CCCXVI. POLYSACCHARIDES SYNTHESISED BY MICRO-ORGANISMS.

II. THE MOLECULAR STRUCTURE OF VARIANOSE PRODUCED FROM GLUCOSE BY *PENICILLIUM VARIANS* G. SMITH.

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A POLYSACCHARIDE unique in its constitution has been prepared by the action of *Penicillium varians* [Smith, 1933] on glucose in Czapek-Dox medium. The mould belongs to the group Biverticillata-symmetrica and the polysaccharide, which has the empirical formula $(C_6H_{10}O_5)_n$, is a white amorphous powder neutral in aqueous solution and having $[\alpha]_D + 15°$. It reduces Fehling's solution slightly and gives no colour with iodine; it is almost unaffected by digestion at 100° with $N/100$ HCl but is hydrolysed readily with $N/10$ HCl at 100° and the scission products contain *d*-galactose to the extent of approximately 70%. The remaining portion consists of glucose (14%) and also an unidentified hexose (14%) which appears to be either *d*-idose or *l*-altrose. It is thus significant that although the only available source of carbon in the original nutrient solution was glucose this has been converted, by the subsequent metabolism involved in the synthesis of the polysaccharide, very largely into galactose and into either *d*-idose or *l*-altrose.

Inasmuch as the polysaccharide was prepared by the agency of *P. varians* it has been given the name *varianose*. Varianose is readily acetylated by the usual methods and the completely acetylated polysaccharide showed $[\alpha]_D^{20°} + 30°$ in chloroform and +38·2° in acetone; the acetate yielded unchanged varianose on deacetylation. Completely methylated varianose was prepared by the action of methyl sulphate and potassium hydroxide on varianose acetate or on the unsubstituted polysaccharide; it was a cream-white powder, soluble in water, chloroform or benzene and showed in these solvents respectively, $[\alpha]_D + 15°$, +20°, +23°. The determination of the structure and chain length of methylated varianose was carried out by the method of Haworth and Machemer [1932]. Methylated varianose was hydrolysed with methyl alcoholic hydrogen chloride; the resulting hexosides were separated first by the use of solvents and finally by careful fractional distillation, yielding 14% of 2:3:4:6-tetramethyl-methyl-glucopyranoside which corresponds to a chain of 8 hexose members of a molecular weight of about 1300 for varianose (see formula I). The remaining hydrolytic products consisted mainly of trimethyl-methylgalactoside which yielded on hydrolysis a liquid 2:3:6-trimethylgalactose, characterised by its oxidation with bromine water to the crystalline 2:3:6-trimethylgalactofuranolactone of Haworth *et al.* [1932]. This yielded on treatment with ammonia a

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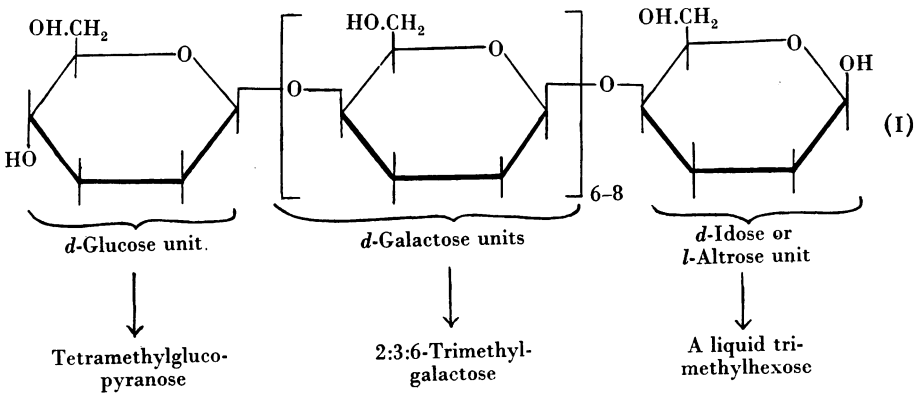
crystalline amide which served further to identify the free sugar. The positions of the three methyl groups were determined by oxidation of the crystalline lactone with nitric acid. The product contained no trace of a mucic acid derivative. From this it is inferred that position 6 in the sugar chain was protected by a methyl group inasmuch as it has been our experience that when the terminal primary alcohol group is unprotected a mucic acid derivative is readily obtained. The crystalline product isolated from this oxidation consisted of *d*-dimethoxysuccinamide and its isolation indicates that positions 2 and 3 in the trimethyllactone are occupied by methyl residues. Inasmuch as the trimethyl-lactone behaved in respect of its rate of hydrolysis exactly as a γ-lactone we also infer that position 4 was not occupied by a methyl group. Moreover, the trimethylsugar showed in its chemical behaviour the capacity to pass either into a furanoside or pyranoside form on glycoside formation. The properties therefore of the isolated trimethylgalactose are in full agreement with our recognition of it as 2:3:6-trimethylgalactose. Complete methylation of the trimethylgalactose yielded a galactoside which, on hydrolysis, gave tetramethylgalactopyranose [Haworth *et al.*, 1924; 1927; Pryde, 1923].



It is evident that a 2:3:6-trimethylgalactose might occur as a scission product of a polysaccharide consisting of hexo-furanose or -pyranose units. At present the behaviour of varianose towards $N/100$ and $N/10$ HCl respectively inclines us to the view that the pyranose form only is present in this polysaccharide. We are supported in this view by the circumstance that we have prepared by means of *P. Charlesii* G. Smith another polysaccharide [Haworth, Raistrick and Stacey, unpublished] which indubitably contains only galactofuranose units and its properties contrast significantly with those of varianose.

From methylated varianose we were also able to isolate about 14% of another trimethyl-methylhexoside which probably represents the hexose residue terminating the polysaccharide chain at the end remote from that occupied by glucose. This portion yielded a liquid trimethylhexose and also a liquid trimethylhexonolactone, but the latter gave easily and quantitatively a crystalline phenylhydrazide not identical with the phenylhydrazide of any known trimethylhexonolactone. Moreover, the lactone itself was laevorotatory and gave a high laevorotatory value in equilibrium with the corresponding acid in water. Its oxidation with nitric acid yielded no mucic acid derivative and no product showing its relationship with galactose. Treatment of the ester of this oxidation product with ammonia yielded, however, *d*-dimethoxysuccinamide, a result

showing the orientation of methyl groups in positions 2 and 3 of the corresponding trimethylhexose. It therefore appears that the hexose representing the reducing end of the polysaccharide chain is either *d*-idose or *l*-altrose. The low value of the specific rotation suggests that varianose is composed largely of β-hexose units.

Experimental.

Preparation of the polysaccharide (with Dr C. G. Anderson). The mould used for the production of the polysaccharide was a new species belonging to the group Biverticillata-symmetrica. It was isolated from cotton in 1927 by Mr G. Smith and named by him *Penicillium varians*. It bears the L.S.H.T.M. Catalogue number A. 91. A culture has been deposited with the Centraalbureau voor Schimmelcultures, Baarn, Holland.

The mould was grown on a standard Czapek-Dox medium containing glucose as the sole source of carbon and of the following composition: glucose, 1750 g.; $NaNO_3$, 70 g.; KH_2PO_4, 35 g.; KCl, 17·5 g.; $MgSO_4,7H_2O$, 17·5 g.; $FeSO_4$, $7H_2O$, 0·35 g.; distilled water, 35 litres.

70 litres of this medium were prepared and placed in 350 ml. quantities in 200 conical flasks of 1 litre capacity. Sterilisation was effected by steaming on each of three successive days and when cold each flask was sown with 2 ml. of a spore suspension prepared from 50 Czapek-Dox agar slopes of *P. varians*.

The flasks were incubated at 24° and the course of metabolism of the glucose was followed by the polarimeter and by the Shaffer-Hartmann iodine titration method with the following results:

Period of incubation at 24° weeks	g. of glucose in solution/100 ml.		g. of polysaccharide (crude)
	Polarimeter	Shaffer-Hartmann	
8	1·60	1·57	0·52 (1 flask)
11	0·52	0·48	0·35 (1 flask)
11½	0·78	0·71	2·46 (5 flasks)

After 12 weeks, the somewhat mucilaginous solution was filtered from the mycelium and concentrated *in vacuo* at 45–50° to 1600 ml. of a dark red syrup. 160 ml. of conc. HCl were added to this syrup and the polysaccharide was then immediately precipitated as a creamy powder by the addition of 6 litres of 95% alcohol. After an hour the clear liquid was decanted off. A little more product separated from the mother-liquor overnight. The product was isolated by centrifuging and dissolved in 1 litre of distilled water. It was precipitated as a sticky mass from this solution by the addition of 5 litres of 95% alcohol. Further purification was effected by repeated precipitations from both acid and neutral aqueous solutions by means of alcohol, until an almost ash-free compound was obtained. Trituration of the sticky mass with absolute alcohol gave the polysaccharide as a white powder which was washed with ether and dried over concentrated sulphuric acid in a vacuum desiccator; yield, 104 g. Precipitation of the polysaccharide from aqueous solution by ethyl alcohol at different concentrations of alcohol gave a series of fractions which showed no differences in rotation or other properties among themselves or from the original material, and the polysaccharide thus appeared to be homogeneous. It showed $[\alpha]_D^{20°}+15·0°$ (*c*, 0·894 in water). (Found (moisture, 7·33%; ash, 2·0%): C, 44·9; H, 6·6%. $(C_6H_{10}O_5)_x$ requires C, 44·4; H, 6·2%.)

In a second experiment 100 flasks each containing 350 ml. of Czapek-Dox medium and incubated in this case at 28° for 7 weeks gave 105 g. of varianose, $[\alpha]_D^{20°}+15·0°$ (*c*, 0·85 in water). The aqueous-alcoholic mother-liquors remaining

after the initial precipitation of the varianose from the evaporated metabolism solution gave, on evaporation, 65 g. of *d*-mannitol.

The polysaccharide was a white amorphous powder moderately soluble in water giving a clear solution which frothed considerably on shaking. In the presence of alcohol it tended to form gummy products. It reduced Fehling's solution slightly on boiling and its aqueous solution was neutral to litmus. It gave no colour with iodine.

Hydrolysis with N/*10 HCl* was carried out at 100°. The initial rotation of +15° changed to +90° in 3 hours. Initial rotation +15·0°, +22·0° (5 min.), +48·3° (20 min.), +75·7° (50 min.), +81·9° (80 min.), +89·4° (120 min.), +90° (180 min., equilibrium value). Equilibrium rotation calculated as hexose $[\alpha]_D$ +81°.

Examination of the hydrolysis products. The polysaccharide (1 g.) was heated for 3 hours at 100° with 30 ml. of *N*/10 HCl. The acid was neutralised with barium carbonate, the solution filtered and evaporated to dryness under reduced pressure at 25°. The syrupy residue was extracted repeatedly with 95 % alcohol which, on removal by distillation, left a viscid pale brown syrup (0·85 g.). This syrup reduced Fehling's solution very strongly.

Formation of galactosazone from the above syrup. The syrup (0·35 g.) was dissolved in water (2 ml.) and heated on the boiling water-bath for 15 min. with phenylhydrazine acetate (0·55 g.); the osazone which separated (0·5 g.) after recrystallisation from water and pyridine had M.P. 195°, unchanged on admixture with authentic galactosazone.

Formation of mucic acid. The syrupy hydrolysis product (0·3 g.) was heated with nitric acid (1·2 ml., sp. gr. 1·2) for 6 min. On dilution with water and standing, a white crystalline substance (0·28 g.) separated. This was filtered off, washed with water and dried *in vacuo* over calcium chloride. It had M.P. 214° (with decomposition) alone or in admixture with an authentic specimen of mucic acid. A sample was also prepared directly from the polysaccharide.

Preparation of crystalline galactose from the polysaccharide. The polysaccharide (2 g.) was hydrolysed by heating it for 30 min. with *N* HCl (100 ml.). The acid was neutralised in the cold with silver carbonate and the solution, filtered from the silver salts, was slowly evaporated at 15° leaving a colourless syrup which crystallised on nucleation with *d*-galactose. It was recrystallised from absolute alcohol. Yield, 1·4 g. (70 %), $[\alpha]_D^{20°}$ +140° → +82° (*c*, 1·1), equilibrium value. A residual syrup (0·5 g.) did not crystallise.

Preparation of varianose acetate. Varianose (1·3 g.) was dissolved in a small amount of hot water (1·5 ml.) and pyridine (25 ml.) was added. The solution was cooled in an ice-bath and acetic anhydride (25 ml.) was dropped in slowly with constant stirring, care being taken to prevent any considerable rise in temperature. After several days at 15° the reaction mixture was poured into warm water (300 ml.) which was vigorously stirred. The acetate was precipitated as a white flocculent powder. It was filtered and freed from excess pyridine and acetic acid by continuous washing with warm water for 6 hours. It was further purified and freed from traces of ash by dissolving it in hot methyl alcohol and filtering through muslin on a hot-jacketed funnel. It was reprecipitated from the alcoholic solution on cooling, filtered, washed with ether and dried in the vacuum oven over calcium chloride at 50°; yield, 2·2 g. (quantitative). It did not reduce Fehling's solution.

Varianose acetate was a finely divided white powder which had M.P. 148–155°. It dissolved readily in chloroform, acetone and hot alcohol, but these solutions could only be filtered through muslin. $[\alpha]_D^{20°}$ in chloroform, +30·0° (*c*, 0·95); $[\alpha]_D^{20°}$ in acetone, +38·2° (*c*, 0·68). (Found: C, 50·3; H, 5·6; OAc, 43·8 %. $[C_6H_7O_2(OCOCH_3)_3]_x$ requires C, 50·0; H, 5·6; OAc, 44·8 %.) Deacetylation of

the acetate with *N* NaOH gave the original ash-free polysaccharide unchanged in properties. $[\alpha]_D$, +15·0° (*c*, 0·96).

Attempted fractionation of varianose acetate. 30 g. of the acetate were made and purified in the manner described. Fractional precipitation was attempted from hot methyl alcohol, from mixtures of chloroform and alcohol, and from chloroform and light petroleum. Acetyl values and rotations were determined for each fraction but in no case could a fraction be obtained which differed in properties from the original acetate. It was concluded that the polysaccharide was homogeneous.

Methylation of the polysaccharide. The polysaccharide (6 g.) was dissolved in water (20 ml.) and 100 g. of KOH in 100 ml. of water added. It was methylated by the gradual addition of methyl sulphate (95 ml.) at 15° over a period of 5 hours. Acetone (100 ml.) was added after 1 hour. The liquid was boiled for 30 min. and poured into 1000 ml. of hot water. The methylated product formed a light brown gum on the surface of the hot water from which it was collected. Its solution in chloroform was washed with water, dried over anhydrous magnesium sulphate and evaporated. The pale yellow viscid syrup which remained was triturated with light petroleum (40–60°) yielding the methylated compound as a fine cream-coloured powder; yield, 7·5 g.; OMe, 36·2%.

One further treatment of this partially methylated product with KOH and methyl sulphate gave a fully methylated derivative (yield, 7·5 g. OMe, 45·9%). It was a white ash-free powder, insoluble in hot water but very soluble in cold water, acetone and chloroform. It was non-reducing and had M.P. 90–100°; $[\alpha]_D^{22°}$ +20° (*c*, 1·10 in chloroform); $[\alpha]_D^{22°}$ +23° (*c*, 0·95 in benzene); $[\alpha]_D^{20°}$ +15° (*c*, 1·08 in water). (Found: C, 53·2; H, 8·0; OMe, 45·9%. (Moisture content 1·08%.) $(C_9H_{16}O_5)_n$ requires C, 52·9; H, 7·8; OMe, 45·6%.)

Hydrolysis with boiling 3% methyl alcoholic HCl. $[\alpha]_D$ +17·1° (initial value); +10·5° (0·25 hours); −8·6° (0·75 hours); −13·6° (1·75 hours, equilibrium value). (*c*, 1·05.)

Hydrolysis of methylated varianose.

30·5 g. of methylated varianose were hydrolysed by boiling gently for 5 hours with 700 ml. of boiling 3% methyl alcoholic HCl. No discoloration took place and no furfuraldehyde derivatives could be detected. The acid was neutralised with silver oxide at 15°. After filtration the solution was evaporated at 35°/18 mm. to a syrup which was dissolved in dry ether to remove traces of silver salt. Evaporation gave a colourless mobile syrup. A portion of this syrup B.P. 120–122°/0·04 mm. had the following properties: $n_D^{18°}$, 1·4514; $[\alpha]_D^{20°}$ −31·8° (*c*, 0·96 in water). (Found: C, 50·5; H, 8·5; OMe, 53·6%. $C_{10}H_{20}O_6$ requires C, 50·8; H, 8·5; OMe, 52·5%.)

Hydrolysis of the mixture of methylated hexosides. After a trial hydrolysis with 2% HCl solution at 100° the following values were obtained (*c*, 0·781) $[\alpha]_D$ −32·5° (initial value); −2·0° (7 min.); +22·0° (20 min.); +44·0° (50 min.); +49·2° (80 min.); +53·0° (120 min., equilibrium value). Equilibrium value calculated as trimethylhexose, +56°. No furfuraldehyde derivatives were detected during hydrolysis and no discoloration took place.

Fractionation of the mixture of methylated hexoses. The mixture of methylated hexosides was hydrolysed by heating it (30 g.) in 300 ml. of 2% HCl at 100° for 5 hours. The HCl was neutralised with barium carbonate, charcoal was added and the solution filtered, the precipitate being well washed with warm water. The aqueous solution (400 ml.) was extracted 10 times with chloroform. The chloroform solution was dried over anhydrous magnesium sulphate, filtered and evaporated. A colourless fairly viscid syrup Fraction A remained (8·1 g.).

The aqueous solution was evaporated to dryness and the solid extracted 10 times with boiling chloroform. This chloroform solution was dried over magnesium sulphate, filtered and the solvent distilled off. A colourless viscid syrup Fraction B remained (21 g.).

Fractionation of Fraction A by means of solvents. Fraction A was dissolved in chloroform to give a thick syrup, and 200 ml. of light petroleum (40–60°) were added to this and the mixture stirred vigorously. The petroleum layer was decanted after standing and this treatment was repeated several times. Removal of the petroleum by distillation gave a highly mobile syrup A_1; yield, 4·5 g. Removal of the chloroform by distillation gave a fairly mobile syrup A_2 (yield, 3·5 g.).

Fractional distillation. Each fraction was now treated separately and converted back into the glycoside stage by the action of boiling methyl alcoholic HCl (2 %).

Distillation of A_1. The highly mobile syrup (4·5 g.) was distilled in high vacuum from a Claisen flask with a wide side limb, the first fraction F_1 being collected in a Widmer flask which was used as a receiver and from which it could be redistilled. In this and succeeding distillations the syrup was distilled very slowly in order to secure the best possible fractionation. In no case was there any decomposition and no furfuraldehyde derivatives could be detected. The following fractions were obtained:

F_1 at B.P. 90°/0·04 mm. (3·5 g. of a colourless highly mobile liquid).

F_2 at bath temperature 140–143°/0·04 mm. (1·0 g. of a colourless mobile syrup).

F_1 (3·5 g.) was now distilled from the Widmer flask fitted with a fractionating column and the following fractions were obtained:

$F_{1\cdot1}$ at bath temperature 120–123°, B.P. 90°/0·06 mm., $n_D^{15°}$ 1·4455, 3·2 g.

$F_{1\cdot2}$ at bath temperature 140–145°/0·06 mm., $n_D^{15°}$ 1·4519, 0·2 g.

$F_{1\cdot1}$, as shown below, was tetramethyl-$\alpha\beta$-methylglucopyranoside. (Found: OMe, 60·0 %. Calc. 62·0 %.)

A portion of it (2 g.) was hydrolysed with boiling 4 % aqueous HCl in the usual manner. The product was a crystalline solid (1·9 g.) which on recrystallisation from light petroleum (B.P. 40–60°) gave, almost quantitatively, long needles of tetramethylglucopyranose; M.P. alone or in admixture with an authentic specimen 88°; $[\alpha]_D^{21°}+84°$ (*c*, 1·1 in water) (equilibrium value). It was further identified by formation of its crystalline anilide by the usual method.

Further fractionation of the residues. Fractions A_2, $F_{1\cdot2}$ and F_2 were mixed (4·7 g.) and distilled as before giving:

F_3 at bath temperature 120–130°/0·06 mm. (A colourless very mobile liquid, 1·5 g.)

F_4 at bath temperature 140–150°/0·06 mm. (A colourless mobile syrup, 3·0 g., $n_D^{15°}$ 1·4559.

F_3 (1·5 g.) was fractionated by means of the Widmer flask and column and the first fraction at bath temperature 120–125° had B.P. 90–92°/0·06 mm. and $n_D^{15°}$ 1·4488; yield, 0·95 g. ($F_{3\cdot1}$). (Found: OMe, 56·5 %.)

The calculated amount of tetramethyl-methylglucopyranoside contained in $F_{3\cdot1}$ was 0·61 g. and the total yield of the latter from 30 g. of methylated hexosides was 3·81 g. The experimental loss from hydrolysis and fractionation as shown by Haworth and Machemer [1932] is not greater than 10 % of the weight of tetramethylglucose. Hence the total estimated yield of tetramethylglucose was 14 %

by weight of the methylated varianose (this gives a chain length of about 8 units) No tetramethylgalactose could be detected.

Examination of the trimethyl portions. (*a*) *Chloroform-soluble.* The residues from the distillations of F_3 and $F_{3\cdot1}$ (0·8 g.) and the fraction F_4 (3 g.) were mixed and distilled (3·8 g.) at bath temperature 140–143°/0·06 mm.; yield, 3·4 g., $n_D^{18°}$ 1·4550 (F_4). (Found: C, 50·8; H, 8·6; OMe, 50·2%. $C_{10}H_{20}O_6$ requires C, 50·8; H, 8·5; OMe, 52·5%. This shows it to be pure trimethyl-methylhexoside.) The residue (0·4 g.) had OMe, 40·7%, $n_D^{16°}$ 1·4615, and probably consisted chiefly of dimethyl-methylhexoside.

(*b*) *Examination of water-soluble Fraction B.* Treatment of a chloroform solution of Fraction B with light petroleum in the manner previously described and subsequent conversion into the glycosides followed by distillation failed to give a low-boiling fraction and hence it contained no tetramethylglycosidic fraction. 21 g. were distilled from a Claisen flask giving 20 g. of a product having B.P. 135–140°/0·05 mm. and $n_D^{18°}$ 1·4550. (Found: C, 50·6; H, 8·6; OMe, 50·2%. $C_{10}H_{20}O_6$ requires C, 50·8; H, 8·5; OMe, 52·5%.) (The residue (1 g.) had OMe, 40·0% and probably consisted mainly of dimethyl-methylglycoside.)

Hydrolysis of trimethyl-methylgalactoside. The syrup was hydrolysed with 2% aqueous HCl at 100°; $[\alpha]_D^{21°}$ 0° (initial value), +14° (5 min.), +46·8° (14 min.), +60·0° (35 min.), +70·4° (60 min., equilibrium value). Equilibrium value calculated as trimethylgalactose is +75°.

Trimethyl-methylgalactoside (8 g.) was hydrolysed by heating with 500 ml. of 2% aqueous HCl at 100° for 2 hours. The acid was neutralised by the addition of silver carbonate in the cold. The solution was filtered and concentrated at 30° to a syrup. This was extracted with ether and the ethereal solution dried over anhydrous magnesium sulphate and filtered. Removal of the ether left a colourless viscid syrup; $[\alpha]_D^{20°}$ +75° (*c*, 0·86 in water), $[\alpha]_D^{21°}$ +36° (*c*, 0·87 in methyl alcohol); $n_D^{17°}$ 1·4660; OMe, 40·1%.

Rate of glycoside formation of trimethylgalactose. The syrup (0·1306 g.) was dissolved in 15 ml. of 3% methyl alcoholic HCl and boiled gently. The reaction was followed polarimetrically; $[\alpha]_D^{21°}$ +35·6° (initial value), −6·4° (5 min.), −29·3° (10 min.), −27·6° (15 min.), −24·7° (20 min.), −24·1° (40 min. constant value). It seemed apparent that both pyranose and furanose forms were present.

Bromine oxidation of trimethylgalactose. The syrup (8 g.) was dissolved in water (200 ml.) and 10 ml. of bromine added. The liquid was heated at 40° for 24 hours and at the end of this time reduced Fehling's solution very faintly. The excess bromine was removed by a vigorous air stream and the acid neutralised with silver carbonate. The solution was filtered, freed from excess silver by the requisite amount of 2*N* HCl, again filtered and evaporated *in vacuo* at 35° leaving a solid mass of crystals (7 g.). The crystals were extracted with ether and the solution was dried over anhydrous magnesium sulphate and filtered. The solid crystallised on concentration of the ethereal solution and was recrystallised from ether-light petroleum (B.P. 40–60°) in clusters of thick rods, M.P. 99°, $[\alpha]_D$ −40° (initial value) in water; it was identical with trimethyl-γ-galactonolactone [Haworth *et al.*, 1932], which was provisionally formulated as 2:3:6-trimethyl-γ-galactonolactone.

By treatment of the mother-liquors (0·5 g.) with phenylhydrazine, a crystalline phenylhydrazide (0·5 g.), M.P. 175°, was isolated. This was identical with the phenylhydrazide prepared from trimethylhexonolactone and described later.

Amide of trimethyl-γ-galactonolactone. This was prepared in the usual manner by the action of concentrated ammonia on the lactone in methyl alcoholic

solution, and recrystallisation of the product from acetone gave long needles, M.P. 135°, $[\alpha]_{5780}^{20°}$ +20·5° (*c*, 0·733). (Found: C, 45·4; H, 8·0; N, 6·2; OMe, 38·7 %. $C_9H_{19}O_6N$ requires C, 45·5; H, 8·1; N, 5·9; OMe, 39·2 %.)

The structure of 2:3:6-trimethylgalactonolactone.

The water-soluble trimethylgalactose (2·5 g.) was converted into the galactoside and redistilled twice in high vacuum at 135°/0·07 mm. in order to free it from the small amount of impurity due to the trimethylhexose B.P. 140°/0·07 mm.

The galactoside was hydrolysed by means of aqueous HCl and the product, trimethylgalactose (2 g.), was carefully dried by heating at 70° for 2 hours. It was dissolved in methyl iodide (15 ml.) and methylated twice by boiling gently with silver oxide (5 g.). The methylated product was isolated in the usual way and purified by ether extraction. It had B.P. 95°/0·05 mm. (bath temperature 120°); yield, 1·8 g. of a colourless mobile syrup, $n_D^{16°}$ 1·4450, OMe, 60·0 %.

Hydrolysis of tetramethyl-methylgalactoside. The syrup (1·8 g.) was dissolved in 2 % aqueous hydrochloric acid (50 ml.) and heated at 100° until the rotation was constant (2 hours). The acid was neutralised with silver carbonate and the solution filtered after the addition of charcoal. The aqueous solution was concentrated at 35°/18 mm. and the residual colourless syrup extracted with ether. Distillation of the ether left a colourless mobile syrup (1·6 g.) which was tetramethylgalactose; $n_D^{18°}$ 1·4523, $[\alpha]_D^{20°}$ +58° (*c*, 0·963 in water), OMe, 51·5 %. This rotation indicated that the tetramethylgalactose was a mixture of the pyranose and furanose forms.

Isolation of tetramethylgalactopyranose anilide. Tetramethylgalactose (1·5 g.) (prepared as described above) was boiled with aniline (0·6 g.) in absolute alcohol solution (50 ml.) for 5 hours. The solution was concentrated to a thick syrup which rapidly crystallised in long needles. These were readily recrystallised from hot ethyl acetate; yield, 1 g.; M.P. and mixed M.P. with an authentic specimen of tetramethylgalactopyranose anilide, 198°. The isolation of the pyranose form shows that position 5 is free in the trimethylgalactose, hence the trimethyl-γ-galactonolactone isolated from it and described previously must be 2:3:6-trimethyl-γ-galactonolactone.

Investigation of the chloroform-soluble trimethylhexoside A. As described previously analysis had shown this fraction to consist of a pure trimethyl-methylhexoside, and accordingly its hydrolysis in 2 % aqueous HCl solution was followed polarimetrically; $[\alpha]_D^{21°}$ −15·6° (*c*, 0·707) (initial value), 0° (5 min.), +17° (12 min.), +27° (20 min.), +30° (35 min.) (constant value). Calculated as trimethylhexose the equilibrium value is +31·6°. The solution reduced Fehling's solution strongly.

2·2 g. of trimethyl-methylhexoside were hydrolysed and the product isolated in the usual manner; yield, 2·1 g. of a viscid colourless syrup, $n_D^{19°}$ 1·4648, $[\alpha]_D^{19°}$ +30·5° (*c*, 0·852), OMe, 40·9 %.

Rate of glycoside formation of trimethylhexose. The rate of glycoside formation of trimethylhexose with 3 % methylalcoholic hydrogen chloride at 100° was followed polarimetrically; $[\alpha]_D^{21°}$ +25·8° (initial value), −21° (5 min.), −29·1° (10 min.), −30·7° (15 min.), (equilibrium value) (*c*, 0·62). These rapid rates of glycoside formation and hydrolysis indicated the presence of the furanose form.

Bromine oxidation of trimethylhexose. The syrup (2 g.) was dissolved in water (75 ml.) and bromine (3 ml.) added. The liquid was well shaken and allowed to stand overnight at 15°. It was then heated for 12 hours at 40°; oxidation was then complete. The excess bromine was removed by a vigorous air stream and the lactone isolated in the usual manner; yield, 1·8 g. of a clear syrup, $n_D^{16°}$ 1·4665.

On nucleation with 2:3:6-trimethyl-γ-galactonolactone the syrup crystallised partially. Ether was added and the crystals filtered off; yield, 0·2 g. (11 %); M.P. and mixed M.P. with 2:3:6-trimethyl-γ-galactonolactone, 99°.

Nothing further would crystallise and the residual syrup (1·5 g.) was treated with phenylhydrazine (0·7 g.) in dry ethereal solution. There was an immediate precipitate and on boiling off the ether a white crystalline mass remained. It was washed with dry ether and crystallised from hot ethyl acetate in the form of fine white needles, M.P. 175°. (Found: C, 54·8; H, 7·6; N, 8·5; OMe, 27·4 %. The phenylhydrazide of a trimethylhexonolactone $C_{15}H_{24}O_6N_2$ requires C, 54·8; H, 7·4; N, 8·5; OMe, 28·4 %.)

Regeneration of pure trimethyl hexonolactone. The phenylhydrazide (2·1 g.) was boiled with the equivalent of *N* HCl for 2 hours. The solution was evaporated to dryness *in vacuo* and the residue extracted repeatedly with ether containing a little chloroform. Evaporation gave a brown syrup which distilled at bath temperature 138–140°/0·04 mm. (B.P. 110–115°) giving 1·4 g. of a pale yellow mobile syrup, $n_D^{16°}$ 1·4628. The syrup immediately gave, with phenylhydrazine, a theoretical yield of the phenylhydrazide, M.P. 175°, described above. (Found: OMe, 41·6. $C_9H_{16}O_6$ requires OMe, 42·3 %.) 0·0759 g. required 3·7 ml. *N*/10 NaOH (theory requires 3·5 ml.); $[\alpha]_D^{20°}$ −62·4° (*c*, 0·99).

Hydrolysis in aqueous solution (*c*, 0·99). $[\alpha]_D^{20°}$ −62·4° (initial value); −60·4° (1 day); −49·8° (3 days); −44·7° (5 days); −44·1° (8 days); −43·0° (12 days); −42·4° (18 days; equilibrium value). It is therefore a γ-lactone.

The rotation of acid → lactone was determined in the usual manner (*c*, 1·15). $[\alpha]_D^{20°}$ −27·8° (initial value as lactone); −37·2° (4 days); −44·1° (9 days); −44·1° (15 days). The proportions of lactone and acid at equilibrium are 55 % and 45 % respectively.

Nitric acid oxidation of trimethylhexono-γ-lactone. The lactone (0·8 g.) purified by regeneration from the phenylhydrazide was heated at 60° for 6 hours with nitric acid (6 ml., sp. gr. 1·2). The excess nitric acid was removed by distillation with the continuous addition of water for 6 hours. The product was dried by heating at 100° for 1 hour and was then esterified in the usual manner by boiling with 4 % methyl alcoholic HCl. It was distilled (0·45 g.) in vacuum giving:

Fraction I. B.P. 80–100°/18 mm., 0·1 g. of a highly mobile liquid, $n_D^{18°}$ 1·4268.
Fraction II. B.P. 100–110°/18 mm., 0·3 g. of a mobile liquid, $n_D^{18°}$ 1·4325.

On treatment with methyl alcoholic ammonia Fraction I yielded 0·07 g. of oxamide immediately and thus consisted chiefly of methyl oxalate. Fraction II yielded 0·04 g. of oxamide which was filtered off. The remainder of the solution after standing for 2 days deposited crystals in the form of clusters of thick rods with pointed ends; yield, 0·25 g., M.P. 284° (decomp.), $[\alpha]_D^{20°}$ +93°. The amide is *d*-dimethoxysuccinamide and its isolation shows that positions 2 and 3 are methylated in the trimethyl-γ-hexonolactone. No methylated mucic acid derivative and no trimethoxyglutaramide could be detected.

Methylation of trimethyl-γ-hexonolactone. The lactone (1 g.) was methylated twice with Purdie's reagents and the product distilled at bath temperature 140–145°/0·02 mm. It had $n_D^{19°}$ 1·4590; $[\alpha]_D^{20°}$ +10·6° (unchanged after several days). 0·0774 g. required 3·0 ml. of *N*/10 NaOH (theory requires 3·3 ml.). (Found: OMe, 50·2 %. Tetramethylhexonolactone requires OMe 53·0 %.)

The methylated compound was methylated again with silver oxide and methyl iodide and distilled. The product distilled at bath temperature 140–143°/0·05 mm., and had $n_D^{16°}$ 1·4610. It did not form a crystalline amide or a stable phenylhydrazide and the conclusion was reached that the trimethylhexono-

lactone was unstable to methylation with silver oxide and methyl iodide. This behaviour is different from that of trimethyl-γ-galactonolactone.

Methylation of trimethylhexose. F_4. A portion of the trimethyl-methylhexoside F_4 was redistilled several times in order to free it from the small amount of trimethyl-methylgalactoside and the fraction (0·6 g.) distilling at bath temperature 143°/0·06 mm. was retained.

This syrup was hydrolysed with 1·5% aqueous HCl to give trimethylhexose (0·5 g.), which was methylated twice with methyl iodide (10 ml.) and silver oxide (4 g.). The product was a colourless and very mobile liquid which had B.P. 90–95°/0·03 mm., $n_D^{14°}$ 1·4448 and $[\alpha]_D^{20°}$ +25·2° (*c*, 1·86 in water). (Found: OMe, 61·6. Tetramethyl-methylhexoside requires OMe, 62·0%.)

Hydrolysis with 0·4% aqueous HCl at 100°. $[\alpha]_D^{21°}$ +25·4° (initial value); +22·7° (5 min.); +21·7° (15 min.); +21·5° (40 min.; equilibrium value). The remainder (0·45 g.) was hydrolysed to give (0·4 g.) of a mobile, colourless and strongly reducing syrup. This syrup was dissolved in water (10 ml.) and bromine (0·25 g.) added. The temperature was maintained at 40° for 24 hours; oxidation was then complete. The lactone was isolated in the usual way. Yield, 0·12 g.; n_D^{16} 1·4500. (Found: OMe, 51·1. $C_{10}H_{18}O_6$ requires OMe, 53%.)

Hydrolysis of the above tetramethylhexonolactone in aqueous solution. $[\alpha]_D^{21°}$ +31·5° (initial value) (*c*, 0·923); +20·2° (30 hours); +15·2° (48 hours); +13·0° (90 hours; equilibrium value).

A phenylhydrazide of the above lactone was prepared. It was a white microcrystalline powder with M.P. 172° but was unstable in moist air. The yield was 36% but there was insufficient material for analysis.

From its behaviour on methylation and from the fact that the trimethylhexono-γ-lactone derived from it gives only *d*-dimethoxysuccinic acid on oxidation it is concluded that the unknown trimethylhexose is a derivative of either *d*-idose or *l*-altrose.

Molecular size of the polysaccharide.

It has been shown by the isolation of 14% of tetramethylglucose from one end of the chain in the methylated polysaccharide that the polysaccharide itself is a long-extended molecule consisting of 8–10 mutually linked hexose units. This value has been confirmed by the estimation of iodine numbers and by determining the mol. wt. of the methylated polysaccharide by Rast's method. Mean mol. wt. of methylated polysaccharide 1622. $(C_9H_{16}O_5)_8$ requires mol. wt. 1632. The iodine numbers of the polysaccharide and of its acetate were determined by the method of Bergmann and Machemer [1930]. Iodine number of the polysaccharide 14 (corresponding to about 9 hexose units). Iodine number of the acetate 8 (corresponding to about 9 acetylhexose units).

SUMMARY.

The molecular structure of varianose, a hitherto undescribed polysaccharide produced from glucose by *Penicillium varians* G. Smith, has been investigated. On acid hydrolysis varianose gives a mixture of *d*-glucose, *d*-galactose and a third hexose which is either *l*-altrose or *d*-idose. Varianose forms acetyl and methyl derivatives which are essentially homogeneous. On treatment with methyl alcoholic HCl the methyl derivative gives a mixture of 14% 2:3:4:6-tetramethyl-methylglucopyranoside, 70% of 2:3:6-trimethyl-methylgalactoside and 14% of a trimethyl-methylhexoside which was identified as a derivative of either *l*-altrose or *d*-idose. From consideration of these hydrolysis products and

from molecular weight determinations, it is shown that varianose is constituted of a chain of 6–8 β-galactopyranose units with a glucopyranose unit at one end of the chain and a unit of either *l*-altrose or *d*-idose at the reducing end. Proof of the structure of 2:3:6-trimethylgalactofuranolactone is given.

REFERENCES.

Bergmann and Machemer (1930). *Ber. deutsch. chem. Ges.* **63**, 316, 2304.
Haworth, Hirst and Jones (1927). *J. Chem. Soc.* 2428.
—— —— and Stacey (1932). *J. Chem. Soc.* 2481.
—— Llewellyn and Westgarth (1924). *J. Chem. Soc.* 2468.
—— and Machemer (1932). *J. Chem. Soc.* 2270.
Pryde (1923). *J. Chem. Soc.* 1808.
Smith (1933). *Trans. Brit. Mycol. Soc.* **18**, 88.