663. *Studies of* Aspergillus niger. *Part XI.* Enzymic Synthesis of a Pseudoaldobiuronic Acid.*

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Two acidic oligosaccharides produced when *A. niger* " 152 " was grown in a medium containing maltose and D-glucurone as the sole sources of carbon have been characterised as 2-*O*-α-D-glucopyranosyl-D-glucuronic acid and *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-glucuronic acid.

PREVIOUS studies [1] have shown that *Aspergillus niger* " 152 " contains a transglucosylase capable of synthesising isomaltose and panose [*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose] from maltose. In the present communication D-glucuronic acid has been used as an alternative acceptor to synthesise enzymically a pseudo-aldobiuronic acid, *i.e.*, a glycosylglycuronic acid. Whereas many aldobiuronic acids (*i.e.*, the isomeric compounds in which the glycuronic acid is the non-reducing moiety) have been obtained as acid-stable hydrolysis products of acidic polysaccharides (*e.g.*, cellobiuronic acid from Type III *Pneumococcus* polysaccharide [2]) the relatively acid-labile pseudoaldobiuronic acids have not been so isolated.

When *Aspergillus niger* " 152 " was grown in a medium containing maltose and D-glucurone, oligosaccharides of both the acidic and the neutral type were detected after 60—70 hr. After 150 hr. the mixture of suggars was fractionated on a charcoal–Celite

* Part X, *J.*, 1958, 2583.

[1] Barker and Carrington, *J.*, 1953, 3588.

[2] Reeves and Goebel, *J. Biol. Chem.*, 1941, **139**, 511.

column [3] by gradient elution with aqueous ethanol.[4] After further purification of certain fractions by passage down Deacidite FF (CO_3^{2-}) resin, two chromatographically pure compounds, A and B, were obtained. On the assumption that oxidation with hypoiodite was quantitative,[5] the molecular weight of compound A was 376 (the sodium salt of a pseudoaldobiuronic acid $C_{12}H_{19}O_{12}Na$ requires 378). Under similar conditions the molecular weight of compound B was 518 (the trisaccharide $C_{18}H_{29}O_{17}Na$ requires 540).

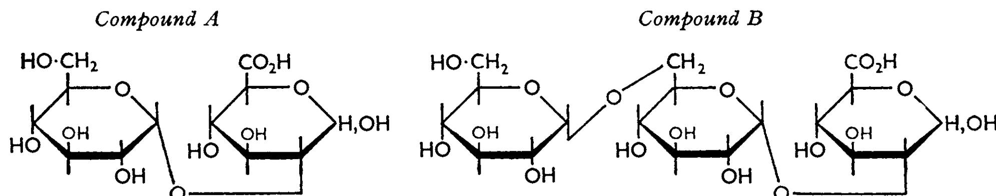
Acidic hydrolysis of compound A gave only D-glucose (characterised as *N-p*-nitrophenyl β-D-glucopyranosylamine) and D-glucuronic acid (characterised as *N-p*-nitrophenyl D-glucuronosylamine). Uronic acid determination [6] showed that a mole of hexuronic acid was contained in 379 g. of compound A; thus the disaccharide contained only one hexuronic acid moiety. The infrared spectra of compound A confirmed the presence of a carboxylic acid group. The sequence of sugar units was determined by oxidation of compound A with hypoiodite and subsequent acid-hydrolysis. Only glucose (characterised as D-glucosazone) and D-glucosaccharic acid (identified by paper ionophoresis in 0·2M-acetate buffer, pH 5) were detected in the hydrolysate, indicating that compound A was a glucosylglucuronic acid. The acid-lability of compound A also indicated this sequence. The high optical rotation ($[\alpha]_D$ +89°) and the infrared spectrum [7] of compound A [type 2a absorption at 852 (Na salt) and 845 cm.$^{-1}$ (free acid)], both indicated an α-glycosidic linkage. Absorption peaks at 919 and 775 cm.$^{-1}$ corresponding to type 1 and type 3 absorption of a pyranose ring were also present.

Preliminary examination suggested the presence of a 1⟶2 linkage in compound A since, like 2-*O*-α-D-glucopyranosyl-D-glucose (kojibiose) and sophorose, this substance did not react with alkaline triphenyltetrazolium chloride [8] under conditions in which D-glucuronic acid, D-glucose, nigerose, maltose, and isomaltose did. A 1⟶2 linkage was also indicated by the low reducing power (56·2% of that calculated for a disaccharide of molecular weight 378) with the cuprimetric Shaffer–Hartmann reagent [9] and its stoicheiometric reaction with alkaline hypoiodite.[5] Compound A was esterified with diazomethane, and the methyl ester then reduced with sodium borohydride. The neutral disaccharide alcohol produced had $[\alpha]_D$ +81°, in confirmation of the assignment of an α-linkage. The alcohol consumed 4·5 mol. of periodate, liberating 2·85 mol. of formic acid and 0·95 mol. of formaldehyde. The theoretical values for 2-*O*-α-D-glucopyranosyl-D-sorbitol are 5, 3, and 1 respectively. As final confirmation of the 1⟶2 linkage, compound A was reduced to kojibiose by a method similar to that applied by Jones and Reid: [10] compound A was converted into its diethyl orthoformate by treatment with triethyl orthoformate and hydrochloric acid and then esterified with diazomethane; reduction of the product with lithium aluminium hydride and removal of the diethyl orthoformate by warm dilute acid afforded a neutral disaccharide. This disaccharide was characterised as kojibiose by (i) its low mobility M_G 0·30 on ionophoresis in borate buffer, (ii) its high optical rotation, $[\alpha]_D$ +121°, (iii) its low reducing power (15·5% of that calculated for a disaccharide) with the Shaffer–Hartmann reagent,[9] (iv) its failure to react with alkaline triphenyltetrazolium chloride,[8] and (v) its almost stoicheiometric reaction (89%) with alkaline hypoiodite.[5] From the above evidence compound A is 2-*O*-α-D-glucopyranosyl-D-glucuronic acid.

The high optical rotation ($[\alpha]_D$ +110°) and infrared spectrum of compound B (type 2a absorption [7] at 835 cm.$^{-1}$; no β-glycosidic absorption at 890 cm.$^{-1}$) indicated that both the glucosidic linkages in this trisaccharide had the α-configuration. The infrared spectrum also showed the presence of a carboxylic acid group (C=O stretching, 1615 cm.$^{-1}$). Partial

[3] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[4] Alm, Williams, and Tiselius, *Acta Chem. Scand.*, 1952, **6**, 826.
[5] Hirst, Hough, and Jones, *J.*, 1949, 928.
[6] Barker, Foster, Siddiqui, and Stacey, *Talanta*, 1958, **1**, 216.
[7] Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171.
[8] Feingold, Avigad, and Hestrin, *Biochem. J.*, 1956, **64**, 351.
[9] Shaffer and Hartmann, *J. Biol. Chem.*, 1921, **45**, 377.
[10] Jones and Reid, *J.*, 1955, 1890.

hydrolysis of compound B liberated glucose, glucuronic acid, isomaltose, and compound A. The sequence of sugar units was determined by oxidation of compound B with hypoiodite and subsequent hydrolysis, the products being glucose, isomaltose, glucosaccharic acid, and a non-reducing acidic disaccharide; no glucuronic acid, gluconic acid or reducing acidic disaccharide was detected. On this evidence compound B was tentatively assigned the structure *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-glucuronic acid (2-*O*-α-isomaltosyl-D-glucuronic acid).



The structures of compounds A and B suggest that the *A. niger* transglucosylase responsible for the synthesis of panose from maltose also synthesised compounds A and B. In this it would resemble closely the transglucosylase obtained from *Betacoccus arabinosaceous* [11] which synthesised panose from a sucrose–maltose mixture but elaborated *O*-β-D-galactopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose in a sucrose–lactose medium.

Experimental

Growth of Aspergillus niger " 152 " *on a Medium containing Maltose*-D-*glucurone*.—Medium (1 l.) was prepared containing ammonium nitrate (2·5 g.), potassium dihydrogen phosphate (1·0 g.), magnesium sulphate heptahydrate (0·25 g.), ferrous sulphate heptahydrate (0·05 g.), zinc sulphate heptahydrate (0·05 g.), N-hydrochloric acid (1 c.c.), maltose hydrate (20 g.), and D-glucurone (20 g.). Erlenmeyer flasks, each containing this medium (50 c.c.), were autoclaved at 15 lb./sq. in. for 20 min., cooled, inoculated with *Aspergillus niger* " 152 " from malt–agar slopes, and incubated at 30°. Aliquot parts (25 μl.), removed under sterile conditions, were analysed by paper ionophoresis in an acetate buffer (pH 5·0), and by paper chromatography with butanol–acetic acid–water (4 : 1 : 5). After 70 hr., a reducing acidic compound, which moved more slowly than D-glucuronic acid on the ionophoretogram, was detected, its concentration increasing to a maximum at 125 hr. After 150 hr., when most of the maltose had been consumed, the culture filtrate and water washings (3 × 50 c.c.) of the mycelium were neutralised, boiled for 10 min. to arrest enzyme action, and cooled. After addition of N-sodium hydroxide until a constant pH of 8·0 was attained, the solution was concentrated *in vacuo* below 40°. Paper ionophoresis (as above) revealed the presence of D-glucuronic acid, two additional acidic compounds (compound A, $M_{\text{glucuronic acid}}$ 0·72; compound B, $M_{\text{glucuronic acid}}$ 0·57; both stained red-brown by aniline hydrogen phthalate [12]), and neutral components. Paper-chromatography (as above) revealed D-glucuronic acid, D-glucose, maltose and at least five oligosaccharides. The solution was freeze-dried (17 g.).

Fractionation of the Mixture.—The mixture (17 g.) in water (150 c.c.) was introduced on to a charcoal–Celite column (length 45 cm.; diameter 6 cm.). Washing with water eluted successively fractions I (350 c.c.; sodium D-glucuronate), II [500 c.c.; sodium D-glucuronate, D-glucose (5·1 g.)], III [600 c.c.; mainly compound A (0·30 g.)], IV [800 c.c.; compound A (0·20 g.)] and V [1700 c.c.; compound A (0·36 g.)]. Gradient elution with 0→5% aqueous ethanol afforded further pure compound A (VI 0·34 g. in 4050 c.c.) and fractions VII (900 c.c.; compound A and neutral oligosaccharides; 0·17 g.) and VIII (150 c.c.; compound A, neutral oligosaccharides, compound B; 0·05 g.). Gradient elution with 5→10% aqueous ethanol eluted the remaining compound B together with neutral oligosaccharides (IX; 0·31 g. in 1600 c.c.) and fraction X (1800 c.c.; neutral oligosaccharides 0·99 g.). Fraction IX (0·28 g.) in water (60 c.c.) was stirred for 24 hr. with Deacidite FF (carbonate form; 12 g.), then packed in a column, and the resin was washed with water. The freeze-dried aqueous eluate yielded

[11] Bailey, Barker, Bourne, Grant, and Stacey, *J.*, 1958, 1895.
[12] Partridge, *Nature*, 1949, **164**, 443.

0·158 g. of neutral oligosaccharides. Elution with N-ammonium carbonate (500 c.c.), treatment of the eluate with Zeo-Karb 225 (H^+), and freeze-drying afforded pure compound B (0·105 g.). Compound A (free acid) was recovered from fraction VII by using the same procedure.

Characterisation of Compound A.—(i) *Optical measurements.* Compound A (sodium salt from IV and VI) was a white hygroscopic powder, having $[\alpha]_D^{18}$ 89·0° (*c* 2 in H_2O). Its infrared spectrum showed peaks (cm.$^{-1}$) at 3380, 2915, 2340, 2115, 1617, 1565, 1545, 1512, 1420, 1362, 1311, 1260, 1207, 1162, 1145, 1050, 955, 919, 852, 775, 670. Compound A (free acid from VII) showed infrared absorption peaks at 3380, 2910, 2330, 2105, 1782, 1645, 1527, 1420, 1355, 1265, 1207, 1155, 1055, 919, 845, and 780 cm.$^{-1}$.

(ii) *Paper chromatography.* Compound A moved as a single component $R_{glucose}$ 0·34 in butanol–acetic acid–water (4 : 1 : 5) and 0·47 in ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4). It could be detected with aniline hydrogen phthalate [12] or silver nitrate–sodium hydroxide,[13] but not with alkaline triphenyltetrazolium chloride.[8]

(iii) *Products of acidic hydrolysis.* Compound A (sodium salt; 50 mg.) was dissolved in 1·5N-sulphuric acid (6 c.c.) and divided into three 2 c.c. portions which were heated at 100° for 1, 2, and 3 hr. severally. Paper chromatography (as above) of the neutralised (barium carbonate) hydrolysates showed only glucose and glucuronic acid in all three hydrolysates. These were combined, concentrated *in vacuo* (40°), and separated into bands on two sheets of Whatman No. 1 paper (46 × 57 cm.) irrigated with butanol–acetic acid–water (4 : 1 : 5) for 3 days.

The strip containing the glucose was eluted and the solution concentrated to a syrup (12·6 mg.). Refluxing this for 15 min. with an aliquot part (0·5 c.c.) of a solution containing *p*-nitroaniline (9 g.), methanol (200 c.c.), and concentated hydrochloric acid (0·14 c.c.) yielded *N*-*p*-nitrophenyl β-D-glucopyranosylamine dihydrate (6·1 mg.). Recrystallisation from methanol yielded pale yellow needles, m. p. and mixed m. p. 184°.

The strip containing the barium glucuronate was eluted, the solution treated with Amberlite I.R.-120 (H^+) to remove barium ions, and concentrated to a syrup (12·8 mg.). Repeated evaporation with methanol and heating for 3 hr. at 40—50° *in vacuo* afforded syrupy glucuronolactone which was treated with *p*-nitroaniline (10 mg.) and methanol containing 0·01% of hydrochloric acid till a clear solution was obtained. After 15 minutes' heating, the solvent was evaporated, and the residue was treated with ether and some spots of methanol and recrystallised to give *N*-*p*-nitrophenyl D-glucuronosylamine, m. p. and mixed m. p. 128—130°. Hamilton, Spriesterbach, and Smith [14] quote m. p. 129—130°.

(iv) *Uronic acid content.* As determined by the method of Barker, Foster, Siddiqui, and Stacey,[6] the weight (g.) of compound A containing one mole of glucuronic acid was 379 ($C_{12}H_{19}O_{12}Na$ requires 378).

(v) *Oxidation by hypoiodite and copper-reducing value.* Under conditions in which maltose was oxidised stoicheiometrically by iodine in alkaline solution (Hirst, Hough, and Jones [5]) compound A, in two determinations, consumed the amount of iodine expected of a reducing disaccharide of molecular weight 376·5 (average of 370 and 383). When analysed by the Shaffer–Hartmann method,[9] compound A (0·965 mg.) had a reducing power equivalent to 0·52 mg. of maltose monohydrate. This corresponded to 56·2% of the calculated value for a disaccharide of molecular weight 378.

(vi) *Hydrolysis of hypoiodite-oxidation product.* Compound A (19 mg.) in water (36 c.c.) was treated with 0·1N-iodine (7·2 c.c.) and a solution (15 c.c.) containing 0·2M-sodium hydrogen carbonate and 0·2M-sodium carbonate. After 2 hr. at room temperature an excess of 30% formaldehyde solution was added and, after 1 hr., the solution was freeze-dried. The residue was dissolved in water and treated with an excess of Amberlite I.R.-120 (H^+). The eluate was neutralised with silver carbonate, concentrated by freeze-drying, then again passed down a column of Amberlite I.R.-120 (H^+). The products (12·4 mg.) obtained by freeze-drying of the eluate were analysed by ionophoresis in 0·2M-acetate buffer (pH 5·0). Non-reducing components were detected with $M_{glucuronic\ acid}$ 0 (weak), 0·97 (weak), and 1·23 (strong). After neutralisation with 0·25N-sodium hydroxide only the component with *M* 1·23 was detected. Oxidised compound A was hydrolysed with 2N-hydrochloric acid at 100° for 2 hr., the hydrochloric acid removed with methyl di-n-octylamine, and the aqueous solution stirred with Deacidite FF (CO_3^{2-}) (0·7 g.) for 20 hr. Elution with water afforded D-glucose (6·8 mg.) which

[13] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.
[14] Hamilton, Spriesterbach, and Smith, *J. Amer. Chem. Soc.*, 1957, **79**, 443.

on being heated for 20 min. at 90° with phenylhydrazine (0·2 c.c.) in 40% acetic acid (1·7 c.c.) gave D-glucosazone, m. p. and mixed m. p. 196—198° (decomp.). The resin was eluted with N-ammonium carbonate (35 c.c.), the eluate treated with Zeo-Karb 225 (H^+), carbon dioxide removed, a few drops of ammonia added, and the solution freeze-dried. Paper-ionophoretic analysis showed that the residue (7·9 mg.) contained only a single compound with the mobility of dipotassium glucosaccharate.

(vii) *Reduction of compound A to* 2-O-α-D-*glucopyranosyl*-D-*glucose*. Compound A (0·38 g.), triethyl orthoformate (10 c.c.), and concentrated hydrochloric acid (0·07 c.c.) were shaken at room temperature for 36 hr. Whenever the suspension became neutral additional amounts of the acid (not exceeding 0·1 c.c. each time) were added up to a total of 0·45 c.c. The mixture was centrifuged, the residue washed with triethyl orthoformate (2 × 25 c.c.), and the supernatant liquid and washings were combined, treated with an excess of diazomethane in ether, and after several hours concentrated *in vacuo* at 40°. The residue was repeatedly dissolved in ethanol, and the solution concentrated to ensure removal of triethyl orthoformate. A solution of the orthoformate derivative of the methyl ester of compound A in dry peroxide-free dioxan (10 c.c.) was stirred for 24 hr. with lithium aluminium hydride (0·3 g.) in dioxan (300 c.c.). The excess of hydride was then destroyed by addition of ethyl acetate (10 c.c.) and afterwards of water (10 c.c.). The filtrate and washings were concentrated *in vacuo* at 40°, and the residue was dissolved in water and freeze-dried (423 mg.). Extraction with acetone and subsequent evaporation gave a syrup (261 mg.) which contained only one non-reducing component, which had $R_{glucose}$ 1·1 in ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4). This orthoformate derivative (260 mg.) was hydrolysed with 0·1N-hydrochloric acid (10 c.c.) at room temperature for 4 days and then at 60° for 5 hr. Paper chromatography in the solvent just mentioned showed components with the mobilities of a disaccharide and glucose. The disaccharide (32·5 mg.) was recovered by streaking the mixture across 8 sheets of Whatman No. 1 paper (46 × 57 cm.), irrigating it with butan-1-ol–ethanol–water–ammonia (40 : 10 : 49 : 1), and eluting the strips containing the disaccharide.

The disaccharide had $[\alpha]_D$ +121° (*c* 0·15 in H_2O) and could be detected on chromatograms with aniline hydrogen phthalate [12] and alkaline silver nitrate [13] but not with triphenyltetrazolium chloride [8]. In the butanol–ethanol–water–ammonia solvent it had $R_{glucose}$ 0·44 and in 0·2M-borate buffer (pH 10) had M_G 0·30. In all these characteristics it was identical with 2-*O*-α-D-glucopyranosyl-D-glucose separated on the same chromatogram. Ionophoresis in 0·2M-acetate buffer of pH 5·0 showed that the disaccharide was neutral and free from acidic impurities. When oxidised by alkaline hypoiodite [5] the disaccharide (2·8 mg.) consumed iodine equivalent to 2·5 mg. of maltose (89% of the theoretical value for a disaccharide). When analysed by the cuprimetric Shaffer–Hartmann reagent,[9] the disaccharide (5·6 mg.) had a reducing power equivalent to 0·87 mg. of maltose hydrate (15·5% of the expected value for a disaccharide, a stoicheiometric reaction being assumed).

(viii) *Reduction of compound* A *to* 2-O-α-D-*glucopyranosyl*-D-*sorbitol*. Compound A (free acid; 190 mg.) in methanol (25 c.c.) was esterified with an excess of diazomethane in ether. The solution was concentrated *in vacuo* at 40°. The residue, dissolved in water (15 c.c.), was added dropwise to sodium borohydride (200 mg.) in water (20 c.c.). After 12 hr. at 18° the excess of hydride was destroyed, Amberlite I.R.-120 (H^+) (10 c.c.) added, and the solution freeze-dried. After removal of boric acid as methyl borate, the freeze-dried residue weighed 123·4 mg. Paper ionophoresis in 0·2M-acetate buffer (pH 5·0) revealed reducing components but showed that the major neutral component was accompanied by some acidic components. These were removed by absorption on Deacidite FF (CO_3^{2-}), and the neutral disaccharide alcohol (45·9 mg.) was recovered by freeze-drying.

The disaccharide alcohol had $[\alpha]_D^{21}$ +81° (*c* 0·3 in water) and moved as a single non-reducing component having $R_{glucose}$ 0·41 in butanol–ethanol–water–ammonia (40 : 10 : 49 : 1) and M_G 0·26 in 0·2M-borate buffer (pH 10). On treatment of the disaccharide alcohol (15 mg.) with 0·04M-sodium periodate (50 ml.) in the dark at room temperature, periodate consumption was 4·4 (4 hr.), 4·5 (14 hr.) and 4·5 (18 hr.) mol. After 18 hr., 2·85 mol. of formic acid were produced and 0·95 mol. of formaldehyde [15] was liberated.

Characterisation of Compound B.—(i) *Optical measurements.* Compound B (sodium salt) had $[\alpha]_D^{19}$ +110° (*c* 0·07 in water). Its infrared spectrum showed peaks (cm.$^{-1}$) at 3420, 2940, 2380, 1615, 1420, 1150, 1110, 1050, 955, 920, 835, and 765.

[15] O'Dea and Gibbons, *Biochem. J.*, 1953, **55**, 580.

(ii) *Paper chromatography.* Compound B moved as a single component of $R_{glucose}$ 0·13 in butanol–acetic acid–water (4 : 1 : 5) and could be detected with aniline hydrogen phthalate [12] and alkaline silver nitrate.[13]

(iii) *Products of partial acid-hydrolysis.* Compound B (14 mg.) was hydrolysed with N-sulphuric acid (7·5 c.c.) at 80—90° for 1 hr. After neutralisation with barium carbonate the solution was freeze-dried (17·5 mg.). Paper ionophoresis in 0·2M-acetate buffer of pH 5·0 showed the presence of neutral compounds, unchanged compound B, an acidic oligosaccharide with the same mobility as compound A, and D-glucuronic acid. Paper ionophoresis in 0·2M-borate buffer (pH 10·0) showed the presence of D-glucuronic acid, D-glucose, compound A, compound B, and probably a disaccharide with the mobility of isomaltose partially overlapping compound B. Paper-chromatographic analysis in butan-1-ol–ethanol–water–ammonia (40 : 10 : 49 : 1) showed D-glucose and isomaltose in addition to three acid compounds near the starting line. The hydrolysate was shaken with Deacidite FF (CO_3^{2-}) (1 g.) for 24 hr., and the filtrate was freeze-dried. Ionophoresis of the residue (5·7 mg.) in 2M-borate buffer (pH 10·0) confirmed that the only neutral compounds present were glucose and isomaltose.

(iv) *Oxidation by hypoiodite.* When oxidised [5] with iodine in alkaline solution, compound B consumed the amount of iodine expected of a reducing trisaccharide of molecular weight 518 (Calc. for $C_{18}H_{29}O_{17}Na$: 540).

(v) *Hydrolysis of hypoiodite-oxidation products.* Compound B (35 mg.) in water (100 c.c.) was mixed with 0·1N-iodine (20 c.c.) and a solution (40 c.c.) containing 0·2M-sodium carbonate and 0·2M-sodium bicarbonate added. After 2 hr., ions, etc., were removed as for compound A, and the oxidised compound B (16 m.) was obtained as a single non-reducing component with $M_{glucuronic\ acid}$ 0·95 in 0·2M-acetate buffer (pH 5·0) detectable with alkaline silver nitrate.[13] The oxidation product was hydrolysed with N-sulphuric acid (2 c.c.) at 90° for 1 hr., and the solution neutralised as above and freeze-dried (17 mg.). Paper ionophoresis in 0·2M-acetate buffer (pH 5·0) showed the presence of reducing neutral components detectable with both aniline hydrogen phthalate [12] and alkaline silver nitrate,[13] and two acidic components only detectable with the latter reagent. One of the acidic components had the same mobility as D-glucosaccharic acid. Paper chromatography in butan-1-ol–acetic acid–water (4 : 1 : 5) showed the presence of, *inter alia,* glucose and isomaltose. No D-glucuronic acid or D-gluconic acid was detected.

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