114. *The Structure of Stachyose.*

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Stachyose from soya-bean meal, on methylation and hydrolysis, yielded tetramethyl D-fructofuranose, tetramethyl D-galactopyranose, 2 : 3 : 4-trimethyl D-galactose, and 2 : 3 : 4-trimethyl D-glucose in equimolecular proportions. These results confirm the structure of stachyose as 6″-α-D-galactopyranosyl-6′-α-D-galactopyranosyl-2-α-D-glucopyranosyl-β-D-fructofuranoside.

The non-reducing tetrasaccharide stachyose, first isolated from *Stachys tubifera* by von Planta and Schulze (*Ber.*, 1890, **23**, 1692), was studied by Onuki (*J. Agric. Chem. Soc., Japan*, 1932, **8**, 445; 1933, **9**, 90; *Proc. Imp. Acad., Tokyo*, 1932, **8**, 496). Mild hydrolysis yielded fructose and a reducing trisaccharide, manninotriose, which on oxidation followed by methylation and hydrolysis gave 2 : 3 : 5 : 6-tetramethyl D-gluconic acid, 2 : 3 : 4-trimethyl D-galactose, and tetramethyl D-galactopyranose. Methylation and hydrolysis of stachyose itself yielded tetramethyl D-fructopyranose, tetramethyl D-galactopyranose, 2 : 3 : 4-trimethyl D-galactose, and a fraction presumed to be 2 : 3 : 6-trimethyl D-glucopyranose, which on complete methylation gave tetramethyl D-glucopyranose. With diazouracil stachyose gives a colour which is believed to be specific for the sucrose linkage (Raybin, *J. Amer. Chem. Soc.*, 1933, **55**, 2603; 1937, **59**, 1402; Purves and Hudson, *ibid.*, 1934, **56**, 709), and fructose is liberated by the action of invertase (Adams, Richtmyer, and Hudson, *J. Amer. Chem. Soc.*, 1943, **65**, 1369). It was also shown that both stachyose and manninotriose were hydrolysed by α-galactosidase, indicating that the linkages of both the galactose residues in the molecule were of the α-form. From the above information it appeared, therefore, that stachyose was 6″-α-D-galactosyl-4′-α-D-galactosyl-2-α-D-glucosyl-β-D-fructoside.

In contradiction to this, however, it was shown that stachyose required seven mols. of periodate for complete oxidation, three mols. of formic acid being liberated (Hérissey, Wickström, and Courtois, *Bull. Soc. Chim. biol.*, 1951, **33**, 642). Samples from three different sources (*Stachys tubifera*, *Plantago maritima*, and *Leucoema glauca*) gave identical results. The structure proposed above would require only six mols. of periodate, while two mols. of formic acid would be produced. The octaldehyde produced in the above reaction, on further oxidation with bromine water, gave a C_8 acid, hydrolysed to glyoxylic, glyceric, and hydroxypyruvic acids. A similar treatment of a compound containing a 1 : 4-linked glucose unit would give D-erythronic acid, but none could be identified. In support of these observations, Hérissey, Wickström, Courtois, and Le Dizet (Congr. Int. Biochim., 1952, Rés. des Comm., p. 311) found that no appreciable amounts of formaldehyde were liberated on periodate oxidation of manninotriose, in accordance with the presence of a 1 : 6-linked glucose unit. Further, manninotriose, on treatment with α-galactosidase for short periods, yielded a disaccharide with the same R_G value on the paper chromatogram as melibiose. These results can only be explained if we assume that, in stachyose, the glucose residue is linked through $C_{(1)}$ and $C_{(6)}$, and hydrolysis of methylated stachyose should, therefore, yield 2 : 3 : 4-trimethyl D-glucose.

In the present investigation soya-bean was used as a source of stachyose. The product, purified by chromatography, showed $[\alpha]_D^{17}$ +102° in water and on hydrolysis yielded galactose (2 mols.), glucose (1 mol.), and fructose (1 mol.). Oxidation with potassium metaperiodate (Halsall, Hirst, and Jones, *J.*, 1947, 1427) in the dark gave formic acid (2·33 mols.). This figure is somewhat lower than that obtained by Hérissey *et al.* (*loc. cit.*); it was found, however, that after prolonged oxidation, the amounts of formic acid produced began to drop, presumably owing to its oxidation. It is thus reasonable to assume that the final value would be higher. Oxidation of stachyose with sodium metaperiodate indicated an uptake of *ca.* 7 mols., and no formaldehyde could be detected.

Stachyose was methylated, first with methyl sulphate and sodium hydroxide, then with the Purdie reagents, to yield a product which showed OMe 48·0%, and $[\alpha]_D^{19}$ +130° in $CHCl_3$ (cf. $[\alpha]_D^{24\cdot5}$ +133·6° in C_6H_6; Onuki, *loc. cit.*). This was hydrolysed and the mixture

of methylated sugars separated by partition on a column of cellulose (Hough, Jones, and Wadman, *J.*, 1949, 2511) into four fractions.

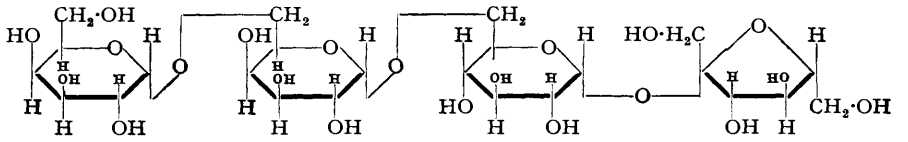
Fraction I (24%; based on weight recovered from the column) gave a spot on the paper chromatogram of the same R_G value as tetramethyl fructofuranose, as well as a small amount of, probably, tetramethyl glucopyranose. Tetramethyl D-fructofuranose was identified by the formation of the crystalline tetramethyl D-fructofuranamide (Avery, Haworth, and Hirst, *J.*, 1927, 2313).

Fraction II (58%) gave spots on the chromatogram corresponding to tetramethyl galactopyranose, 2 : 3 : 4-trimethyl glucose, and a small amount of 2 : 3 : 4-trimethyl galactose. On treatment with methanolic hydrogen chloride, it yielded mixed methylglycosides which after fractionation by solvent extraction (Brown and Jones, *J.*, 1947, 1344) gave syrups (*a*) and (*b*). Hydrolysed (*a*) contained 2 : 3 : 4 : 6-tetramethyl galactose, identified as the aniline derivative, and 2 : 3 : 4-trimethyl glucose; hydrolysed (*b*) contained 2 : 3 : 4-trimethyl glucose and 2 : 3 : 4-trimethyl galactose.

Fraction III (7%) was found by paper chromatography to be a mixture of trimethyl glucose and trimethyl galactose. This was combined with (*b*) and the sugars were separated on a column of cellulose into approximately equal amounts of 2 : 3 : 4-trimethyl D-glucose and 2 : 3 : 4-trimethyl D-galactose. The syrupy glucose derivative (OMe, 41·0%), $[\alpha]_D^{18}$ +60·5°, with periodate (Bell, *J.*, 1948, 992) gave formaldehyde (27% of theory), and authentic 2 : 3 : 4-trimethyl D-glucose gave a 35% yield. Demethylation (Hough, Jones, and Wadman, *J.*, 1950, 1702) afforded only glucose. Methylglycoside formation, both at room temperature and at 75°, followed similar routes {$[\alpha]_D^{18}$ +60° ⟶ −18°; values quoted for 2 : 3 : 4-trimethyl D-glucose are: free sugar, +60°; β-methylglycoside, −21° (Peat, Schluchterer, and Stacey, *J.*, 1939, 581)}. Treatment of the syrup with aniline in ethanol gave crystalline 2 : 3 : 4-trimethyl *N*-phenyl-D-glucosylamine. Examination of the free sugar on the chromatogram confirmed the presence of 2 : 3 : 4-trimethyl D-glucose. No 2 : 3 : 6-trimethyl glucose could be detected.

Fraction IV (11%) crystallised and was fully identified as 2 : 3 : 4-trimethyl D-galactose.

Thus, hydrolysis of methylated stachyose yielded tetramethyl D-fructofuranose, tetramethyl D-galactopyranose, 2 : 3 : 4-trimethyl D-glucose, and 2 : 3 : 4-trimethyl D-galactose in approximately equimolecular proportions. This result is in agreement with the views of Hérissey *et al.* (*loc. cit.*), and stachyose is represented by the annexed formula.



Experimental

Evaporations were conducted under diminished pressure. Temperatures recorded are bath-temperatures. Fractions from the cellulose column were evaporated to dryness, dissolved in water, digested with charcoal, and filtered hot; the aqueous solution was then evaporated to dryness and exhaustively extracted with boiling acetone, and the extracts were evaporated to dryness.

Extraction of Stachyose.—Soya-bean meal (250 g.) was ground in a hand mortar and extracted with boiling aqueous ethanol (70%; 1 l.) with continuous stirring for 6 hours. After filtration the residue was re-treated as above and the extracts were combined. The precipitate which settled out on cooling was removed by filtration, and the filtrate evaporated to a syrup. This procedure was repeated with three further 250-g. portions of meal, and the syrups were combined and dissolved in water (1 l.). The solution was heated to 95°, cadmium sulphate (10%; 100 c.c.) and sodium hydroxide (0·5N; 50 c.c.) were added, and the mixture was kept at 95° for a further 3 minutes. After cooling to room temperature and filtration, de-ionisation with Amberlite resins (I.R.-100 and IR-4B) yielded a solution from which the last traces of protein were removed by shaking with chloroform and butanol (Sevag, Lackmann, and Smollens, *J. Biol. Chem.*, 1938, **124**, 425). The product was warmed with a small amount of charcoal and filtered.

The volume of the extract was reduced to *ca.* 100 c.c. and added slowly with continuous stirring to ethanol (900 c.c.), to yield a brown gummy precipitate. This was separated on the

centrifuge, washed with absolute ethanol, and dried in a vacuum-desiccator ($CaCl_2$). This material showed $[\alpha]_D^{17}$ +67° (*c*, 0·9 in H_2O). Examination on the paper chromatogram indicated the presence of sucrose, a trisaccharide, and stachyose. After several reprecipitations from 90% ethanol the product showed $[\alpha]_D^{19}$ +85° in H_2O.

Preparation and Use of the Charcoal Column (cf. Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677).—Decolourising charcoal (May and Baker; 50 g.) and "Hyflo Supercel" (50 g.), made into a paste with water, were poured into a column. The mixture was allowed to drain till the liquid level was *ca.* 1—2 cm. from the top of the column, which was never allowed to run dry, and then washed with water (1 l.).

The oligosaccharide mixture, in water (200 c.c.), was passed through the column. The sucrose and most of the trisaccharide which remained on the column were removed by elution with 5% aqueous ethanol. The tetrasaccharide contaminated with a little trisaccharide was recovered from the column by elution with 20% aqueous ethanol, and the eluate evaporated to dryness, dissolved in water, and purified as before on a second column. The final solution was evaporated to dryness and dissolved in water (230 c.c.), and the stachyose was precipitated by the addition of ethanol (2 l.). The product was a white powder (6·0 g.). A further crop of stachyose (0·8 g.) was obtained by concentrating the mother-liquors from the above precipitation and adding an excess of ethanol. The precipitates were combined, dissolved in water, and reprecipitated in 95% ethanol. This gave pure stachyose (6·5 g.), $[\alpha]_D^{17}$ +102° (*c*, 1·6 in H_2O). On examination by means of the paper chromatogram, with butanol–pyridine–water–benzene (5 : 3 : 3 : 1) as solvent (de Whalley, Albon, and Gross, *Analyst*, 1951, **76**, 287), only one spot was obtained (travelled 1 cm.; sucrose on same paper travelled 15·6 cm.). With amyl alcohol–pyridine–water (7 : 7 : 6) (Jeanes, Wise, and Dimler, *Analyt. Chem.*, 1951, **23**, 415) a single spot was again obtained (10·7 cm. from starting line). A test for the sucrose linkage with diazouracil (Raybin, *loc. cit.*) gave a positive result.

Hydrolysis.—Stachyose (*ca.* 10 mg.) was heated with sulphuric acid (3%; 4 c.c.) in a sealed tube at 100° for 6 hours. After neutralisation and filtration, the filtrate was evaporated to dryness and the sugars were estimated by paper chromatography (Nelson, *J. Biol. Chem.*, 1944, **153**, 375; Laidlaw and Reid, *J. Sci. Food Agric.*, 1952, **3**, 19) (Found, in two concordant analyses: galactose, 0·19 mg.; glucose, 0·09 mg.; fructose, 0·09 mg.).

Periodate Oxidation.—Stachyose (0·1 g.) was dissolved in aqueous sodium metaperiodate (0·2M; 10 c.c.). The periodate uptake, determined by the arsenite method, was 5·81 (2 hr.), 6·33 (21 hr.), 6·67 (44 hr.), 7·28 (5 days), expressed as moles of sodium periodate consumed per mole of stachyose.

Oxidation with potassium periodate (*loc. cit.*) gave the following results (expressed as moles of formic acid liberated per mole of stachyose): 0·95 (1 day); 1·97 (4 days); 2·09 (6 days); 2·34 (11 days). With a higher concentration of stachyose the values obtained were 0·86 (21 hours); 2·31 (4 days). In a third trial stachyose (0·1338 g.) was dissolved in water (20 c.c.), and sodium metaperiodate (0·075M; 30 c.c.) was added. The formic acid produced was 1·33 (1·25 hr.), 1·68 (4 hr.), 2·21 (1 day), 2·33 (2 days). In each case the titre fell slightly on prolonged storage.

The solution from the third trial above (3 c.c.), after 3 days at room temperature, was treated with the dimedon reagent (Reeves, *J. Amer. Chem. Soc.*, 1941, **63**, 1475). No formaldehyde–dimedon compound was produced.

Methylation.—Stachyose (4·5 g.) was dissolved in water (50 c.c.), and sodium hydroxide (30%; 50 c.c.) added. Methyl sulphate (100 c.c.) and sodium hydroxide (30%; 200 c.c.) were run in during 4 hours at room temperature with continuous stirring. The solution was then set aside overnight. Solid sodium hydroxide (40 g.) was added and methyl sulphate (70 c.c.) run in during 4 hours. The solution was again left overnight. Water (500 c.c.) was added and the solution was extracted with chloroform (4 × 500 c.c.). The extracts were combined, washed twice with water, dried (Na_2SO_4), and evaporated to a clear syrup (2·8 g.). To the residual sodium hydroxide solution was added solid sodium hydroxide (50 g.), and methyl sulphate (100 c.c.) was run in during 6 hours. Extraction of the product and treatment as above yielded a further 0·7 g. of methylated material. The combined syrups were methylated in chloroform (50 c.c.) with silver oxide and methyl iodide at 45°. After three further such methylations the product was dissolved in water, and the solution heated with charcoal, filtered, and evaporated to dryness. The residue was extracted with acetone and the extracts were evaporated to yield a non-reducing syrup (2·68 g.), n_D^{20} 1·4692 (Found: OMe, 48·0. Calc. for $C_{38}H_{70}O_{21}$: OMe, 50·3%). A further methylation with methyl iodide did not increase the methoxyl content. The product showed $[\alpha]_D^{19}$ +130° (*c*, 1·07 in $CHCl_3$).

Hydrolysis and Fractionation.—The syrup (2·43 g.) was heated with sulphuric acid (2% w/v; 70 c.c.) at 100°. $[\alpha]_D^{18}$ were +132° (1 hr.), +129° (2 hr.), +122° (4 hr.), +116° (6 hr.). Concentrated sulphuric acid (2·3 c.c.) was added (total acid concentration 8%) and hydrolysis was continued {$[\alpha]_D^{18}$ +115° ($\frac{3}{4}$ hr.), +113° (2 hr.), +110° (3 hr.), +109° (4 hr., const.). The solution was neutralised with calcium carbonate, filtered, and evaporated to dryness. Extraction of the residue with boiling acetone and evaporation of the extracts yielded a syrup which was fractionated on a column of cellulose (20″ × 1·3″) (*loc. cit.*), with butanol–light petroleum (b. p. 100—120°) (1 : 2) saturated with water as solvent, giving fractions: I, 0·45 g.; II, 1·10 g.; III, 0·14 g.; IV, 0·21 g.

Fraction I contained tetramethyl fructofuranose and a very small amount of tetramethyl glucose and showed $[\alpha]_D^{18}$ +29° (*c*, 1·32 in H_2O). The syrup (0·2 g.) was oxidised with nitric acid (Avery, Haworth, and Hirst, *loc. cit.*). Esterification, methylation, and distillation of the product gave a non-reducing syrup (0·1 g.) which on treatment with methanolic ammonia yielded tetramethyl fructofuranamide. After recrystallisation from light petroleum containing 2% of ethyl acetate, this showed m. p. 100°, not depressed on admixture with an authentic specimen.

Investigation of fraction II on the paper chromatogram indicated it to be a mixture of tetramethyl galactose, 2 : 3 : 4-trimethyl glucose, and a smaller amount of 2 : 3 : 4-trimethyl galactose. The syrup (1·05 g.) was heated in methanolic hydrogen chloride (2%; 100 c.c.) under reflux: $[\alpha]_D^{18}$ were +79° (zero time), +98° (2 hr., const.). The solution was neutralised with silver carbonate, filtered, and evaporated, to yield a non-reducing syrup which was dissolved in water (50 c.c.). This solution was extracted continuously with light petroleum (b. p. 40—60°) (Brown and Jones, *loc. cit.*) to yield fractions: (i) 3·75 hr., 0·20 g., n_D^{18} 1·4509, (ii) +4 hr., 0·10 g., n_D^{20} 1·4512; (iii) +11 hr., 0·10 g., n_D^{20} 1·4510; (iv) +8 hr., 0·08 g., n_D^{20} 1·4510. These were combined to give (*a*) 0·48 g. A further fraction (*b*) 0·49 g., n_D^{20} 1·4570, was obtained by evaporation of the aqueous solution.

Syrup (*a*) (0·45 g.) was hydrolysed with sulphuric acid (2%; 25 c.c.) at 100°. $[\alpha]_D^{18}$ were +102° (zero time), +107° (2 hr.), +106° (4 hr., const.). The reducing syrup (0·41 g.) obtained was treated with aniline in ethanol. This gave tetramethyl *N*-phenyl-D-galactopyranosylamine, m. p. 195°, not depressed on admixture with an authentic specimen, and some syrupy material. The residual syrup was heated at 100° with sulphuric acid (2%; 25 c.c.) for 1 hour, then neutralised with barium carbonate, the solution extracted several times with ether, and the aqueous solution evaporated to yield a syrup (0·15 g.). Examination of this product on the paper chromatogram, with benzene (167)–ethanol (47)–water (16) as solvent, indicated it to be mainly composed of 2 : 3 : 4-trimethyl glucose with a small amount of 2 : 3 : 4-trimethyl galactose.

Syrup (*b*) was heated in sulphuric acid (2%; 30 c.c.) at 100°. $[\alpha]_D^{18}$ were +140° (zero time), +131° (6 hr.). The syrup was recovered from the solution in the usual manner and subjected to further hydrolysis with nitric acid (2%; 50 c.c.) at 100°. $[\alpha]_D^{18}$ were +106° (zero time), +98° (3 hr.), +96° (5 hr., const.). Examination of the product on the paper chromatogram as above indicated the presence of 2 : 3 : 4-trimethyl glucose and 2 : 3 : 4-trimethylgalactose. No tetramethyl galactose could be detected.

Fraction III was combined with hydrolysed (*b*) and fractionated on a column of cellulose to give 2 : 3 : 4-trimethyl D-glucose (0·23 g.) and 2 : 3 : 4-trimethyl D-galactose (0·25 g.).

Identification of 2 : 3 : 4-*trimethyl* D-*glucose*. The syrup showed $[\alpha]_D^{18}$ +60·5° (*c*, 0·8 in H_2O) and had OMe, 41·0 (Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

The syrup (8 mg.) with hydrobromic acid (48%) (Hough, Jones, and Wadman, *loc. cit.*) gave glucose, identified on the paper chromatogram.

The syrup (8·43 mg.) on oxidation with periodate (Bell, *loc. cit.*) yielded formaldehyde, identified as the dimedon compound (3·96 mg., 27% of theory), m. p. and mixed m. p. 188—190°. Authentic 2 : 3 : 4-trimethyl D-glucose (10·6 mg.) gave the dimedon compound (6·56 mg., 35%).

The syrup (0·0215 g.) was dissolved in methanolic hydrogen chloride (1%; 5 c.c.). The rotational changes of the solution at room temperature and at 75° were observed: at room temp., $[\alpha]_D^{18}$ +53·5° (5 min.), +46° (35 min.), +40° (50 min.), +30° (2 hr.), +14° (4 hr.), −16° (22 hr., const.); at 75°, $[\alpha]_D^{18}$ +23° (45 min.), +7° (1½ hr.), −12° (3½ hr.), −18° (4½ hr., const.).

The syrup (0·1 g.) with aniline in ethanol yielded 2 : 3 : 4-trimethyl *N*-phenyl-D-glucosylamine, m. p. 140°. On examination on the paper chromatogram with amyl alcohol (4)–acetic acid (1)–water (5), one spot only was obtained, having R_G 0·79 corresponding to authentic 2 : 3 : 4-trimethyl glucose. 2 : 3 : 6-Trimethyl D-glucose, run on the same paper, had R_G 0·74. Similar results were obtained with benzene–ethanol–water as solvent.

Fraction IV crystallised as the monohydrate (cf. McCreath and Smith, *J.*, 1939, 387). After

being washed with light petroleum (b. p. 40—60°) it showed m. p. 75°. When dried in a vacuum-desiccator (P_2O_5), the crystals tended to become syrupy. Prolonged trituration with ethyl acetate–light petroleum gave a product with m. p. 83° (previous softening). The crystals showed $[\alpha]_D^{18}$ +135° (15 min.) ⟶ +114·5° (3 hr., const.) (*c*, 0·3 in H_2O).

Demethylation with hydrogen bromide (*loc. cit.*) gave galactose only.

Oxidation with periodate (Bell, *loc. cit.*) gave formaldehyde, identified as the dimedon compound. Treatment of fraction IV with aniline in ethanol gave 2 : 3 : 4-trimethyl *N*-phenyl-D-galactosylamine, m. p. 165—167°, unchanged on admixture with an authentic specimen.

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