eluted with the butanol–light petroleum solvent (1 l.) used previously. The upper 15 cm. of packing were then removed and extracted with water. After evaporation at 40°/15 mm. the residue was esterified with methanolic hydrogen chloride (15 c.c.; 2%) for 9 hours at 65°, the solution neutralised with silver carbonate, and silver in solution removed as sulphide. On removal of the methanol a brown syrup remained which was transferred to a distillation flask. Distillation gave fractions (*w*) bath temperature 90—120°/0·01 mm. (40 mg.) (Found: OMe, 53·0%), and (*x*) bath temperature 120—200°/0·01 mm. (170 mg.). Fraction (*x*) was contaminated with sulphur which was removed by extraction with hot water, filtration, and evaporation, to give (*y*) (50 mg.) (Found: OMe, 45·0%).

Amide formation. Fraction (*w*) was dissolved in methanolic ammonia (3 c.c.) and kept at 0° for 4 days. Crystals of (—)-D-dimethoxysuccinamide separated, having m. p. 283°, $[\alpha]_D^{16}$ —90° (*c*, 1·2 in water) (Found: C, 40·7; H, 6·7; OMe, 34·0. Calc. for $C_6H_{12}O_4N_2$: C, 40·9; H, 6·8; OMe, 35·2%). It was concluded that (—)-D-dimethoxysuccinic acid had been produced from 3 : 4 : 6-trimethyl D-fructose by degradative oxidation, owing to the prolonged contact with bromine.

The syrupy residue obtained on evaporating the methanolic solution was added to the syrupy amide from (*y*).

Fraction (*y*) was treated as above with methanolic ammonia, and a further small quantity of the crystalline dimethoxysuccinamide was removed. The accompanying syrup (49 mg.) had OMe, 35·0% (Calc. for $C_9H_{19}O_6N$: OMe, 36·7%). The syrupy amide (7·4 mg.) in water (2 c.c.) was treated with N-sodium hydrogen carbonate (2 c.c.), followed by periodic acid (2 c.c.; 0·3M.). After 1 hour at 15° hydrochloric acid (3 c.c.; N.) and sodium arsenite (2 c.c.; N.) were added. When the precipitate and yellow colour had disappeared sodium acetate (2 c.c.; N.) and dimedon reagent (1 c.c. containing 85 mg./c.c. in 95% ethanol) were added. A control experiment with gluconamide (8·2 mg.) was carried out. This gave 8·4 mg. of the formaldehyde–dimedon complex (70% of theory), but the amide under investigation gave no formaldehyde.

The amide (12·0 mg.) was tested by the Weerman reaction, with gluconamide as a control: no hydrazodicarbonamide was obtained. To the amide (18·0 mg.) in water (1·5 c.c.) was added sodium periodate (2 c.c.; M/4), and the mixture kept for 5 hours at 15°. A control with 2 : 3 : 6-trimethyl gluconamide

(15·2 mg.) and a water blank were similarly treated. At the end of the experiment the excess of periodate was determined by titration with sodium arsenite solution. The control used 0·95 mole of sodium periodate per $C_9H_{19}O_6N$ and the unknown syrup 0·274 mole.

Oxidation of Inulin by Periodate.—Inulin (*ca.* 50 mg.) was treated with sodium metaperiodate (10 c.c.; M/8) for varying periods, titration with sodium arsenite solution in the presence of potassium iodide being used to determine the amount of periodate remaining. Found: 18 hours, 0·665; 47 hours, 0·874; 85 hours, 0·925; 130 hours, 1·03 moles/$C_6H_{10}O_5$ (final value).

The method of Halsall, Hirst, and Jones (*J.*, 1947, 1427) was used to determine the formic acid liberated by oxidation with potassium periodate; samples of 200—300 mg., potassium chloride (1 g.), and sodium metaperiodate (10 c.c.; M/4) diluted to 50 c.c. were used, with shaking at room temperature in the dark. Samples (5 c.c.) were withdrawn at intervals and titrated with sodium hydroxide (N/100) to methyl-red after destruction of periodate with ethylene glycol. The following figures represent the number of $C_6H_{10}O_5$ residues per mole of formic acid: 138 hours, 62; 192 hours, 57; 213 hours, 49; 305 hours, 47·9; 336 hours, 47·6.

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