413. *Protozoal Polysaccharides. Structure of a Polysaccharide produced by* Cycloposthium.

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The ciliated protozoon *Cycloposthium* found in the colon and cæcum of a horse synthesises as reserve material a polysaccharide of high molecular weight, which contains only glucose residues. Examination of this material by the methylation method and by the periodate oxidation procedure has shown that its highly branched molecular structure is closely similar to that of amylopectin. The unit chains contain on an average 23 α-1 : 4-linked glucopyranose residues and are joined by $C_{(1)}$–$C_{(6)}$ linkages.

It has been shown that certain intestinal protozoa synthesise glucose-containing polysaccharides which are stored intracellularly as food reserve materials (Oxford, *J. Gen. Microbiol.*, 1951, **5**, 83; Heald and Oxford, *Biochem. J.*, 1953, **53**, 506). These materials are stained reddish-brown by iodine, and a chemical examination of a sample produced by a free-living protozoan *Tetrahymena piriformis* led to the conclusion that it was a typical animal glycogen of molecular weight about 10^7, possessing an average unit chain length of 13

glucose residues (Ryley, *Biochem. J.*, 1951, **49**, 577; Manners and Ryley, *ibid.*, 1952, **52**, 480). It is now clear, however, that considerable differences in structure may occur between the glucose-containing polysaccharides synthesised by protozoa. The present paper is concerned with a study of a polysaccharide isolated from the large protozoon *Cycloposthium* (Class, Ciliophora; Sub-Class Ciliata) which was found in large numbers in the colon and cæcum of a horse (see Adam, *Parasitology*, 1951, **41**, 301, concerning the distribution of ciliate protozoa in this animal's large intestine). The protozoa, separated from the liquid contents of the colon and cæcum, were so rich in polysaccharide as to be stained almost black by iodine. This intracellular carbohydrate occurs in the form of coherent masses which are structural features characteristic of the cell. After extraction by chloral hydrate it was obtained as a white powder in a yield of 50% by weight of the dried protozoa. By this method of extraction the morphologically discrete character of the polysaccharide cannot be preserved but little or no alteration of the main chemical structure is brought about. The material gave a red-purple colour with iodine and had a specific rotation ($[\alpha]_D$) of $+190°$ in 30% aqueous perchloric acid and $+154°$ in N-sodium hydroxide, the values being similar to those of amylopectin. The low " blue value " (Hassid and McCready, *J. Amer. Chem. Soc.*, 1943, **65**, 1154; Haworth, Peat, Bourne, and Macey, *J.*, 1948, 924) indicated that material of amylose type was present in negligible amount. Hydrolysis with acid gave glucose as the only sugar. The amount of formic acid produced when the polysaccharide was oxidised by potassium periodate corresponded to the presence of one non-reducing terminal glucose residue for every 23 glucose units in the molecule. The reaction proceeded without complications and followed exactly the course observed with amylopectin (Brown, Halsall, Hirst, and Jones, *J.*, 1948, 27). After oxidation by periodate the residual polysaccharide was subjected to acid hydrolysis and the products were examined chromatographically. Only a trace of glucose was present and it is therefore clear that the proportion of glucose residues not accessible to attack by periodate must be very small, certainly less than 1%. There can be few, if any, glycosidic linkages involving either $C_{(2)}$ or $C_{(3)}$ of any of the glucose residues, and the great majority of the inter-unit bonds must be of the 1 : 6-type.

Methylation of the polysaccharide was carried out in nitrogen by methyl sulphate and sodium hydroxide. The trimethyl derivative had properties very similar to those of methylated amylopectin. It showed a relatively high specific viscosity in *m*-cresol, corresponding to a molecular weight of about 200,000 when the constant given by Hirst and Young (*J.*, 1939, 1471) was used. This trimethyl derivative was heated with methanolic hydrogen chloride, and the methylated methylglucosides so obtained were hydrolysed with aqueous hydrochloric acid. The mixture of reducing sugars was examined chromatographically both on paper strips and on a column of powdered cellulose. The substances present were 2 : 3 : 4 : 6-tetramethyl D-glucose, 2 : 3 : 6-trimethyl D-glucose, 2 : 3-dimethyl D-glucose, and a mixture of 2 : 6- and 3 : 6-dimethyl glucose which probably owed their origin to incomplete methylation of the polysaccharide or to some slight demethylation during hydrolysis. Only traces of monomethyl glucose and free glucose were found.

The yield of tetramethyl glucose corresponded to the presence of one non-reducing terminal glucose residue for every 23 glucose units in the molecule. This value is identical with that found by the periodic acid method and it follows that the polysaccharide from *Cycloposthium* has an average unit chain length of 23 glucose residues. In this respect and in its high specific viscosity in solution it resembles amylopectin much more closely than it does glycogen. It clearly possesses a highly branched structure of the amylopectin type but in the absence of detailed enzymic studies on the lines employed by Cori and by Peat for glycogen and starch no estimate of the extent of multiple branching can yet be made. In conclusion it may be remarked that in the present state of knowledge it would be preferable not to designate polysaccharides of this type as glycogen or amylopectin solely by reason of the unit chain length present in their molecules. Amylopectins generally differ from glycogen in having high specific viscosities. This, as has been indicated previously (Halsall, Hirst, Hough, and Jones, *J.*, 1949, 3203), may imply some fundamental variation in molecular structure and must be taken into account in any detailed formulation of the arrangement of the unit chains. In the particular case now investigated the high viscosity would point to a structure resembling that of amylopectin, despite the fact

that the biological relations of the protozoa would have rendered a glycogen type of structure more probable for the reserve material.

EXPERIMENTAL

Isolation of a Polysaccharide from the Colon and Cæcum of a Horse.—The contents (*ca.* 25 l.) of the ventral colon and the cæcum of a freshly slaughtered horse were filtered through three layers of muslin, the brown filtrate being set aside until the solid material had settled. The coarse material retained on the muslin was suspended in distilled water, refiltered, and then discarded. After the combined filtrates had been kept for 2 hr. the supernatant liquor was decanted, leaving on the bottom of each container a fine white residue. These residues were combined, washed with distilled water, and allowed to settle, and the supernatant liquor was decanted. After three such treatments, the moist solid was found, under the microscope, to consist almost entirely of the protozoon *Cycloposthium.* Each individual protozoon was stained dark brown or black by iodine. As far as could be observed, such impurities as other protozoa, parasitic worms, or their eggs were absent. After centrifugation to remove most of the adherent water the product was dried over phosphoric oxide in a desiccator at room temperature.

This material (8·0 g.) was extracted with chloral hydrate as described by Meyer and Bernfield (*Helv. Chim. Acta*, 1940, **23**, 875). The product (3·8 g.) was obtained as a colourless, easily powdered polysaccharide, the residue (3·9 g.) consisting of coarse, dark brown, fibrous material which was not investigated further.

Properties of the Polysaccharide.—A suspension of the polysaccharide in water gave a purple colour with iodine. In boiling 4% sulphuric acid (200 parts) the substance (1 part) yielded 86% of the theoretical amount of glucose (quantitative chromatographic determination). No sugar other than glucose was detected. The material had $[\alpha]_D^{14}$ $+154°$ (*c*, 1·0 in N-NaOH), $+190°$ (*c*, 0·4 in 4·8N-$HClO_4$). The blue value, determined in the standard manner (*locc. cit.*), was 0·10.

Oxidation by Periodate.—The sample (0·293 g.) was treated with sodium metaperiodate and potassium chloride (Brown, Halsall, Hirst, and Jones, *loc. cit.*), the acid titre after 150 hr. corresponding to 0·041 mole of formic acid from 162 g. of polysaccharide, *i.e.*, 1 mol. per 24 glucose residues. A duplicate experiment yielded one mol. of formic acid per 23 glucose units.

After completion of the formic acid determinations, the solutions were combined, excess of periodate being destroyed by the addition of excess of ethylene glycol (overnight shaking). The insoluble oxidised polysaccharide was filtered off, washed with water till free from iodate, and then dried by washing with ethanol and ether. This material was hydrolysed with sulphuric acid (10 ml.; 0·5N) at 95° for 8 hr. After neutralisation with barium carbonate, followed by filtration and evaporation to dryness, the residue was examined in the normal way by paper chromatography. No glucose or other reducing sugar was observed.

Methylation of the Polysaccharide.—The sample (1·8 g.) was methylated under the conditions used by Hirst, Jones, and Roudier (*J.*, 1948, 1779), 18 additions of reagents being made. The product (1·24 g.) was purified by precipitation from chloroform with light petroleum (b. p. 100—120°). The product had $[\alpha]_D^{17}$ $+207°$ (*c*, 1·0 in $CHCl_3$), $\eta_{sp.}/c$ 1·27 (*c*, 0·4 in *m*-cresol), corresponding to an apparent $M = 175{,}000$ (Found : OMe, 43·1%).

Hydrolysis of the Methylated Polysaccharide.—(*a*) The material (50 mg.) was hydrolysed in a sealed tube by the method of Hough, Hirst, and Jones (*J.*, 1949, 928). Analyses by paper chromatography with butanol–light petroleum saturated with water as mobile phase revealed the 2 : 3 : 4 : 6-tetramethyl glucose R_G 1·0 (5·3%), 2 : 3 : 6-trimethyl glucose R_G 0·81 (84%), and dimethyl glucoses R_G 0·63, 0·54 (12%). Traces of monomethyl glucose and of glucose were also observed. These results indicate the presence of one non-reducing terminal group per 21 $\pm$ 1 glucose residues.

(*b*) The material (0·88 g.) was hydrolysed by boiling methanolic hydrogen chloride (40 ml.; 1%) for 9 hr. After neutralisation with silver carbonate, treatment with hydrogen sulphide, etc., and concentration, the resulting syrup was boiled with hydrochloric acid (40 ml.; 2%) for 8 hr. After neutralisation with silver carbonate, and concentration, a syrup (0·88 g.) was obtained which partly crystallised. This mixture of methylated glucoses was separated on a column (50 × 3 cm.) of powdered cellulose (Whatman No. 1 ashless filter tablets) (Hough, Jones, and Wadman, *J.*, 1950, 1702; Chanda, Hirst, Jones, and Percival, *J.*, 1950, 1289). By elution with 2 : 3 *n*-butanol–light petroleum (b. p. 100—120°) saturated with water, four fractions were isolated, *viz.*, (1) 0·105, (2) 0·452, (3) 0·050, and (4) 0·020 g. By elution of the

column with water, a further fraction (0·008 g.) was obtained. Paper chromatography indicated that the last fraction, which was not further investigated, contained monomethyl glucose and glucose. The total recovery of sugars was 91%.

Fraction (1). Paper chromatography indicated the presence of a single substance (R_G 1·0) which corresponded to tetramethyl glucose. A small portion was hydrolysed with boiling 4% sulphuric acid, and after neutralisation was re-examined by paper chromatography. The presence of two substances was then indicated corresponding to 2 : 3 : 4 : 6-tetramethyl glucose and 2 : 3 : 6-trimethyl glucose (R_G 0·81). Hypoiodite oxidation indicated that the fraction (1) contained 25% of tetramethyl glucose. Fraction (1) was therefore rehydrolysed with hydrochloric acid (10 ml.; 1%) for 7 hr. The hydrolysate was separated on a fresh cellulose column (50 × 1·5 cm.), giving fractions (1*a*) 0·027 and (1*b*) 0·066 g. (93% recovery). Fraction (1*a*) partly crystallised and was found by hypoiodite oxidation to be 98% pure. After crystallisation twice from light petroleum (b. p. 40—60°) it had m. p. 84—85° (not depressed on admixture with authentic 2 : 3 : 4 : 6-tetramethyl D-glucopyranose), $[\alpha]_D^{14}$ + 84° (*c*, 0·5 in H_2O) (Found: OMe, 52·1. Calc. for $C_{10}H_{20}O_6$, 52·5%). From these results it followed that the polysaccharide contained one non-reducing terminal group per 22 ± 2 glucose units.

Fraction (1*b*) did not crystallise. Paper chromatography indicated the presence of a single substance (R_G 0·81) corresponding to 2 : 3 : 6-trimethyl glucose. It had $[\alpha]_D^{14}$ + 69° (*c*, 1·0 in H_2O), +67° (initial), dropping to −38° after 8 hr. in 2% methanolic hydrogen chloride (*c*, 1·0) (Found : OMe, 40·9. Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

Fraction (2), which partly crystallised, was recrystallised twice from butyl acetate, and had m. p. 117°, alone or admixed with authentic 2 : 3 : 6-trimethyl D-glucose. The crystalline material had $[\alpha]_D^{16}$ +87° (initial), +68° (const.) (*c*, 1·0 in H_2O), and $[\alpha]_D^{14}$ +67° (initial), −38° (8 hr.) (*c*, 1·0 in 2% methanolic hydrogen chloride) (Found : OMe, 41·4%).

Fraction (3) was obtained as a syrup which failed to crystallise during several weeks in the cold. The R_G value (0·62) was identical with that of 2 : 3-dimethyl D-glucose. The material had $[\alpha]_D^{15}$ +106° (initial), +65° (final) (*c*, 1·0 in H_2O) (Found : OMe, 29·3. Calc. for $C_8H_{16}O_6$: OMe, 29·7%).

Fraction (4) was a syrup which failed to crystallise during several weeks in the cold. Paper chromatography indicated the presence of 2 : 6- or 3 : 6-dimethyl D-glucose (R_G 0·54) or a mixture of the two, contaminated with traces of 2 : 3-dimethyl D-glucose. It had $[\alpha]_D^{13}$ +83° (initial), +62° (final) (*c*, 1·0 in H_2O), +62° (initial), −12° (final, 12 hr.) (*c*, 0·7 in 2% methanolic hydrogen chloride) (Found : OMe, 29·1%). The fraction was converted into the methyl glucosides and treated with sodium metaperiodate by Bell's method (*J.*, 1948, 992). The uptake of sodium metaperiodate indicated that the fraction contained 70% of 2 : 6-dimethyl D-glucose.

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