CCLII. POLYLAEVANS FORMED BY THE CARBOHYDRATE METABOLISM OF CERTAIN BACTERIA

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ØRSKOV [1930; 1931; 1936; 1938; Ørskov & Poulsen, 1931] has stated that when certain Gram-negative organisms found in milk and certain soil bacteria (*Actinomycetes*) are grown on sucrose- or raffinose-containing subtrates, polysaccharides are formed abundantly. The substances formed have, however, not been examined chemically, and their nature as polysaccharides has been deduced from their appearance alone. It was therefore thought to be of interest to examine them more thoroughly, and the investigation here reported shows that the three micro-organisms hitherto studied do in fact form polylaevans and most probably the same laevan in each case.

(1) *Substance from milk bacteria [Ørskov, 1931]*

The micro-organisms were grown on 2% sucrose-agar. After 2 days the bacteria were separated by filtration and the "colonies" of polysaccharide remaining in the somewhat opalescent liquid were precipitated by addition of alcohol and ether. The supernatant liquid was decanted and the precipitate was washed with physiological salt solution, separated by centrifuging and dried to constant weight in a vacuum desiccator over conc. H_2SO_4.

Analysis showed that the substance isolated had the composition of a polysaccharide, contaminated with a small amount of protein possibly originating from residual bacteria or from cellular nuclei in the "colonies" of polysaccharide. (Found: C, 44·32; H, 6·41; N (Kjeldahl), 0·59%. $(C_6H_{10}O_5)_n$ requires C, 44·42; H, 6·23%.) The polysaccharide is practically insoluble in all solvents. It does not reduce Fehling's solution, but after hydrolysis with boiling dilute acid it gives strongly reducing products.

0·54 g. of the polysaccharide was heated for 3 hr. on the steam bath with 40 ml. water and 5 ml. *N* HCl. The solution was left at room temperature overnight; next day it was made up to 50 ml. in a measuring flask, filtered from a trace of flocculent precipitate and its optical rotation determined; $\alpha_D^{20°}$ was $-1·77°$ (c (calc. as monosaccharide) $= 1·2$, $l = 2$), whence $[\alpha]_D^{20°} = -74°$. This is a minimum value and it is some 20% lower than the specific rotation of equilibrium fructose, but as already pointed out the polysaccharide was not N-free; it may therefore be assumed with some probability that the polysaccharide is a (slightly impure) fructose anhydride (laevan).

In order to confirm this supposition, a solution of 1·5 g. polysaccharide was neutralized with NaOH, concentrated in a vacuum at 35–40° to low volume and finally dried in a vacuum desiccator over H_2SO_4. The residue, 1·2 g., was treated with dry acetone and conc. H_2SO_4 as recommended by Ohle & Koller [1924] for the preparation of β-diacetonefructose. 0·9 g. of a compound with M.P. 95–96° and $[\alpha]_D^{20°} = -38·6°$ (alcohol, $c = 1·166$) or $-34·3°$ (water, $c = 1·808$) was obtained. Ohle & Koller [1924] obtained from 10 g. of fructose 6·5 g. of diacetonefructose

with M.P. 97° and $[\alpha]_D^{20°} = -36{\cdot}69°$ (alcohol, $c = 1{\cdot}172$) or $-26{\cdot}17°$ (water, $c = 1{\cdot}856$); they indicate, however, that the specific rotation in aqueous solution depends on the concentration; for $c = 3{\cdot}161$ they found $[\alpha]_D^{20°} = -32{\cdot}9°$.

For comparison we prepared β-diacetonefructose from authentic fructose. From 2·5 g. we obtained 1·4 g. of a preparation with M.P. 96–97°, i.e. nearly the same yield as that quoted by Ohle & Koller. The specific rotation of this preparation in aqueous solution did not depend on the concentration: for $c = 3{\cdot}193$ $[\alpha]_D^{20°}$ was $-34{\cdot}0°$ and for $c = 1{\cdot}811$ $[\alpha]_D^{20°}$ was $-34{\cdot}1°$, in agreement with Ault *et al.* [1935] who found $[\alpha]_D^{21°} = -34°$ (water, $c = 0{\cdot}9$). For an alcoholic solution with $c = 1{\cdot}183$ we found $[\alpha]_D^{20°} = -37{\cdot}97°$ for the product from fructose. The values found by us for authentic diacetonefructose are thus identical with those found for the acetone derivative of the product of hydrolysis of the polysaccharide, which therefore may be assumed to be fructose.

Further confirmation of this assumption has been obtained by the method of Bridel [1930] for detection of glucose or galactose in plant materials, namely, solution of the substance in 70% methyl alcohol and examination of the influence of addition of emulsin (β-glucosidase). If a sugar, which is able to form a methyl glycoside under the influence of emulsin is present, an alteration of the optical rotation of the solution will take place.

The monosaccharide obtained from 4·65 g. of the polysaccharide was dissolved in 100 ml. 70% methyl alcohol and 0·2 g. of an emulsin preparation with sal. f. 0·091 was added. The solution was rotated mechanically for 14 days at 30°. Before addition of emulsin the solution had $\alpha_D - 5{\cdot}17°$ and the rotation remained unaltered during the experiment. From this it may be concluded at least that glucose and galactose are absent from the solution, and the assumption that fructose is the sole product of hydrolysis gains in probability.

(2) *Substance from soil* Actinomycete, *strain 38.* [*Ørskov, 1938*]

The bacteria were grown as above. The polysaccharide formed in this case, although corpuscular, did not form "colonies"; it was isolated in the same manner as previously. 2 g. were hydrolysed by heating on the steam bath for 3 hr. with 150 ml. water and 5 ml. $N/10$ HCl. The solution was left overnight at room temperature and then neutralized with NaOH, filtered, evaporated in a vacuum at 40° to low volume and finally dried in a vacuum desiccator over H_2SO_4. The residue was a syrup, which was taken up in 30 ml. abs. alcohol; only a very little hygroscopic substance was left undissolved. The alcoholic solution was evaporated to dryness and 1·5 g. of non-hygroscopic substance were obtained. The M.P. was very unsharp, 80–95° (decomp.). Pure fructose melts at 102° or 95°. 0·0997 g. was dissolved to make 10 ml. aqueous solution. In a 2 dm. tube α_D was $-1{\cdot}57°$, and next day had diminished to $-1{\cdot}52°$, i.e. $[\alpha]_D^{20°} = -76{\cdot}2°$. 1·5 g. of the substance yielded 1·05 g. of a diacetone derivative with M.P. 96–97° and $[\alpha]_D^{20°} = -38{\cdot}76°$ (alcohol, $c = 1{\cdot}193$). This polysaccharide also seems therefore to be a laevan.

(3) *Substance from soil* Actinomycete, *strain 47.* [*Ørskov, 1938*]

The bacteria were grown and the substance isolated as in the case of strain 38. 2·0715 g. were heated for 1 hr. on the steam bath with 85 ml. water and 5 ml. N HCl. Next day the solution was filtered and made up to 100 ml. The rotation of the solution in a 2 dm. tube was $-3{\cdot}24°$, whence $[\alpha]_D^{20°} = -70{\cdot}4°$.

The solution was neutralized with NaOH and the monosaccharide was isolated as above. Yield *ca.* 2 g. Without purification the material was trans-

formed into a diacetone derivative, of which 1 g. was obtained with M.P. 95·5–96·5 and $[\alpha]_D^{20°} = -38·67$ (alcohol, $c = 1·170$). In this case too fructose seems to be the sole product of hydrolysis.

Comparison with other naturally occurring laevans. Acetylation and methylation

Polylaevans are rather commonly met with in nature. Inulin is the classical example, but in the last few years Schlubach and co-workers [1936; 1937, 1, 2, 3] have isolated a series of fructose anhydrides from different plants, and, which is particularly significant for us, Harrison *et al.* [1930] and Hibbert and co-workers [1930; 1931, 1, 2] have found polylaevans to be synthesized by various micro-organisms when they are grown in sucrose solutions.

The micro-organisms examined by Hibbert *et al.* were *Clostridium gelatigenosum, Semiclostridium commune, Bacillus laevaniformans, B. hemiphloriae, Aspergillus sydowi*; the *subtilis* and *mesentericus* groups were specially prone to synthesize polylaevans and the most effective seems to have been *B. mesentericus* Trevisan which can bring about the synthesis from sucrose or raffinose but not from melezitose, lactose, maltose, xylose, glucose or fructose. Ørskov [1938] has found the same for the micro-organisms examined by him.

The polylaevans examined by Schlubach *et al.* were all soluble in water. Those isolated by Hibbert *et al.* were soluble in hot and slightly so in cold water. In most organic solvents they were insoluble, but they dissolved in hot glycerol or ethyleneglycol. These laevans, with acetic anhydride and pyridine, gave triacetates which, after treatment first with methyl sulphate and NaOH and finally with Purdie's reagent, gave trimethyl derivatives. The triacetates and trimethyl derivatives of the different laevans differed in solubility, specific rotation and M.P., the most important difference being that the triacetates of the plant laevans were laevorotatory, whilst those of the bacterial laevans isolated by Hibbert *et al.* were dextrorotatory.

We have therefore prepared the triacetates and trimethyl derivatives of our laevans and have compared their properties with those mentioned above.

(1) *Substance from milk bacteria.* The acetylation was carried out as indicated by Schlubach & Loop [1936]. A triacetate was formed which softened at 90–100° but was completely molten only at 160–170°. (Found: C, 49·73; H, 5·15; CH_3CO, 43·5 %. $(C_{12}H_{16}O_8)_n$ requires C, 50·00; H, 5·60; CH_3CO, 44·9 %.)

The triacetate was nearly insoluble in all solvents. Glacial acetic acid dissolved a small amount when warm, but most of it separated again on cooling; the cooled solution was dextrorotatory, having $\alpha_D + 0·06°$. In chloroform the product had $[\alpha]_D^{20°} + 11·2$, which is to be regarded with reserve as a minimum figure.

The methylation of the triacetate was carried out as indicated by Schlubach & Loop [1936]. The trimethyl derivative was dissolved in benzene and precipitated by light petroleum. A white, non-hygroscopic, amorphous powder was obtained, which softened at 122–125° and was completely molten at 140° $[\alpha]_D^{20°} = -60·9°$ ($CHCl_3$, $c = 2·086$). (Found: OCH_3 44·6 %. $(C_9H_{16}O_5)_n$ requires OCH_3 45·6 %.)

(2) *Substance from soil* Actinomycete, *strain 38.* The triacetate and trimethyl derivative were prepared as mentioned above. The trimethyl derivative softened at 122–125° and was completely molten at 138°. $[\alpha]_D^{20°} = -57·2°$ ($CHCl_3$, $c = 1·958$, $l = 2$). (Found: OCH_3, 46·3 %. Calc. OCH_3, 45·6 %.)

(3) *Substance from soil* Actinomycete, *strain 47.* The trimethyl derivative prepared as above had M.P. 138–140° (softening at 120–125°), $[\alpha]_D^{20°} = -58·8°$ ($CHCl_3$, $c = 1·360$, $l = 2$). On account of lack of material no methoxyl determination was made.

From the properties of the methyl derivatives it appears that all three preparations of polysaccharide are identical. In the table below we have collected the data for different laevans described in the literature.

			Triacetate		Trimethyl derivatives	
	M.P.	$[\alpha]_D$	M.P.	$[\alpha]_D$	M.P.	$[\alpha]_D$
Inulin [Hibbert *et al.*, 1931]	—	−40° water	102–103°, soft 95°	−42·55°, CH_3COOH	138–140°	−50·2°, $CHCl_3$
Laevan [Hibbert *et al.*, 1931]	—	−45·3° water	190°, soft 106°	+21°, $CHCl_3$	145–147°	−87 to −91°, $C_2H_2Cl_4$
Triticin [Schlubach & Peitzner, 1937]	—	−51·4° water	115° or 191°	−15·5°, $CHCl_3$	141–151°	−61·2°, $CHCl_3$
Sinistrin [Schlubach & Loop, 1936]	—	−44°	—	−23·5°, C_6H_6	—	−57°, $CHCl_3$
Asparagosin [Schlubach & Böe, 1937]	197–198° soft 170°	−32·4°	93°, soft 80°	−20·1°, $CHCl_3$	—	−47·8°, $CHCl_3$
Aspholedin [Schlubach & Lendzian, 1937]	—	−30·5°	—	−16·6°	—	—
Own preparations	—	—	160–170°, soft 90°	> +11°, $CHCl_3$	140°, soft 122°	−57 to −60°, $CHCl_3$

The dextrorotation of the triacetate points to the identity of our preparations with the laevans of Hibbert *et al.*, whilst the rotations of the trimethyl derivates resemble more those of the plant laevans examined by Schlubach *et al.* Hibbert *et al.* states that their trimethyllaevans on hydrolysis yield 1:3:4-trimethylfructose, whereas trimethylinulin on hydrolysis yields 3:4:6-trimethylfructose. The amount of trimethyl derivative at our disposal did not permit an examination of the products of hydrolysis, but the investigation is being continued in this direction. We wish to point out, however, that the solubility of our preparations seems to differ both from that of plant laevans and from that of bacterial laevans hitherto described.

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