galactonyl chloride pentanitrate. It was too unstable for purification and and characterization but was found to decompose in moist air to yield crystalline D-galactonic acid pentanitrate (m.p. 130–133°). Attempts to utilize this substance in esterification reactions were unsuccessful. Likewise, suitable conditions could not be found for the direct esterification of D-galactonic acid pentanitrate with methanol under acid catalysis.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Constitution of Stachyose[1]

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Partial hydrolysis of stachyose by almond emulsin gives D-galactose and raffinose, sucrose and galactobiose. Acid hydrolysis of stachyose to D-fructose and manninotriose, followed by reduction of the manninotriose to manninotriitol and partial acid hydrolysis gives D-galactose, melibiitol, D-sorbitol and galactobiose. Periodate oxidations of manninotriitol and manninotriose 1-phenylflavazole confirm the presence of a 1,6-linkage between the D-galactose and D-glucose units in stachyose. Stachyose is O-α-D-galactopyranosyl-(1 → 6)-O-α-D-galactopyranosyl-(1 → 6)-O-α-D-glucopyranosyl β-D-fructofuranoside.

Suggested structures[2–7a] for stachyose are at variance on whether there is a 1,4- or a 1,6-linkage between the D-galactose and D-glucose units. In order to distinguish between these possible alternatives, we have subjected stachyose to partial enzymic hydrolysis by almond emulsin, which contains α-galactosidase but which is relatively free from invertase.[8] By interrupting the hydrolysis at an intermediate stage, it has now been possible[9] to identify raffinose as a partial hydrolysis product (others include D-galactose, sucrose and galactobiose). The raffinose was isolated in crystalline form and its identity confirmed by comparison of its X-ray powder pattern with that of an authentic specimen.

In another experiment, stachyose was hydrolyzed by dilute acid to D-fructose and manninotriose,[10] which was reduced to manninotriitol. Manninotriitol, obtained in crystalline form, was subjected to partial acid hydrolysis. Products indicated by paper chromatography were melibiitol, D-galactose, D-sorbitol and galactobiose. The melibiitol was isolated in crystalline form and identified by comparing its properties, including X-ray powder pattern, with those of authentic melibiitol.[11,12]

Periodate oxidations of manninotriitol and of manninotriose-1-phenylflavazole[13] have also indicated that the hexose units in manninotriose are all linked by 1,6-bonds. Further, the large and regular increases in molecular rotation in going from sucrose ($[M]_D$ +22,700) to raffinose ($[M]_D$ +62,400) to stachyose ($[M]_D$ +99,900) indicate that in each case the D-galactose units have an α-configuration.

Experimental

Isolation of Sucrose, Raffinose and Stachyose from *Stachys tuberifera*.—*Stachys tuberifera* (*S. sieboldii*) rhizomes[14] (256 g.), were disintegrated in a Waring Blendor with methanol. Filtration gave 370 ml. of solution (ρ = 57.5° S, 2 dm.) and a pulp which was boiled briefly with water and filtered (400 ml. of filtrate, ρ = 10° S, 1 dm.). The combined filtrates were evaporated to a sirup in a warm air stream. Paper chromatography of the sirup showed small amounts of sucrose and raffinose, much stachyose, small amounts of verbascose and higher molecular weight saccharides.

Sucrose was obtained from a small portion of the sirup by the charcoal column fractionation method.[15] The evaporated 5% ethanol eluate crystallized by addition of glacial acetic acid and butanol. The sucrose was identified by paper chromatography and by comparison of its X-ray diffraction pattern with that of authentic sucrose.[16]

Another portion of the sirup was separated into fractions by large scale paper chromatography. The sirup was placed in a narrow band near one edge of many sheets of filter paper (Eaton and Dikeman 613, 8 × 10"), the papers were stapled in the form of cylinders with the sirup band near the bottom edge and the cylinders were developed in

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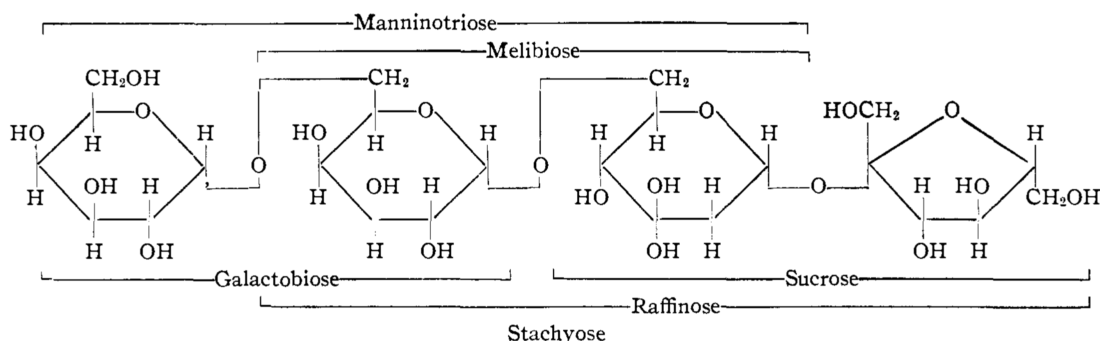

Stachyose

water:pyridine:butanol (3:4:6 parts by volume) using the multiple ascent technique.[17] After eight ascents the raffinose and stachyose bands were cut out and eluted with water. The raffinose eluate was evaporated to a sirup and crystallized by adding glacial acetic acid. It was positively identified as raffinose by comparison with authentic raffinose on a paper chromatogram before and after partial acid hydrolysis and by comparison of its X-ray diffraction powder pattern with that of authentic raffinose hydrate: 8.58,[18] 70[19]; 7.16, 100; 6.91, 50; 6.65, 50; 6.28, 60; 5.43, 70; 5.03, 30; 4.64, 50; 4.31, 100; 3.97, 40; 3.81, 15; 3.66, 25; 3.51, 60.

The stachyose eluate was concentrated to a sirup (1.8 g.) and crystallized by adding glacial acetic acid. Its X-ray diffraction powder pattern was identical with that of a sample of stachyose from soy beans[20]: 11.32,[18] 45[19]; 7.79, 100; 6.83, 15; 6.48, 35; 6.06, 35; 5.50, 80; 5.27, 20; 4.98, 50; 4.68, 10; 4.54, 65; 4.17, 45.

Preparation of Stachyose.[20a]—The bulk of the *Stachys tuberifera* sirup (from the preceding section) was diluted to 1 liter and decolorized by passing it through a 48 × 85 mm. column of charcoal–Celite (1:1 by weight). The small amount of stachyose adsorbed on the column was eluted with 15% ethanol. The sirup after evaporation was treated with glacial acetic acid and butyl alcohol, and seeded with stachyose hydrate crystals. Partial crystallization gave 11 g. of stachyose, leaving a considerable amount of stachyose in the mother liquors. The stachyose was recrystallized by dissolving it in water and adding a mixture of methyl and butyl alcohol. On standing the solution gradually deposited crystals which were removed by filtration, washed with dilute alcohol and air dried: $[\alpha]^{26}D$ +131 (c 3, H_2O).

X-Ray Data on Stachyose.—Stachyose hydrate crystals were obtained by slow evaporation of a solution in 70% n-propyl alcohol. The crystal density was determined by the flotation method in a mixture of nitrobenzene and carbon tetrachloride: found, 1.483, 1.487, av. 1.485. X-Ray data obtained using filtered copper radiation and Weissenberg and precession techniques, indicated the crystal to be orthorhombic with no systematic absent reflections except odd orders of (100) and (010). Since stachyose is optically active the space group indicated is $P\,2_12_12$, which requires four asymmetric molecules per unit cell (or two molecules having 2-fold symmetry). The unit cell dimensions are $a_0 = 12.76 \pm 0.02$, $b_0 = 23.78 \pm 0.08$, $c_0 = 10.90 \pm 0.06$. The observed X-ray molecular weight of stachyose hydrate is (10.90)(12.76)(23.78)(1.485)(0.6023)/4 = 739.5; calcd. for $C_{24}H_{42}O_{21}\cdot 4H_2O$, 738.6.

Emulsin Hydrolysis of Stachyose.—Pure stachyose (raffinose-free, 1.6 g.) was dissolved in water and treated with 6 ml. of an almond emulsin preparation[8,12] and made up to a total volume of 80 ml. The solution was incubated at 35° with thymol as a preservative. After 10 days the rotation of the rather turbid reaction mixture had dropped from *ca.* 16 to 13.8 (saccharimeter reading, 2-dm. tube). The solution was boiled and evaporated to a sirup in a warm air stream. Paper chromatography showed the presence of a trace of D-fructose, much D-galactose, some sucrose and raffinose, a small amount of galactobiose, and most of the stachyose still unchanged. The sirup was fractionated by large-scale paper chromatography. The raffinose bands, which overlapped the galactobiose bands, were cut out and eluted with warm water. The eluate was placed on a charcoal–Celite column (5:5 g.) and separated by elution with water, 5% ethanol and 10% ethanol. The 5% ethanol eluate contained the bulk of the galactobiose. The first 50 ml. of the 10% ethanol eluate contained a trace of galactobiose and mostly raffinose. After the 10% ethanol eluate was evaporated to a sirup (0.25 g.), it was treated with glacial acetic acid. The crystalline material which separated (0.19 g.) was identified as raffinose by chromatography before and after partial acid hydrolysis and by comparing its X-ray diffraction pattern with that of authentic raffinose hydrate.

Manninotriitol Acetate.—Four grams of crystalline stachyose was dissolved in 20 ml. of 1 M sulfuric acid and allowed to hydrolyze at 35°. After 45 hours the rotation had dropped from 14.6 to a constant value of 10.2 (observed saccharimeter readings in a 2-dm. tube). The acid was then neutralized with sodium hydroxide and the mixture of D-fructose and manninotriose was placed on a charcoal–Celite column (70:70 g.). Elution with 5 l. of water gave D-fructose and inorganic salts. Elution with 2 l. of 15% ethanol gave manninotriose, which was evaporated to a sirup. The sirup was taken up with 50 ml. of water and treated with 0.5 g. of sodium borohydride.[21] After the reaction mixture had stood overnight, reduction did not appear to be complete as judged by the positive alkaline copper test (possibly because the sodium borohydride had not been adequately protected against moisture during storage) and the overnight treatment with 0.5 g. of sodium borohydride was repeated. The solution was evaporated to a dry sirup and acetylated with 1.5 g. of sodium acetate and 15 ml. of acetic anhydride at the boiling temperature for 30 min. The acetic acid and anhydride were evaporated in a hot air stream and the residue was extracted with benzene. The filtered extract was evaporated to a dry sirup which was taken up in warm propanol. On cooling a gel-like semi-crystalline aggregate appeared. After two reprecipitations from propanol, manninotriitol acetate showed $[\alpha]^{26}D$ +103.6° (c 2, $CHCl_3$) and m.p. 97–100° (cor., not increased by two additional recrystallizations).

Manninotriitol.—Two grams of manninotriitol acetate was dissolved in 50 ml. of methanol and treated with 0.5 g. of potassium hydroxide dissolved in 10 ml. of methanol. The solution was boiled gently for 15 min. and the methanol was evaporated in an air stream. The residue was treated with 15 ml. of water and heated to boiling for 30 min.; then it was evaporated to dryness. The residue was dissolved in a small volume of water, neutralized with acetic acid and evaporated to dryness in a warm air stream. The residue was taken up in boiling methanol. After filtration and standing at room temperature the solution deposited fine tufts of manninotriitol crystals, which after recrystallization from methanol gave $[\alpha]^{26}D$ +147° (c 2, H_2O) and m.p. 190–191° (cor.). The X-ray diffraction powder pattern gave: 10.91,[18] 7[19]; 9.50, 20; 6.32, 10; 5.57, 50; 4.99, 5; 4.70, 5; 4.62, 100; 4.44, 40; 4.26, 15; 3.99, 20; 3.75, 10; 3.46, 15; 3.21, 12.

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(18) d value in Å.

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Complete hydrolysis of manninotriitol was effected by refluxing a solution in 0.07 N hydrochloric acid overnight. Products identified by paper chromatography were D-galactose (62% by polarimetric analysis, 65% by iodometric analysis)[22] and D-sorbitol (identified as the hexaacetate, m.p. 96–99°, mixed m.p. 97–99°; after recrystallization from butanol, m.p. 99).

Periodate Oxidation of Manninotriitol.—Manninotriitol (10^{-4} mole) was treated with sodium periodate solution (15 $\times$ 10^{-4} mole, adjusted to the methyl red end-point) in a total volume of 30 ml., and allowed to stand 3 days at room temperature. Titration showed that 8.2 $\times$ 10^{-4} mole of periodate had been consumed with the production of 4.6 $\times$ 10^{-4} mole of formic acid (methyl red end-point). In another sample similarly treated with periodate the acid produced was neutralized frequently. After 30 min., acid production corresponded to 3.2 moles per mole of manninotriitol (theor., 3.0 for oxidation of the sorbitol moiety) and the reaction mixture was divided into two equal parts. Half the mixture was treated at once with 0.1 g. of dimedon and allowed to stand overnight. The dimedon–formaldehyde compound which formed amounted to 0.96 mole per mole of manninotriitol. The other half of the reaction mixture was allowed to stand 24 hr. longer when the formic acid production amounted to 4.9 moles per mole of manninotriitol. Treatment with dimedon as before gave 1.02 moles of dimedon–formaldehyde compound per mole of manninotriitol.

Melibiitol from Partial Acid Hydrolysis of Manninotriitol.—One gram of manninotriitol was dissolved in 20 ml. of 0.16 N hydrochloric acid, heated to boiling and kept at 100° for 45 min. During this time the rotation dropped from 45.7 to 32.4 (observed saccharimeter reading, 2-dm. tube). The solution was then cooled and neutralized with sodium hydroxide solution. Paper chromatography showed the presence of D-galactose and galactobiose as the only reducing sugars and also D-sorbitol, melibiitol and manninotriitol as substances which darken ammoniacal silver nitrate solution. The mixture was separated on a 20-g. charcoal:20-g. Celite column; washing the column with 2 l. of water removed D-sorbitol and D-galactose as well as inorganic salts. Treating the column with 500 ml. of 5% ethanol eluted nearly all the melibiitol and some of the galactobiose. After evaporation of the 5% ethanol eluate to dryness, the residue was dissolved in 90% methanol, filtered, and again evaporated to dryness. The dry residue was treated with dry methanol and allowed to stand overnight. Crystals of melibiitol (0.07 g.) which formed were removed by filtration and identified by their characteristic X-ray diffraction powder pattern,[12] and after recrystallization from methanol melted at 177° (cor.) and showed $[\alpha]^{26}D$ +114 $\pm$ 2° (c 1, H_2O).[11]

Manninotriose 1-Phenylflavazole.—Manninotriose (0.8 g.) from stachyose was heated with 1.44 g. of phenylhydrazine hydrochloride, 0.22 g. of o-phenylenediamine and 0.5 ml. of glacial acetic acid in 20 ml. of water at 100° in a stoppered tube. After 3 hr. the reaction mixture was cooled and neutralized with 40 ml. of 0.5 N sodium hydroxide. The trisaccharide flavazole was isolated by fractional extraction of the solution with ether (to remove organic bases, etc.), methyl ethyl ketone (to remove traces of monosaccharide and disaccharide flavazoles as well as other colored impurities) and butanol (which extracts trisaccharide flavazoles). Each step in the fractionation process was followed by paper chromatography of the fractions using water-saturated methyl ethyl ketone as the developing solvent. The flavazoles fluoresce brightly in ultraviolet light. Monosaccharide flavazoles have an R_f greater than 0.9, R_f values of disaccharide flavazoles vary considerably, but fall in an intermediate R_f range, and trisaccharide flavazoles have R_f less than 0.2. The final butanol extract (free from colored substances other than trisaccharide flavazole) was allowed to crystallize by evaporation in a warm air stream The crystalline residue was suspended in a small amount of butanol, filtered and washed with a small amount of ethanol. The material was redissolved in methanol, treated with a small amount of charcoal and filtered, and the filtrate treated with butanol. The bright yellow crystals which formed by evaporation at room temperature were removed by filtration and washed with acetone and ethanol (yield 0.18 g.). The X-ray powder pattern gave: 9.71,[18] 12[19]; 7.55, 3; 6.57, 3; 6.18, 2; 5.56, 2; 4.87, 2; 4.67, 1; 4.39, 100 (broad); 4.12, 2; 3.88, 4; 3.70, 2; 3.41, 20.

Periodate Oxidation of Manninotriose 1-Phenylflavazole.—A sample of 10^{-4} mole of manninotriose 1-phenylflavazole was dispersed in 10 ml. of a solution containing 7.5 $\times$ 10^{-4} mole of sodium periodate and 3 ml. of methanol. After 3 days at room temperature the reaction mixture was diluted to 100 ml. and the insoluble flavazole aldehyde (0.98 $\times$ 10^{-4} mole) was removed by filtration: m.p. 141–142°, without recrystallization; mixed m.p. with authentic 1-phenyl-flavazole-3-aldehyde, 141–143°; literature[13] value 144°. The filtrate was tested for the presence of formaldehyde by adding 0.2 g. of dimedon and allowing to stand overnight. A small amount of precipitate appeared, about 0.3 $\times$ 10^{-4} mole, probably representing formaldehyde which was formed by slow oxidation of the methanol present. Comparison oxidations of glucose flavazole, maltose flavazole, melibiose flavazole and a blank containing methanol run under the same conditions indicated that formaldehyde is always produced in excess of the theoretical amount, but that the flavazole aldehyde is formed only when position 4 (and 5) of the original saccharide is unsubstituted. In the case of maltose, for instance, there is produced a rather soluble flavazole trialdehyde of indefinite melting point ($ca.$ 120–160°), which in contrast to the water-insoluble flavazole aldehyde is not readily extracted from an aqueous solution by benzene. Since the trisaccharide flavazole is appreciably soluble in water, periodate oxidation was carried out with 10^{-5} mole in solution in a reaction mixture containing 2 $\times$ 10^{-4} mole of sodium periodate in a total volume of 10 ml. Immediately after combining the reactants the solution began to cloud with the formation of a silky precipitate of flavazole aldehyde (maltose flavazole, similarly treated did not give a crystalline precipitate). After standing overnight the insoluble yellow crystals were removed by filtration: wt. 1.4 mg., theor. 2.7 mg., m.p. 143–144°. The filtrate was tested for formaldehyde by adding 0.08 g. of dimedon and allowing to stand overnight. A definite turbidity developed, but the characteristic dimedon–formaldehyde precipitate did not form. On the other hand, 10^{-5} mole of formaldehyde similarly treated gave a substantial precipitate of fine long needles.

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