A Structural Re-examination of the Levans formed by Pseudomonas prunicola, *Wormald, and* Bacillus subtilis, *BG2 F1.*

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Both micro-organisms, grown on sucrose as the main source of carbon, produce levans of high molecular weight, presumably by *trans*-fructosidation. Both fructosans are shown to comprise highly branched molecules. The predominant linkage is that of the typical levan (2 : 6′) while the branching linkage is (2 : 1″) (" inulin type "). The anomeric form of the glycosidic link is assumed to be β, from rotational and enzymic evidence.

Levans may be defined as fructosans in which the 2β : 6′-inter-radical linkage predominates, while inulins may be likewise considered to be those fructosans in which 2β : 1′-links unite the major fraction of the molecule. Levans of small molecular weight, unbranched or of very low degrees of branching, are found in quantity in the aerial portions of the Gramineae (Laidlaw and Reid, *J.*, 1951, 1830; Bell and Palmer, *J.*, 1952, 8763; Aspinall, Hirst, Percival, and Telfer, *J.*, 1953, 337; Schlubach and his collaborators, numerous papers in *Annalen*). Levans of large molecular weight are formed, exocellularly, by enzymes (" levansucrases "; Hestrin and Aveneri-Shapiro, *Biochem. J.*, 1944, **38**, 2) secreted by spore-forming aerobes such as *Bacillus subtilis*, *B. mesentericus*, and *B. megatherium*. The predominant inter-radical link in these fructosans is 2β : 6′, as was first shown by Hibbert and his co-workers (cf. Evans and Hibbert, *Adv. Carbohydrate Chem.*, 1946, **2**, 204) by the isolation and characterisation of 1 : 3 : 4-tri-*O*-methyl-D-fructose. This sugar was obtained from *B. subtilis* levan in over 98·5% yield. If this sugar was in fact uncontaminated it would indicate the possibility that levans handled by Hibbert and his colleagues were long-chain, and possibly unbranched, polysaccharides. Challinor, Haworth, and Hirst (*J.*, 1934, 676), using fractional distillation of the methanolysis products of methylated levan (*B. mesentericus*), estimated a chain length of 10—12 units. Some uncharacterised dimethyl-

fructose was detected in these experiments, which could be taken to indicate a highly branched structure. Lyne, Peat, and Stacey, by the same procedure, deduced similar chain lengths for levans from *B. megatherium*, *Phytomonas pruni*, and *Pseudomonas prunicola* (*J.*, 1940, 237).

Palmer (*Biochem. J.*, 1951, **48**, 389), using D-glucose oxidase, detected about one D-glucose molecule in 500 derived by hydrolysis of levan from *B. subtilis* (a different strain from that in the present work.) Hydrolysed levan from *Ps. prunicola*, Wormald contained no glucose detectable by the enzyme. It is possible that the *prunicola* levan either was so large that glucose liberated on hydrolysis is swamped by the accompanying fructose molecules, or had lost its glucose radical by enzyme action during isolation or before. That the bacterial levans might be derived by chain-lengthening transfer of β-fructofuranosyl (or β-fructofuranosido) radicals from a donor molecule to an acceptor, *ab initio* sucrose, would account for the presence of a glucose moiety as a molecular component. Such a process of biological synthesis seems very likely in the instances of the plant levans and inulin.

Confirmation of this speculation has been afforded by Dedonder and Noblesse (*Ann. Inst. Pasteur*, 1953, **85**, 356) who showed the formation of an ascending series of D-glucose-containing oligosaccharides during enzymic synthesis of levan by the levan sucrase of the strain of *B. subtilis* used in the present work.

Using silica column chromatography (Bell and Palmer, *J.*, 1949, 2522) we have investigated the structures of two levans produced respectively by *Ps. prunicola*, Wormald, and *B. subtilis* BG2 F1. The former material is the sample used by Palmer (*loc. cit.*) and by

TABLE 1. *Summarised comparison of the two levans and their trimethyl ethers.*

	Levan of *Ps. prunicola*	Levan of *B. subtilis*
Purified levan: $[\alpha]_D$ in H_2O	−71°	−54°
Presence of D-glucose after hydrolysis	Negative	Negative
Trimethyl levan sol. in dioxan (%)	100	14 *
OMe (%)	45·6	—
$[\alpha]_D$ in $CHCl_3$	−63°	—
Hydrolysate		
(*a*) Paper qual. analysis: sugars found	Fructose Me_4, Me_3, and Me_2 ethers	Fructose Me_4, Me_3, and Me_2 ethers
(*b*) Silica column quant. analysis and identification; sugars found: molar ratios		
D-Fructose 1 : 3 : 4 : 6-Me_4 ether	1	—
,, 1 : 3 : 4-Me_3 ,,	7—8	—
,, 3 : 4-Me_2 ,,	1	—
Trimethyl levan insol. in dioxan (%)	0	86
OMe (%)	—	45·2
$[\alpha]_D$ in $CHCl_3$	—	−58°
Hydrolysate		
(*a*) Paper qual. analysis: sugars found	—	Fructose Me_4, Me_3, and Me_2 ethers
(*b*) Silica column quant. analysis and identification; sugars found: molar ratios		
D-Fructose 1 : 3 : 4 : 6-Me_4 ether	—	1
,, 1 : 3 : 4-Me_3 ,,	—	7—8
,, 3 : 4-Me_2 ,,	—	1

* The total material soluble in dioxan was obtained as "Fractions I and III" (see Experimental section).

Beattie and Bell (*Quart. J. Physiol.*, 1953, **38**, 1); the strain of the latter organism is that, from the collection of Mlle. Delaporte, used by Dedonder and Noblesse (*loc. cit.*). Beattie and Bell, examining the excretion of certain fructosans by the dog kidney, concluded that *prunicola* levan has a very high molecular size. *B. subtilis* levan is likewise of large size since the bulk of it can be centrifuged down at 25,000 **g**; work is in progress (at the Institut Pasteur) to determine its molecular weight.

Neither of the hydrolysates of these levans contained D-glucose detectable by paper chromatography. The use of D-glucose oxidase, however, showed the presence of D-glucose radicals in very small relative proportions.

Using the procedure of Bell and Palmer (*J.*, 1949, *loc. cit.*), Murphy (*Canad. J. Chem.*, 1952, **30**, 872) analysed the hydrolysis products of the methylated levan of *B. polymyxa* and obtained 1 : 3 : 4 : 6-tetra-, 1 : 3 : 4-tri-, and 3 : 4-di-*O*-methyl-D-fructoses in the ratios of 1 : 4 : 0·66. From the author's description of his fractions we conclude that they may not have been completely pure; none the less, traces of impurities would not affect his conclusion that this polysaccharide is very highly branched, and that the unit-chain length is considerably shorter than that of other bacterial fructosans.

As will be seen from Table 1, the " average chain " structures of both of our highly branched molecules are not to be distinguished by our chemical methods. However, the specific rotations, in water, of the two levans are markedly different, as are the solubilities in dioxan of their methylated derivatives, but we are unable yet to explain these differences.

Since methylation of both levans is accompanied by some losses (~6% per treatment) we feel justified in claiming only that the bulk of their molecules are composed of unit-chains having average lengths of 9—10 radicals. This is rather shorter than that deduced from the earlier analyses noted above which, however, were carried out by fractional distillation and when the characterisation of fructose derivatives had not reached its present efficiency. Since the silica-column chromatogram yields sharply defined fractions with no overlap we feel justified in believing that our results are not inaccurate. We draw attention to the fact that we have isolated both terminal radicals (tetramethyl fructose) and branch-point radicals (dimethylfructose) in homogeneous and equimolar amounts. The di-*O*-methyl-D-fructose is the 3 : 4-derivative, indicating that the linkage between the 2 : 6′-linked chains is of the inulin type and may be described as 2 : 1″. So far no bacterial fructosan has been found containing proved linkages other than 2 : 6′ or 2 : 1″, and there is no evidence that these are other than β in configuration.

Experimental

Evaporation of solvents was done below 40°. Optical rotations were measured in a 2-dm. tube.

Isolation of the Levans.—(*a*) The levan from *Ps. prunicola* (Wormald) was the preparation made, and used, by Beattie and Bell (*loc. cit.*). It had $[\alpha]_D$ −54° in H_2O.

(*b*) The medium (Grelet, *Ann. Inst. Pasteur*, 1951, **81**, 43; with sucrose 6 g./100 ml.) in which the *B. subtilis* was grown was first cleared of bacteria and solid debris by passage through a Sharples centrifuge. The resulting fluid was treated first with barium acetate at pH 8·2, filtered, and then saturated with barium hydroxide. The levan–barium complex was decomposed by carbon dioxide, and the fructosan isolated by freeze-drying (Beattie and Bell, *loc. cit.*). It had $[\alpha]_D$ −71° in H_2O and contained 0·3% of nitrogen.

Hydrolysis of the Levans.—This was done by heating solutions in 0·02N-sulphuric acid for 30 min. on the boiling-water bath. The recovery of fructose (colorimetric determination) was 96—97%.

Search for D-Glucose in the Hydrolysates.—(a) *By paper chromatography.* *n*-Butanol–ethanol–water (4 : 1·1 : 1·9) (Dedonder, *Bull. Soc. chim.*, 1952, **19**, 874) being used as solvent, portions (2 cu. mm.) of hydrolysates corresponding to 5, 10, and 20% solutions of the levans, after development, showed no detectable amounts of aldose when sprayed with aniline hydrogen phthalate.

(b) *By D-glucose oxidase* (cf. Palmer, *loc. cit.*). Data are given in Table 2. We consider that no significance can be attached to results obtained with D-glucose oxidase when the concentration of D-glucose is below 1 millimole in presence of 500 millimoles of other hexoses.

Table 2. *Examination of levan hydrolysates with D-glucose oxidase.*

Substrates	*Ps. prunicola* levan hydrolysate		*B. subtilis* levan hydrolysate		D-Fructose control	D-Glucose control
Amount of hexose examined (millimoles)	494	494	500	500	500	5
O_2 consumed (millimoles)	0	0·25	0·15	0	0	2·5
D-Glucose assayed from O_2 consumed	0	0·5	0·3	0	0	5
Moles of D-glucose per 100 moles of D-fructose	0	0·1	0·06	0	0	1

Methylation of the Levans.—The polysaccharides were treated with methyl sulphate and 30% (w/v) sodium hydroxide in presence of dioxan, and the products purified, as described by Bell and Palmer (*J.*, 1952, 3763) for grass levans.

(a) *Prunicola* levan (8·8 g., dry/wt.), after two methylations, was completely soluble in dioxan. The product, obtained by distilling off the dioxan in presence of an excess of water, amounted to 8·5 g. (86·2%; mean loss per methylation treatment, 6·7%). $[\alpha]_D$ was −63° in $CHCl_3$ (Found: OMe, 45·6%).

(b) *Subtilis* levan. The levan (4·0 g.) was methylated three times. The product was only partly soluble in dioxan (500 ml.). The insoluble material was separated on a glass filter bearing a layer of Celite; concentration of the filtrate to small bulk, followed by hot-water precipitation, yielded fraction I (174 mg.; 4% of final total). The residual solid was dissolved in chloroform (500 ml.), and the solution washed with an equal volume of water and then dried (Na_2SO_4). Light petroleum (b. p. 40—60°; 800 ml.) was added till no more precipitate formed. The resulting product (fraction II) weighed 3·80 g. (86% of final total) and had $[\alpha]_D$ −58° in $CHCl_3$ (Found: OMe, 45·2%). The chloroform–light petroleum mother-liquor was evaporated; the residue was soluble in dioxan from which water precipitated fraction III (425 mg.; 10% of final total).

The total yield of material accounted for was 4·4 g. (87·5%; mean loss per methylation treatment, 4·1%).

Paper-chromatographic Examination of the Methylated Levan Hydrolysates.—Samples of the methylated levans were hydrolysed by the method used for quantitative analysis. [Fractions I, II, and III from *B. subtilis* were examined separately. With aqueous butanol for development, each hydrolysate showed the presence of tetra-, tri-, and di-methylhexuloses (urea–HCl spray).] With aniline hydrogen phthalate spray no aldose derivatives were found.

TABLE 3. *Quantitative analysis of the hydrolysed methylated levans.*

Source of material	*Ps. prunicola*	*B. subtilis*
Wt. hydrolysed (mg.)	2566	1988
Total wt. of fractions recovered from column (mg.)	2530	1915
Recovery (%)	90·5	88·7
1 : 3 : 4 : 6-Tetra-*O*-methyl-D-fructose fraction :		
Wt. (mg.)	275	225
OMe (%) (distilled material)	52·1	52·1
$[\alpha]_D^{20}$ (H_2O) ,, ,,	+28°	+30°
n_D^{20} ,, ,,	1·4506	1·4502
Purity of fraction by oxidation with alkaline 3 : 5-dinitrosalicylate (%) [a]	100	100
Ketose spots detected on paper chromatography	Me_4 only	Me_4 only
Molar ratio	1	1
1 : 3 : 4-Tri-*O*-methyl-D-fructose fraction (crystalline) :		
Wt. (mg.)	1922	1512
OMe (%)	—	41·0
$[\alpha]_D^{20}$ (H_2O)	−56·3°	−55·8°
n_D^{20}	1·4666	1·4665
CH_2O from IO_4^- oxidation [b] (mol.)	0·98, 0·96	0·97
Ketose spots detected on paper chromatography	(*a*) Me_3 (*b*) ?Me_2 trace *	Me_3
Molar ratio	7—8	7—8
3 : 4-Di-*O*-methyl-D-fructose fraction :		
Wt. (mg.)	263	178
OMe (%)	29·6	29·6
$[\alpha]_D^{20}$ (H_2O)	−50·9°	−60·6°
n_D^{20}	1·4843	1·4855
CH_2O from IO_4^- oxidation [b]	1·51	1·63
Phenylosazone, m. p.	—	127—128°
Mixture with 3 : 4-di-*O*-methyl-D-glucosazone, m. p.	—	127—128°
Ketose spots detected on paper chromatography	(i) Me_2 (ii) trace of faster-moving material *	Me_2
Molar ratio	1	1

* These traces may be due to a tetramethyldifructose dianhydride formed by autocondensation of two molecules of 3 : 4-di-*O*-methylfructose (Bell, unpublished work).

[a] Bell, Manners and Palmer, *J.*, 1952, 3760. [b] Bell, Palmer, and Johns, *J.*, 1949, 1537.

Quantitative Analysis of the Hydrolysis Products.—By Bell and Palmer's procedure (*J.*, 1949,

2522), hydrolysates of the methylated levans (fraction II from *B. subtilis* only) were analysed on 15-g. columns of silica. The results are summarised in Table 3.

Paper Chromatography. Differentiation of Certain Methylated Fructoses by Alkaline Triphenyltetrazolium Spray.—Sprayed with an alkaline triphenyltetrazolium solution (cf. Wallenfels, *Naturwiss.*, 1950, **37**, 491; Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444) and kept at 50° for 45 min. in an atmosphere with water vapour, papers bearing spots of the methylated fructoses noted provide a simple means of differentiating between 3 : 4-dimethyl- and 3 : 4 : 6-trimethyl-fructose on the one hand (red spots) and 1 : 3 : 4-trimethyl- and 1 : 3 : 4 : 6-tetramethyl-fructose on the other (no reaction with reagent).

When a paper chromatogram of the crystalline trimethylfructose fraction from *prunicola* levan was treated as above, a red colour appeared on the most advanced edge of the sugar spot. This indicated the contamination of the 1 : 3 : 4-tri-*O*-methylfructose by a reducing isomer. The fraction was therefore crystallised from carbon tetrachloride; the contaminant was found to be concentrated in the mother-liquor. Dr. D. H. Northcote kindly examined the material isolated from the carbon tetrachloride, by borate-paper electrophoresis (Consden and Stanier, *Nature*, 1952, **169**, 783). From Table 4 it will be noted that the substance is very probably

TABLE 4. *Paper-electrophoretic examination of trimethylfructose contaminant in boric acid, with* 3 : 4 : 6-*tri*-O-*methyl*-D-*glucose as standard.*

Sugar	Presence or absence of vicinal (*cis*)-OH groups	Electrophoretic displacement from start-line
2 : 4 : 6-Tri-*O*-methylglucose	Absent	0
1 : 3 : 4-Tri-*O*-methylfructose	,,	0
3 : 4 : 6-Tri-*O*-methylglucose	Present	1·0 (Marker)
3 : 4 : 6-Tri-*O*-methylmannose	,,	1·45
3 : 4 : 6-Tri-*O*-methylfructose	,,	2·4
Contaminant	?	2·4

3 : 4 : 6-tri-*O*-methylfructose. No attempt was made to assay the amount of this contaminant; its origin is obscure. It appeared, from spray reaction, to be a hexulose derivative.

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