314. *The Constitution of a Levan produced from Sucrose by* Pseudomonas mors-prunorum (*Wormald*).

By Violet E. Gilbert and M. Stacey.

The polysaccharide produced from sucrose by *Pseudomonas mors-prunorum* (Wormald) is shown to be a typical levan, the repeating unit of which is a terminated chain composed of about twelve fructofuranose units mutually joined through the 2 and 6 positions. Experiments on the chromatography of methylated fructoses are described.

Pseudomonas mors-prunorum is the casual agent of bacterial canker in plum trees (Wormald, *Ann. Appl. Biol.*, 1930, **17**, 725; *Trans. Brit. Mycol. Soc.*, 1932, **17**, 157). Some biochemical characteristics of the organism were studied by Erikson (*Ann. Appl. Biol.*, 1945, **32**, 44, 112, 117), who succeeded in cultivating it on a simple synthetic medium containing sucrose as the sole source of carbon.

Among the more interesting metabolic products produced during the growth were a yellow-green fluorescent pigment and a polysaccharide. The effect of injections of the polysaccharide, both alone and together with the *Pseudomonas mors-prunorum* bacterial cells, on the resistance of certain plum trees to bacterial canker was also studied by Erikson (*loc. cit.*, p. 117). Interesting results were obtained, and the preliminary findings of this mode of approach to problems of bacterial infections of fruit trees appears to be worthy of further development. Miss Erikson kindly placed at our disposal a sample of the polysaccharide, and the present paper describes the investigation of its structure. The polysaccharide was obtained in an essentially homogeneous water-soluble form, $[\alpha]_D$ — 47°, giving opalescent solutions of low viscosity.

On hydrolysis with dilute acid the polysaccharide was shown to be constituted of fructose residues only. The polyfructose was methylated, and on methanolysis gave rise to *ca.* 9% of 1 : 3 : 4 : 6-tetramethyl fructofuranoside " end residue ", the remainder consisting of 1 : 3 : 4-trimethyl methylfructofuranoside. Thus the *Pseudomonas mors-prunorum* polysaccharide contains a repeating unit which is composed of a terminated chain of 10—12 fructofuranose units mutually joined through the 2 and 6 positions. It belongs to the same class as other plant and bacterial levans, notably those from certain plant pathogens (Challinor, Haworth, and Hirst, *J.*, 1934, 1560; Lyne, Peat, and Stacey, *J.*, 1940, 237) which have been investigated previously in these laboratories.

During the course of this work attempts were made to separate various mixtures of tetramethyl and trimethyl fructofuranosides and fructopyranosides by chromatographic methods in order to develop a micro-method of assaying the chain length of fructosans. It has been possible to achieve separation of trimethyl from tetramethyl fructosides in the qualitative sense, but so far a convenient chromatographic technique for separating them quantitatively has eluded us.

Experimental.

Production of the Polysaccharide (D. Erikson).—The basal medium contained the following salts: dipotassium hydrogen phosphate (1 g./l.), magnesium sulphate (hydrated) (0·5 g./l.), and potassium chloride (0·5 g./l.). After being adjusted to pH 7 the solution was autoclaved, and when cold there was added a sterile aqueous solution of sucrose to make a final concentration of 10 g./l.

After inoculation with a culture of *Pseudomonas mors-prunorum* the medium was incubated at 25° for periods up to 20 days. The polysaccharide formed was precipitated from the metabolism solution by addition of several volumes of alcohol and was purified and obtained free from nitrogen-containing bacterial debris, ash, etc., by fractional precipitation from aqueous solution by addition of various solvents.

A typical ash-free sample had $[\alpha]_D^{20°}$ — 45° (*c*, 1·0 in water) and could not be separated into fractions having different properties.

It was very readily hydrolysed by acids. Thus on being kept at 50° with N/10-sulphuric acid the specific rotation changed from $[\alpha]_D^{20°}$ — 43° to $[\alpha]_D^{20°}$ — 90° (equilibrium value) in 55 minutes. After removal of the acid the hydrolysate gave crystalline fructose in good yield, and determination of ketose sugar (Nyn, *Chem. Abs.*, 1925, **19**, 1236) showed that only fructose residues were present in the polysaccharide.

Methylation.—The procedure adopted was a slight modification of that described by Challinor, Haworth, and Hirst (*loc. cit.*) followed by treatment with silver oxide and methyl iodide. The product was a fine white powder soluble in acetone and chloroform and insoluble in ligroin, and was separated into fractions by use of these solvents. The main bulk of the methylated polysaccharide (*ca.* 60% yield) had OMe, 44·0%, $[\alpha]_D^{18°}$ — 56° (*c*, 0·5 in chloroform) (cf. Lyne, Peat, and Stacey, *loc. cit.*). It could not be separated into further fractions and so was used for the chain-length estimation.

Chain-length Estimation.—The various samples from the attempted fractionation were hydrolysed separately with oxalic acid in aqueous methanol. In a typical experiment the methylated polysaccharide (0·87 g.) was dissolved in methanol (30 c.c.), and oxalic acid (0·4 g.) in water (10 c.c.) added. On being

kept at 80° the rotation $[\alpha]_D^{29°} - 40°$ changed to $[\alpha]_D - 20°$ (equilibrium value) in 12 hours. The solution was neutralised with barium carbonate and filtered, and the solvent distilled off. By being kept in methanolic hydrogen chloride (100 c.c. of 0·25%), the residual sugar (0·85 g.) was converted into the methylglycosides, which were fractionally distilled in a high vacuum. The distillation behaved very similarly to that described previously for the methylated derivatives of the *Bact. pruni* levan (Lyne, Peat, and Stacey, *loc. cit.*). In the initial fractions (b. p. 80°/0·04 mm., $n_D^{15°}$ 1·4455, OMe, 58·0%) no furfuraldehyde or unsaturated decomposition products were detected. The percentage of " tetra " was estimated from the methoxyl contents of the fractions, for it was considered that the possibility of the occurrence of mixed pyranose and furanose forms made less reliable the result of calculations from the refractive indices of the fractions. Allowing a 10% correction for losses it was found that 3·00 g. of methylated levan yielded 0·265 g. of " tetra ", *i.e.*, *ca.* 9% yield, which amount corresponds to a chain length of about 12 units. The tetra- and tri-methyl fructosides were identified as 1 : 3 : 4 : 6-tetra- and 1 : 3 : 4-tri-methyl fructofuranose respectively by the methods previously described (Challinor, Haworth, and Hirst, *loc. cit.*).

Attempted Quantitative Separation of Tetra- and Tri-methyl Fructose by Chromatographic Methods.—A large number of experiments were carried out in this unsuccessful work, but only some typical findings are herein reported. For most of the attempts the hydrolysate (OMe, 4·38%) from a sample of trimethyl inulin (OMe, 44·0%) served as the mixture of tetra- and tri-methyl fructoses. By a simple carbon tetrachloride–water partition method it was possible readily and consistently to separate a fraction in 10% of the mixture having OMe, 49·0% (Calc. for tetramethyl fructose : OMe, 52·0%). For further separation of the fractions (0·35 g.) by the use of essentially the column as described by Gordon, Martin, and Synge (*Biochem. J.*, 1941, **35**, 1338; 1943, **37**, 80; Gilbert, Smith, and Stacey, *J.*, 1946, 622) with carbon tetrachloride as eluting agent the following typical result was achieved :

Fraction.	Column-lengths of solvent required.	Wt. of fraction (g.).	OMe (%).
1	13 of carbon tetrachloride + 1 of 2% butanol in carbon tetrachloride	0·107	37·9
2	1 of 2·5% butanol in carbon tetrachloride	0·085	44·7
3	2 of 2·5% butanol in carbon tetrachloride	0·095	50·6
4	Extruded column extracted with boiling acetone (50 c.c.)	0·034	45·6
	Recovery	0·321	

In further experiments various types of continuous extraction devices were employed, and from the inulin hydrolysate a range of fractions having OMe, 19·8—47·2% were directly obtained, but in no case so far has a sharp quantitative separation been achieved.

Paper-strip Chromatography (with Dr. P. W. Kent).—The paper-chromatogram technique for the qualitative separation of reducing sugars (Partridge, *Nature*, 1946, **158**, 270) has been extended for qualitative separation of mixtures of partly methylated sugars. By using strips of filter paper (10 × 25 cm.) and butanol–water or butanol–acetic acid as the eluting agents, it was shown that a mixture of 2 : 3-dimethyl, 2 : 3 : 4-trimethyl, and 2 : 3 : 4 : 6-tetramethyl glucose could be readily separated. No separation of mixtures of 2 : 3 : 4-, 2 : 3 : 6-, and 3 : 4 : 6-trimethyl glucose could, however, be achieved. There was some separation of trimethyl glucoses from trimethyl mannoses and of trimethyl hexoses from trimethyl pentoses (cf. Flood, Hirst, and Jones, *Nature*, 1947, **160**, 86).

Mixtures of partly methylated sugars obtained by hydrolysis of methylated polysaccharides were examined by the technique, and by this means it was possible to identify a number of the constituents. Thus the hydrolysis mixture from the methylated *Pseudomonas mors-prunorum* levan (described above) showed two constituents only corresponding in R_F value (Partridge, *loc. cit.*) to 1 : 3 : 4 : 6-tetra- and 1 : 3 : 4-tri-methyl fructofuranose.

The University, Edgbaston, Birmingham 15. [*Received, October 20th*, 1947.]