47. *Polysaccharides. Part XXXIX. The Constitution of Certain Levans formed by Bacterial Action.*

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The polysaccharides produced from sucrose by three different organisms, namely, *B. megaterium*, *Bact. pruni* and *Bact. prunicola*, are polyfructoses of the levan type. From an examination of the products of hydrolysis of the methylated levans it was demonstrated that each levan could be structurally represented by a terminated chain of 10—12 contiguous fructofuranose units mutually linked through positions 2 and 6 as in the levan formed by *B. subtilis*; thus certain differences in properties between the levans were probably due to varying degrees of aggregation of the "repeating unit." Anomalies in the optical rotations of levan acetates have been shown to arise from incomplete acetylation of the levan.

In an endeavour to determine whether plant-gums of the type of cherry- or damson-gum are of plant or of microbiological origin numerous attempts have been made in these laboratories to cultivate, in artificial media, organisms isolated from these gums or from the bark of infected trees. Occasionally certain of these organisms were able to produce polysaccharides, but in such cases the products were invariably polyfructoses of the levan type and not complex gums.

Levans formed from sucrose by the action of *B. subtilis* and *B. mesentericus* were isolated in purified form and in considerable quantities by Harrison, Tarr, and Hibbert (*Canadian J. Res.*, 1930, **3**, 449). The chemical structure of these "bacterial" levans, which are water-soluble, acid-labile analogues of inulin, was studied by Hibbert, Tipson, and Brauns (*ibid.*, 1931, **4**, 221) and investigated in greater detail by Challinor, Haworth, and Hirst (J., 1934, 676). Levans of a similar, if not identical, constitution are widely distributed in plants and they appear to represent the form in which carbohydrate is stored in certain species. Certain of these levans have been investigated previously (see, *e.g.*, Challinor, Haworth, and Hirst, J., 1934, 1560; Haworth, Hirst and Lyne, *Biochem. J.*, 1937, 31, 786; Schlubach *et al.*, *Annalen*, 1936, **130**, 523; 1937, 532, 191). Further interest in levans arose when Cooper and Preston (*Biochem. J.*, 1935, **29**, 2267), during the course of an investigation of polysaccharide synthesis by bacteria, demonstrated that a number of micro-organisms, particularly those in the plant-pathogen group, were able to produce from sucrose acid-labile polyfructoses of the levan type.

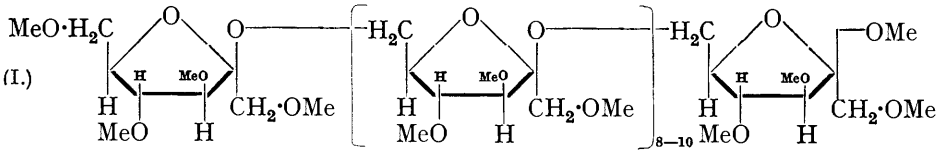
For the purpose of the present investigation quantities of levans were prepared from sucrose by the action of three different organisms (Cooper and Preston, *loc. cit.*). Those employed were: (a) *B. megaterium*, an aerobic bacillus closely related to the *B. subtilis* group; (b) *Bact. pruni* (*Phytomonas pruni*), the plant pathogen which causes "black spot" and canker in peaches, plums, etc.; (c) *Bact. prunicola* (*Phytomonas prunicola*), the plant pathogen which is the causative agent of "bacterial shot wilt" of the plum tree.

The levans were purified by repeated precipitation by methyl alcohol from an aqueous solution. The rotations of the levans in water were almost identical and each on hydrolysis with dilute acid yielded only fructose residues. Whereas the levans from *Bact. pruni* and

B. megaterium appeared to be identical in physical properties with previously known levans, the product from *Bact. prunicola* was considerably less soluble in water and gave solutions of pronounced viscosity and opalescence.

Previous work (Challinor, Haworth, and Hirst, *loc. cit.*) has shown that the methylated *B. mesentericus* levan can be structurally represented by (I).

(I.)



In this formulation the molecular structure is shown to be built up on the plan of 10—12 contiguous fructofuranose units, mutually linked through positions 2 and 6, forming a terminated chain molecule, and its relationship to inulin has been discussed. In the present work, carried out separately on each levan, the methods followed closely the lines laid down by Challinor, Haworth, and Hirst (*loc. cit.*). Direct methylation, with sodium hydroxide and methyl sulphate, proceeded smoothly to give fully methylated levans which were homogeneous and apparently identical with one another. By the " end-group " method it was found, within the limits of experimental error, that in each case (I) could be taken to represent the molecular constitution of the " repeating unit " of the methylated levan. The terminal residue, obtained in 10—12% yield of the scission products, was recognised as 1 : 3 : 4 : 6-tetramethyl methylfructoside, since it was readily converted into 2 : 3 : 4 : 6-tetramethyl fructuronamide (Avery, Haworth, and Hirst, J., 1927, **2313**). The trimethyl methylfructoside constituent was identified by its conversion into crystalline 1 : 3 : 4-trimethyl fructose. Such differences of properties as existed between the levans examined appeared to be attributable to the different degrees of aggregation of the repeating unit and in this respect a parallelism between the mode of constitution of levan and of starch and glycogen is apparent (Haworth, Hirst, and Isherwood, J., 1937, 577).

Challinor, Haworth, and Hirst (*loc. cit.*) had observed that both the levan formed by *B. subtilis* and that occurring in rough-stalked meadow grass (*Poa trivialis*) gave acetates the rotations of which varied according to the mode of preparation. Similar results had previously been recorded by Haworth and Streight (*Helv. Chim. Acta*, 1932, **15**, 609) for inulin acetate. Using certain bacterial levans, we have been able to examine the problem more closely. The general method used for preparing the levan acetates involved the addition of a small quantity of water to dissolve the levan, prior to the addition of the acetylating reagents, namely, pyridine and acetic anhydride. It was observed that the rotations of these acetates varied in a remarkable manner according to the proportion of water present. That these products were not the result of polysaccharide degradation was proved by the facts that (*a*) each acetate on de-acetylation by sodium hydroxide gave the original levan unchanged in properties; (*b*) each acetate on methylation yielded a methyl levan identical with that produced directly from the original levan; (*c*) each acetate on further acetylation by the aqueous pyridine–acetic anhydride method gave acetates with rotations which varied according to the amount of water present. A study of the acetyl contents of the products revealed the interesting fact that within certain limits a decreased quantity of water present in the acetylation mixture gave a product with an increased acetyl content and that the more highly acetylated products showed a higher positive rotation than those which were incompletely acetylated. For example, a fully acetylated levan (OAc, 44·8%) had $[\alpha]_D$ +23° in chloroform, whereas an acetylated levan with acetyl content of 43·2% showed $[\alpha]_D$ −13° in chloroform. Each of these acetates yielded the same levan on de-acetylation.

Experimental.

Bact. Pruni *Levan*.—The polysaccharide was obtained as a pinkish-white, non-reducing powder. Fractionation from aqueous solution by alcohol showed it to be essentially homogeneous, an average sample having ash 3·0%, moisture 5·0%, and showing $[\alpha]_D^{20°}$ −45° in water.

It was labile towards acid reagents inasmuch as heating with N/100-hydrochloric acid at 50° changed the specific rotation from $[\alpha]_D^{20°}$ −40° to $[\alpha]_D^{20°}$ −86° (equilibrium value) in 140 minutes. An aliquot portion of the hydrolysate, on warming with phenylhydrazine acetate, gave glucosazone in quantitative yield, and in a second portion of the hydrolysate, the estimation of total reducing sugar by Fehling's solution and of ketose sugar by Nyn's method (*Chem. Abstr.*, 1925, **19**, 1236) indicated that the levan was constituted of fructose residues only.

Methylation. The method used was that described by Challinor, Haworth, and Hirst (*loc. cit.*). The product (20 g.) (OMe, 44·8%) was dissolved in chloroform–acetone and separated by the addition of light petroleum into the following fractions:

Fraction.	Weight, g.	OMe, %.	$[\alpha]_D^{20°}$ in $CHCl_3$.	$\eta_{sp.}$ in *m*-cresol.
I	1·0	38·0	−57°	0·18
II	4·1	43·7	−57	0·93 (contained 0·5% of ash)
III	11·0	44·9	−56	0·31
IV	2·0	44·2	−54	0·30
V	1·0	44·3	−54	0·29

Determinations of the chain-length by the methods of Challinor, Haworth, and Hirst (*loc. cit.*) were carried out on fraction II and on a sample from the combined fractions III, IV, and V. No furfural or unsaturated decomposition products could be detected during the operations of hydrolysis, glycoside formation, or distillation of these or subsequent fractions described later. From fraction II (4·02 g.) the following products were isolated by distillation at 0·01 mm.:

Fraction.	Weight, g.	$n_D^{20°}$.	Bath temp.	OMe, %.
1	0·3	1·4450	115°	59·7
2	0·1	1·4510	115—120	57·1
3	2·16	1·4570	120—130	51·1
4	0·17	1·4610	130	50·2

The estimated amount of tetramethyl methylfructoside (contained in fractions 1 and 2) was 0·385 g. This amount was derived from 4·02 g. of levan and corresponds to a chain length of 11—12 fructose units.

A repetition of the hydrolytic procedure applied to the combined fractions III, IV, and V (6·72 g.) yielded the following monosaccharide fractions:

Fraction.	Weight, g.	$n_D^{20°}$.	Bath temp.	OMe, %.
a	0·24	1·4450	130°	60·3
b	1·39	1·4550	130—135	53·7
c	2·07	1·4585	135—140	51·2
d	0·5	1·4612	above 140	49·0

The amount of tetramethyl methylfructoside from this material was estimated to be 0·66 g. and it corresponds to a chain length of 11—12 units.

In both cases the tetramethyl methylfructoside was shown to be 1 : 3 : 4 : 6-tetramethyl methylfructofuranoside inasmuch as it was converted (in yield comparable with that from authentic tetramethyl fructofuranose) into the crystalline amide of 2 : 3 : 4 : 6-tetramethyl fructuronic acid, m. p. 96° alone or when mixed with an authentic specimen (Avery, Haworth, and Hirst, *loc. cit.*).

The fractions with properties corresponding to those of a trimethyl methylhexoside gave, on hydrolysis, 1 : 3 : 4-trimethyl fructose, m. p. 72° alone or in admixture with an authentic specimen.

Bact. Prunicola *Levan.*—This polysaccharide was a white powder, non-reducing in Fehling's solution; it was moderately readily soluble in water, in which it showed $[\alpha]_D$ −40° (ash, 1·8%; moisture, 2·2%). It was hydrolysed by N/100-hydrochloric acid at 60°, the rotation ($[\alpha]_D$ −40°) changing to $[\alpha]_D$ −93° in 240 minutes. Estimations of the reducing sugars in the hydrolysate by Fehling's solution and by Nyn's method proved that the polysaccharide contained only fructose residues. Hydrolysis of the levan with N-acetic acid, followed by the addition of phenylhydrazine, gave glucosazone in quantitative yield.

Methylation. Five methylations of *Bact. prunicola* levan (10 g.) by the method previously indicated gave a methylated product (8 g.) (OMe, 44·5%). 30 G. of material were prepared in this way and separated in the usual manner into the following fractions:

Fraction.	Weight, g.	OMe, %.	$[\alpha]_D$ in $CHCl_3$.	$\eta_{sp.}$ in *m*-cresol.
A	1·6	41·0	−53°	insol.
B	8·3	43·0	−59	0·6
C	12·3	43·0	−59	0·37
D	2·6	44·7	−60	0·33

Estimations of the chain length by hydrolysis with acid methyl alcohol, followed by fractional distillation of the methylated fructosides so formed, was carried out in the usual way.

Fraction B (5·06 g.) yielded the following main fractions: (a) 0·27 g., b. p. 110°/0·02 mm., $n_D^{20°}$ 1·4465 (OMe, 58·0%); (b) 1·24 g., b. p. 115°/0·02 mm., $n_D^{20°}$ 1·4515 (OMe, 53·7%); (c) 2·30 g., b. p. 120°/0·02 mm., $n_D^{20°}$ 1·4550 (OMe, 50·9%); residues 1·5 g. The amount of tetramethyl methylfructoside contained in (a) and (b) (0·58 g.) was *ca.* 11% of the weight of methylated levan hydrolysed and corresponded to a chain length of 10—11 units.

Fraction C (10·55 g.) yielded the following main fractions: (a′) 0·55 g., b. p. 110°/0·03 mm., $n_D^{20°}$ 1·4440 (OMe, 59·0%); (b′) 1·13 g., b. p. 120°/0·04 mm., $n_D^{20°}$ 1·4480 (OMe, 58·0%); (c′) 4·92 g., b. p. 125°/0·03 mm., $n_D^{20°}$ 1·4550 (OMe, 52·0%); residues 4·0 g. (OMe, 50·0%). The amount of tetramethyl methylfructoside in (a′) and (b′) (1·12 g.) was *ca.* 10% of the weight of methylated levan hydrolysed and as before corresponded to a chain length of 10—12 units.

From both fractions the 1 : 3 : 4 : 6-tetramethyl methylfructoside was characterised by conversion into 2 : 3 : 4 : 6-tetramethyl fructuronamide, and the trimethyl methylfructoside by its hydrolysis to crystalline 1 : 3 : 4-trimethyl fructose.

B. Megaterium *Levan.*—This polysaccharide resembled closely the *Bact. pruni* levan in its physical properties. It was a water-soluble white powder, $[\alpha]_D$ −40°, ash 2·0%, moisture 3·0%. After hydrolysis with N-acetic acid, followed by treatment with phenylhydrazine, it yielded a quantitative amount of glucosazone. On heating with N/50-hydrochloric acid at 60° its rotation $[\alpha]_D$ −40° changed to $[\alpha]_D$ −85° (equilibrium value) in *ca.* 30 minutes.

Methylation. Three treatments by the method previously indicated sufficed to give a methylated levan with methoxyl content 44·6%. A sample (26 g.) was separated into the following fractions by addition of light petroleum to a chloroform–acetone solution:

Fraction.	Weight, g.	OMe, %.	$[\alpha]_D$ in $CHCl_3$.	$\eta_{sp.}$
1	1·7	43·0	−55°	0·17
2	10·4	44·0	−57	0·18
3	10·4	44·0	−56	0·11
4	2·6	45·6	−54	0·10

Chain-length determinations were carried out on fractions 2 and 3. From fraction 2 (8·5 g.) the following fractions of methylfructosides were obtained: (e) 0·29 g., $n_D^{21°}$ 1·4440 (OMe, 60·0%); (f) 1·25 g., $n_D^{21°}$ 1·4510 (OMe, 55·0%); (g) 4·5 g., $n_D^{21°}$ 1·4550 (OMe, 51·0%); (h) 1·33 g., $n_D^{21°}$ 1·4582 (OMe, 50·0%). The amount of tetramethyl methylfructoside in fractions (e) and (f) was 9% of the weight of methylated dextran hydrolysed and corresponded to a chain length of 12—13 units. From fraction 3 (9·05 g.) the following fructoside fractions were prepared: (e′) 0·15 g., $n_D^{20°}$ 1·4450 (OMe, 61·0%); (f′) 1·68 g., $n_D^{20°}$ 1·4510 (OMe, 55·0%); (g′) 2·91 g., $n_D^{20°}$ 1·4570 (OMe, 52·0%); (h′) 2·17 g., $n_D^{20°}$ 1·4630 (OMe, 50·0%). The amount of tetramethyl methylfructoside (1·09 g.) in (e′) and (f′) was *ca.* 12% of the weight of the methylated levan hydrolysed and corresponded to a chain length of about 10 units.

In this distillation it was noted that certain fractions (h′ and the residue) were more viscid than usual. Accordingly they were combined and subjected to further hydrolysis by contact with 0·5% methyl-alcoholic hydrogen chloride during 50 hours at room temperature. The product, isolated in the usual way, now distilled readily, had $n_D^{20°}$ 1·4550, and was shown to be 1 : 3 : 4-trimethyl methylfructoside. It would appear from these observations that fraction (h′) and the residues contained a difructosan derivative (cf. Haworth, Hirst, and Percival, J., 1932, 2384). The tetra- and tri-methyl methylfructosides were characterised by the methods previously described.

Levan Acetates.—B. pruni *levan acetate. Method I.* Levan (1·0 g.) was dissolved in water (2 c.c.) and pyridine (20 c.c.), and the solution cooled in ice. Acetic anhydride (20 c.c.) was then added cautiously to the well-stirred solution. After being kept for 2 days at room temperature, the gel-like mass was poured into water; acetyl levan then separated as a white powder. It was thoroughly washed with water and dried in a vacuum. Purification was effected by the addition of light petroleum to a solution in chloroform. The acetate had $[\alpha]_D$ +20° in chloroform and $\eta_{sp.}$ 0·50 (in *m*-cresol).

Method II. The acetylation was carried out as above, except that the acetylating reagents were allowed to react for 8 hours only. The acetate had $[\alpha]_D$ +21° in chloroform, $\eta_{sp.}$ 0·59 (in *m*-cresol). On the addition of light petroleum to a solution of the acetate in chloroform the following fractions were separated: (A) 4·1% yield, $[\alpha]_D$ +21° in chloroform; (B) 31·2% yield, $[\alpha]_D$ +21° in chloroform; (C) 28·0% yield, $[\alpha]_D$ +21° in chloroform; (D) 20·0% yield, $[\alpha]_D$ +21° in chloroform.

A sample (0·9 g.) on de-acetylation with sodium hydroxide yielded levan (0·33 g.) having $[\alpha]_D$ −45° in water. This material, when re-acetylated by method II, gave an acetate having $[\alpha]_D$ +20° in chloroform, whereas another sample, acetylated by method III (below), formed an acetate having $[\alpha]_D$ −10° in chloroform.

Acetyl levan prepared by method II on treatment with sodium hydroxide and methyl sulphate gave a methylated derivative (OMe, 44·4%) having $[\alpha]_D$ −59° in chloroform.

Method III. This acetylation was carried out by method I, but with the further addition of 4 c.c. of water. The reaction was allowed to proceed for 8 hours. The product had $[\alpha]_D$ −10° in chloroform and $\eta_{sp.}$ 0·44. On fractionation by the addition of ether and light petroleum to the chloroform solution the following fractions were separated :

Fraction	A	B	C	D
Yield, %	10	44	20	11
$[\alpha]_D$	± 0°	−10°	− 6°	− 2°

On de-acetylation, a sample of this acetate (0·7 g.) gave levan (0·30 g.) having $[\alpha]_D$ −44° in water. This material on re-acetylation by method II gave an acetate having $[\alpha]_D$ +20° (in chloroform), and a further sample on re-acetylation by method III yielded an acetate having $[\alpha]_D$ −10° (in chloroform).

B. megaterium *levan.* Comparable results were obtained with the *B. megaterium* levan. A series of acetates was prepared having rotations varying from $[\alpha]_D$ −26° to $[\alpha]_D$ +23° (in chloroform) depending upon the amount of water present during the acetylation. Determination of the acetyl contents of these products gave the following values :

$[\alpha]_D$ of acetate in $CHCl_3$	+23°	+15°	+14°	+ 4°	− 1°	−13°	−18°	−30°
% Acetyl	44·8	44·4	44·4	44·8	44·0	43·2	38·1	36·4

It would thus appear that incomplete acetylation, due to the presence of an excess of water during acetylation, provides the reason for the differences in rotation of the levan acetates.

Acetyl levan prepared by method III, on methylation in the usual way, gave a methylated derivative having OMe 44·2% and $[\alpha]_D$ −58° in chloroform.

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