437. *Protozoal Polysaccharides. Structure of the Polysaccharide produced by the Holotrich Ciliates present in Sheep's Rumen.*

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The holotrichously ciliated protozoa present in the rumen of sheep synthesise *in vivo* and *in vitro* an intracellular storage polysaccharide from glucose, fructose, or sucrose. This polysaccharide is stained reddish-purple by iodine, contains only glucose residues, and has a high molecular weight. It has one terminal glucose residue for every 22 glucose residues in the molecule. The unit chains, of average chain length 22, consist of α-1 : 4-linked D-glucose residues and are united by $C_{(1)}$–$C_{(6)}$ linkages. The amylose content is negligible and the polysaccharide therefore has a highly branched molecular structure closely similar to that of amylopectin.

SOME protozoa are known to synthesis polysaccharides of the glucosan type. One such, from *Tetrahymena piriformis*, has been examined by Manners and Ryley (*Biochem. J.*, 1952, **52**, 480) who found it to be structurally similar to animal glycogen, with a unit chain length of 13 α-linked glucose residues and a molecular weight of about 10^7. Another, found in the ciliate *Cycloposthium* present in the colon and cæcum of a horse, has a different structure, resembling that of amylopectin. This polysaccharide has a unit chain length of 23 α-linked glucose residues and a molecular weight of 200,000 or more (Forsyth, Hirst, and Oxford, preceding paper). It has been found recently by Oxford that the holo-

trichously ciliated protozoa [*Isotricha* (two species) and *Dasytricha* (one species)] present in sheep's rumen and found nowhere in Nature but in ruminants, can ferment either *in vitro* or in the rumen glucose, fructose, or sucrose, but not (*in vitro* at any rate) other simple sugars such as glucuronolactone, mannose, galactose, xylose, arabinose, sorbose, lactose, and maltose. During the process these protozoa store immense numbers of granules of a glucose-containing polysaccharide which is stained purple with iodine (Oxford, *J. Gen. Microbiol.*, 1951, **5**, 83). The chemical structure of this material forms the subject of the present paper and the results show that here again the polysaccharide is closely similar to amylopectin in possessing a unit chain length of 23 α-linked glucose residues, a highly branched structure, a high molecular weight, and a high intrinsic viscosity in solution.

We are indebted to Dr. Oxford for a supply of the polysaccharide which had been liberated as granules when the cells of the protozoa were disrupted by shaking them with a buffered solution of "Teepol XL" (Oxford, *loc. cit.*). In bulk these granules formed a greyish-white powder which on acid hydrolysis gave glucose as the only sugar. For subsequent investigations the material was purified by the chloral hydrate method (Meyer and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 875) and was obtained as a colourless powder. It gave a purple colour with iodine. The absence of material of the amylose type was shown by the low "blue value" (0·5) (Hassid and McCready, *J. Amer. Chem. Soc.*, 1943, **65**, 1154; Haworth, Peat, Bourne, and Macey, *J.*, 1948, 924), the results of potentiometric iodine titration (see the Experimental section), and the negative results obtained on application of the Nussenbaum test for amylose (*Analyt. Chem.*, 1951, **23**, 1478). Only glucose was obtained on hydrolysis.

Acetylation of the polysaccharide, carried out by the mild method of Pacsu and Mullen, gave a triacetate which was indistinguishable from acetylated amylopectin and had a high intrinsic viscosity in *m*-cresol. The trimethyl derivative of the polysaccharide was prepared by the action of methyl sulphate and aqueous sodium hydroxide in an atmosphere of nitrogen, followed by methylation in liquid ammonia by Freudenberg and Boppel's method (*Ber.*, 1938, **71**, 2527). It appeared that methylation to a methoxyl content of 44% could be effected by this procedure without appreciable degradation of the molecule. The trimethyl derivative had $\eta_{sp.}/c$ 1·42 in *m*-cresol, corresponding to a molecular weight of *ca.* 170,000, the constant given by Hirst and Young (*J.*, 1939, 1471) being used.

After methanolysis of the trimethyl derivative with methyl alcohol containing hydrogen chloride, followed by acid hydrolysis of the methylated methyl glucoside so obtained, paper partition chromatography revealed the presence of four substances corresponding in R_G values with 2 : 3 : 4 : 6-tetramethyl glucose, 2 : 3 : 6-trimethyl glucose, and two dimethyl glucoses. Quantitative results showed that the yield of tetramethyl glucose was 5·3%, equivalent to the presence of one non-reducing terminal glucose residue for every 21 glucose residues in the molecule. The experiment was then repeated on a larger scale, the methylated glucoses being separated by partition chromatography. Crystalline 2 : 3 : 4 : 6-tetramethyl D-glucose was obtained in yield corresponding to a unit chain length of 22 glucose residues in the polysaccharide. The identity of the 2 : 3 : 6-trimethyl D-glucose was confirmed and it was proved that a considerable portion of the dimethyl glucose was the 2 : 3-derivative. Some 2 : 6-dimethyl D-glucose appeared to be present also, but the structural significance of this is uncertain since it could have arisen from incomplete methylation of the polysaccharide. Monomethyl glucoses and glucose were found only in negligible proportions.

The occurrence of 1 : 6-linkages as inter-unit bonds was further indicated by the results of oxidation with periodate. Determination of the amount of formic acid produced when the polysaccharide is oxidised by potassium metaperiodate showed that there is one non-reducing terminal glucose residue for every 22 glucose residues in the molecule. This is in agreement with the value obtained by the methylation method and proves the absence of glucose residues linked solely through $C_{(1)}$ and $C_{(6)}$. The absence of glucose amongst the products obtained by hydrolysis of the periodate-oxidised polysaccharide shows that by far the most, if not all, of the inter-unit bonds are of the 1 : 6-type. Linkages of the $C_{(1)}$–$C_{(2)}$ or $C_{(1)}$–$C_{(3)}$ type must be almost totally absent.

This evidence points to a molecular structure for this protozoal polysaccharide very similar to that of amylopectin. The molecule must be highly branched and consists of a large number of units chains, each containing on an average 22 α-1 : 4-linked glucopyranose residues, the unit chains themselves being joined at some point (or points) in their length by 1 : 6-glucosidic linkages. The molecular structure is therefore strikingly similar to that found to be present in the polysaccharide produced by *Cycloposthium* in the colon and cæcum of the horse.

Experimental

Whatman No. 1 filter paper was used for chromatography, with butanol–light petroleum saturated with water as mobile phase, unless otherwise stated. R_G values refer to rates relative to that of tetramethyl glucose.

Purification of the Polysaccharide.—The material was provided by Dr. A. E. Oxford, Rowett Research Institute, Aberdeenshire. (For method of preparation see Masson and Oxford, *J. Gen. Microbiol.*, 1951, **5**, 664.) It was a colourless powder which gave a reddish-purple colour with iodine. After hydrolysis with acid, paper chromatography revealed that glucose only had been formed. Purification of the polysaccharide was effected by Meyer and Bernfeld's method (*loc. cit.*) using chloral hydrate. The resulting product (86% recovery) was a colourless powder which on acid hydrolysis gave 89% of the theoretical amount of glucose. The polysaccharide had $[\alpha]_D^{17}$ +171° (*c*, 1·0 in N-NaOH), $[\alpha]_D^{20}$ +201° (*c*, 0·4 in 30% $HClO_4$). The blue value, determined by Hassid and McCready's method (*loc. cit.*) as modified by Bourne, Haworth, Macey, and Peat (*loc. cit.*), was 0·049.

Potentiometric Iodine Titration of the Polysaccharide.—The method of Bates, French, and Rundle (*J. Amer. Chem. Soc.*, 1943, **65**, 142) as modified by Wilson, Schoch, and Hudson (*ibid.*, p. 1381) was used. No break in the curve of free-iodine concentration plotted against iodine uptake was found. The amylose content was therefore negligible.

Acetylation.—Pacsu and Mullen's method was used (*J. Amer. Chem. Soc.*, 1941, **63**, 1487). After two treatments the product was a colourless powder, soluble in chloroform, having $[\alpha]_D^{17}$ +168° (*c*, 1·0 in $CHCl_3$), $\eta_{sp.}/c$ 2·1 in *m*-cresol (Found: Ac, 43·9. Calc. for $C_{12}H_{16}O_8$: Ac, 44·8%).

End-group Determination by Periodate Oxidation.—The substance was treated with sodium metaperiodate and potassium chloride (Brown, Halsall, Hirst, and Jones, *J.*, 1948, 27), the acid titre after 150 hr. corresponding to the formation of 0·045 mole of formic acid from 162 g. of the polysaccharide. This corresponds to the presence of one non-reducing terminal group per 22 ± 1 glucose residues.

After completion of the formic acid determination (see above) ethylene glycol was added to the solution to destroy excess of periodate. The oxidised material was filtered off, washed with water till free from oxidant (tested with diphenylamine and sulphuric acid), and dried. It was then hydrolysed with sulphuric acid (20 c.c.; 0·5N) at 95° for 10 hr. After neutralisation with barium carbonate, followed by filtration, the solution was examined by paper chromatography for the presence of glucose. No glucose or other reducing sugar was found.

Methylation of the Polysaccharide.—The material (10 g.) was methylated under conditions similar to those used by Hirst, Jones, and Roudier (*J.*, 1948, 1779). After 37 additions of the reagents, the product showed $\eta_{sp.}^{20}/c$ 1·01 (*c*, 0·32 in *m*-cresol) (Found: OMe, 40·7%). A sample (4 g.) of this material was further methylated by Freudenberg and Boppel's method (*Ber.*, 1938, **71**, 2527) as modified by Hodge, Hilbert, and Harjala (*J. Amer. Chem. Soc.*, 1951, **73**, 3312). After three additions of sodium and methyl iodide, the product (2·9 g.) was isolated as a cream-coloured powder by precipitation from chloroform by light petroleum (b. p. 40—60°) (Found: OMe, 43·2%). This product was fractionated by the solution method, with successive mixtures of chloroform and light petroleum. The following fractions were obtained.

Fraction	Petroleum : Chloroform	Yield	OMe (%)	$[\alpha]_D^{20}$ (*c*, 1·0 in $CHCl_3$)	$\eta_{sp.}^{20}/c$ (*c*, 0·4 in *m*-cresol)
1	50 : 0	0·0024 g.	—	—	—
2	45 : 5	0·0033 g.	—	—	—
3	40 : 10	0·8490 g.	43·9	+205°	1·24
4	35 : 15	0·9390 g.	43·7	+204°	1·42
5	30 : 20	0·6940 g.	43·6	+204°	1·21
6	25 : 25	0·4420 g.	42·0	—	1·49

Hydrolysis of the Methylated Polysaccharide and Separation of Methylated Glucoses.—Fractions 3, 4, and 5 (see above) were combined (2·21 g.) and hydrolysed under reflux by boiling

methanolic hydrogen chloride (80 c.c.; 1%). After neutralisation with silver carbonate, treatment with hydrogen sulphide, filtration, and removal of the solvent, a non-reducing syrup was obtained. This was boiled under reflux with hydrochloric acid (70 c.c.; 2%) for 7 hr. After neutralisation with silver carbonate, filtration, and concentration, a syrup (1·97 g.) was obtained which partly crystallised. Preliminary analysis of a small portion by paper chromatography revealed 2 : 3 : 4 : 6-tetramethyl glucose R_G 1·0 (5·3%), 2 : 3 : 6-trimethyl glucose R_G 0·81 (84—85%), and dimethyl glucoses R_G 0·59, 0·50 (9·0—10·0%). Traces of monomethyl glucose and glucose were also observed. The yield of tetramethyl glucose indicates the presence of one non-reducing terminal group per 21 ± 1 glucose residues.

Separation of Methylated Glucoses on a Cellulose Column.—The mixture of methylated glucoses (1·13 g.) was separated on a column of powdered cellulose (powdered Whatman No. 1 ashless filter tablets; 50 × 3 cm.) in the usual way (Hough, Jones, and Wadman, *J.*, 1949, 2511; Chanda, Hirst, Jones, and Percival, *J.*, 1950, 1289). By elution with a mixture of 1 : 1 light petroleum (b. p. 100—120°)-butanol, saturated with water, fractions (1) (0·247 g.), (2) (0·688 g.), (3) (0·081 g.), and (4) (0·023 g.) were isolated. By subsequent elution with water, a further fraction was obtained which was shown by paper chromatography to contain traces of monomethyl glucose and glucose. This was not further investigated. The total recovery was 91%.

Fraction (1). Paper chromatography revealed the presence of a single spot (R_G 1·0), corresponding to tetramethyl glucose. A small portion (10 mg.) was hydrolysed with boiling sulphuric acid (2%) for 7 hr. Re-examination by paper chromatography revealed the presence of an additional substance corresponding to 2 : 3 : 6-trimethyl glucose. Fraction (1) (0·244 g.) was, therefore, hydrolysed with hydrochloric acid (10 c.c.; 1%) for 7 hr. The hydrolysate was separated on a fresh cellulose column (50 × 1·5 cm.), as before, to give fractions (1*a*) (0·051 g.) and (1*b*) 0·180 g. (recovery 94%). Fraction (1*a*) partly crystallised. After three recrystallisations from light petroleum (b. p. 40—60°) it had m. p. 85° (not depressed on admixture with authentic 2 : 3 : 4 : 6-tetramethyl D-glucopyranose), $[\alpha]_D^{18}$ +84° (*c*, 0·5 in H_2O) (Found : OMe, 52·0. Calc. for $C_{10}H_{20}O_6$: OMe, 52·5%). The anilide had m. p. 137° (alone or admixed with authentic 2 : 3 : 4 : 6-tetramethyl D-glucose anilide) (Found : N, 4·4; OMe, 39·4. Calc. for $C_{16}H_{25}O_5N$: N, 4·5; OMe, 39·9%). From the above results it was estimated that the methylated polysaccharide contained one non-reducing terminal group per 22 ± 2 glucose units.

The combined fractions partly crystallised. The material was recrystallised from butyl acetate and had m. p. 117° (alone or admixed with an authentic sample of 2 : 3 : 6-trimethyl D-glucose), $[\alpha]_D^{16}$ +87° (initial), +69° (final) (*c*, 1·0 in H_2O), and $[\alpha]_D^{14}$ +67° (initial), −36° (12 hr.) (*c*, 1·0 in 2% methanolic hydrogen chloride) (Found : OMe, 41·5. Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

Fraction (3) was a pale yellow syrup which failed to crystallise after several weeks. The R_G value (0·59) on a paper chromatogram was identical with that of 2 : 3-dimethyl D-glucose. The material had $[\alpha]_D^{15}$ +107° (initial value), +61° (final) (*c*, 1·0 in H_2O) (Found : OMe, 29·0. Calc. for $C_8H_{16}O_6$: OMe, 29·7%). On oxidation by bromine water 2 : 3-dimethyl gluconic acid was obtained and was identified as the phenylhydrazide (Evans, Levi, Hawkins, and Hibbert, *Canad. J. Res.*, 1942, **20**, *B*, 175), m. p. 160° (Found : N, 8·6; OMe, 18·9. Calc. for $C_{14}H_{22}O_6N_2$: N, 8·9; OMe, 19·7%).

Fraction (4) was a pale yellow syrup, $[\alpha]_D^{15}$ +74° (initial value), +60° (final) (*c*, 1·0 in H_2O), $[\alpha]_D^{13}$ +60° (initial value), −10° (after 12 hr.) (*c*, 0·75 in cold 2% methanolic hydrogen chloride) (Found : OMe, 28·9. Calc. for $C_8H_{16}O_6$: OMe, 29·7%). The fraction was boiled with 1% methanolic hydrogen chloride, and the mixed methyl glucosides were isolated in the usual way. This mixture was then oxidised by sodium metaperiodate (see Bell, *J.*, 1948, 992). The uptake of sodium metaperiodate was 74% of the amount required for one mol. of periodic acid per $C_9H_{18}O_6$ unit, indicating that the fraction contained approx. 74% of 2 : 6-dimethyl D-glucose.

The authors gratefully record the interest taken by the late Dr. E. G. V. Percival in the early stages of this work. They thank Dr. A. E. Oxford for a supply of the polysaccharide, the Department of Scientific and Industrial Research for a maintenance allowance (awarded to G. F.) and the Distillers Company Limited for a grant.

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[*Received, March 18th,* 1953.]