407. *An Amylopectin-type Polysaccharide Synthesised from Sucrose by* Cl. butyricum.

By P. N. Hobson and H. Nasr.

The iodophile polysaccharide synthesised in the interior of the cells of *Cl. butyricum* grown in a sucrose medium has been partly purified. It has been shown to be a polyglucose of the amylopectin type.

The presence of substances in the interior of the bacterial cell giving conspicuous colour reactions with iodine has long been known to bacteriologists and, on the basis of these and other reactions, they have been presumed to be starch-type polysaccharides. Precise information regarding the chemical constitution of these substances has, however, rarely been obtained, although Carlson and Hehre (*J. Biol. Chem.*, 1949, **177**, 281) have examined the starch synthesised from glucose-1 phosphate by diphtheria bacilli, and Hehre and Hamilton (*ibid.*, 1946, **166**, 777) and Hehre (*ibid.*, 1949, **177**, 267) have also studied the extra-cellular polysaccharide synthesised from sucrose by the intact bacteria and by a cell-free enzyme from a strain of *Neisseria perflava*. In addition Barker, Bourne, and Stacey (*J.*, 1950, 2884) have shown that the polysaccharide produced by a different strain of *N. perflava* is ramified and has an average basal chain length intermediate between that of amylopectin and glycogen. The present work deals with the iodine-reacting substance synthesised in the interior of the cells of *Clostridium butyricum* grown in a sucrose medium. The strain employed was isolated from the cæcum of pigs fed on raw potato starch and is listed in the National Collection of Type Cultures (culture No. 7423). This strain is known to be primarily responsible for the breakdown of starch occurring in the pig's cæcum (Baker, Nasr, Morrice, and Bruce, *J. Path. Bact.*, 1950, **62**, 625). Nasr and Baker (*Nature*, **1949**, **164**, 745) have presented preliminary evidence which indicates that a phosphorylating mechanism may be involved in the observed synthesis of iodine-reacting substance, although the presence of an enzyme system of the " amylosucrase " type (Hehre, *loc. cit.*) cannot be entirely discounted.

The dried bacterial cells were stained purple by iodine and were largely insoluble in hot dilute sodium hydroxide. Acid hydrolysis by the method given below showed that the cells contained 32·8% of acid-hydrolysable polysaccharide of which 5—6% was in the form of oligosaccharides not precipitated by acetone. Small portions of the dried bacteria were treated by six different methods designed to yield the polysaccharide. Except for the fractions extracted by autoclaving, the acid-hydrolysable polysaccharide in the extracts was not more

than 5·3% of the original weight of bacteria, the remaining polysaccharide being in the bacterial residues. The properties of the fractions extracted by treatment of the bacteria with chloral hydrate solution (fraction C1) and by autoclaving in the presence of barium carbonate (fractions A1 and A2) are shown in the table. Fractions C1 and A1 were white powders almost entirely soluble in hot dilute sodium hydroxide, but giving a positive Millon's test.

The polysaccharide synthesised from sucrose by Cl. butyricum.

Fraction.	Acid-hydrolysable polysaccharide as % of dried bacteria.	Acid-hydrolysable polysaccharide as % of extracted material.	Polysaccharide–iodine solutions. Blue value.†	Polysaccharide–iodine solutions. Wave-length of peak absorption, mμ.	Conversion into maltose by β-amylase (%).† 5 hr.	Conversion into maltose by β-amylase (%).† 25 hr.
Dried bacteria	32·8	—	—	—	—	—
C1 *	5·3	51·1	0·204	540	63·5	69·0
A1 *	7·1	40·9	0·212	540	62·6	70·7
A2 *	3·3	53·3	0·173	—	53·1	57·3
Potato amylopectin ...	—	—	0·155	540	48·0	51·1
Potato starch	—	—	0·474	570	56·0	60·3

* See Experimental section for the preparation of these fractions.
† The values are determined from the weight of acid-hydrolysable polysaccharide in the fraction.

The polysaccharide was characterised as a polyglucose by paper chromatography of the products of acid hydrolysis, and its hydrolysis by β-amylase provided strong evidence that the primary linkage is of the α-1 : 4-glucosidic type. The low blue value and purple iodine stain are characteristic of amylopectin, although the limiting conversion into maltose by β-amylase is somewhat higher than that of potato amylopectin. In this respect it resembles the " Fraction B " of the polysaccharide isolated from diphtheria bacilli by Carlson and Hehre (*loc. cit.*). That it was not an amylose whose properties were masked by lipoid material was shown by the exhaustive extraction of fraction A1 with 85% aqueous methanol, which did not affect its properties.

The action of amylose-precipitants cannot be determined with the protein-containing fractions at present available, but the nature of the iodine stain renders it unlikely that the polysaccharide is a mixture of amylose and amylopectin such as is found in potato starch.

The protein impurity in the extracts appears to be firmly bound to the polysaccharide, for attempts to remove it resulted in co-precipitation of polysaccharide and protein.

The polysaccharide remaining in the residues cannot be extracted by a repetition of the procedures used here, but the material is stained purple by iodine and it seems likely that the polysaccharide is similar to that contained in the extracts.

Further studies are in progress.

Experimental.

The weights quoted refer to polysaccharide fractions dried at 60° in a vacuum over phosphoric oxide. Reducing sugars were determined by Somogyi's copper reagent (*J. Biol. Chem.*, 1945, **160**, 61) in a measured volume of the supernatant liquid after the solutions had been neutralised with a previously determined volume of N-sodium hydroxide and deproteinised with zinc sulphate and barium hydroxide by the method given by the same author (*ibid.*, p. 69).

Potato Amylopectin.—The amylopectin was prepared by thymol fractionation of potato starch, followed by purification of the amylopectin fraction on a cotton-wool column (Hobson, Whelan, and Peat, *J.*, 1950, 3566).

Preparation of the Dried Bacteria.—Cultures were grown in modified Beijerinck-sucrose medium, containing sucrose (10 g.), peptone (10 g.), precipitated chalk (6 g.), disodium hydrogen phosphate (0·1 g.), magnesium sulphate (0·1 g.), sodium chloride (0·1 g.), and water (200 c.c.), until gas production ceased, whereafter excess of calcium carbonate was removed by filtration and the micro-organisms were removed on a centrifuge at 3000 r.p.m., washed, and dried *in vacuo* over calcium chloride.

Analysis of the Polysaccharide Fractions.—(a) *Hydrolysis by aqueous acid.* A portion (about 0·030 g.) of the polysaccharide was wetted with alcohol, and 0·1N-sodium hydroxide (10 c.c.) was added. The material was dissolved as far as possible by heating it in a boiling water-bath for not more than 9 minutes (longer heating caused degradation of starch fractions) with intermittent shaking, and the solution cooled, neutralised with N-sulphuric acid, and diluted to 25 c.c. [solution (*a*)]. After centrifugation of this solution, 5 c.c. of the supernatant liquid were transferred to a dry flask, 5N-sulphuric acid (2·15 c.c.) was added, and the flask lightly stoppered and heated in boiling water for 3 hours. The solution was diluted to 10 c.c. after cooling and neutralisation, and the reducing sugars in suitable duplicate aliquots were determined as glucose. A correction was made for loss of glucose during the hydrolysis (see Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224), and the amount of starch type polysaccharide in the original solution was then calculated.

(b) *Blue value.* A portion of the neutral solution [(*a*) above] containing 0·5 mg. of acid-hydrolysable polysaccharide was stained with iodine under the standard conditions prescribed by Bourne, Haworth, Macey, and Peat (*J.*, 1948, 924).

(c) *β-Amylolysis.* Soya-bean β-amylase, prepared as described by Bourne, Macey, and Peat (*J.*, 1945, 882), was used. The activity units were defined by Hobson, Whelan, and Peat (*J.*, 1950, 3566). A further volume (15 c.c.) of solution (*a*) was incorporated in a digest containing in addition 0·2M-acetate buffer (pH 4·8; 3 c.c.), β-amylase solution (2 c.c.; 880 units in 0·2M-acetate buffer, pH 4·8), and water to 25 c.c. The digest was incubated at 35° and the reducing power, as maltose, of aliquots determined at intervals. A blank digest containing water in place of the polysaccharide solution was incubated in parallel and from this a correction was made for the reducing power of the enzyme alone. The β-amylase digest was made up immediately after centrifugation of the polysaccharide solution, as such neutral solutions showed a tendency to deposit a slight precipitate after some hours. The β-amylolysis of amylopectin and potato starch was carried out under the same conditions, a portion of a neutral solution prepared as in (*a*) containing 0·0100 g. of polysaccharide being incorporated in the digest.

(d) *Protein test.* Millon's mercuric nitrite reagent was used as a test for protein in the polysaccharide fractions.

Determination of the Total Polysaccharide in the Original Bacteria and in the Residues remaining after Extraction of the Soluble Polysaccharide.—These materials were largely insoluble in aqueous sodium hydroxide, so the following procedure was adopted. A portion (about 0·040 g.) was treated with 0·1N-sodium hydroxide as above and then neutralised with 5N-sulphuric acid, and a further volume (4·3 c.c.) of acid added. The whole was heated in boiling water for 3 hours, whereupon most of the residue dissolved. After being cooled and neutralised the solution was diluted to 25 c.c., and the reducing sugars in suitable duplicate aliquots were determined as glucose.

Extraction of the Bacteria with Chloral Hydrate Solution.—The method is essentially that described by Meyer and Bernfeld (*Helv. Chim. Acta*, 1940, **23**, 875). The dried bacteria (0·9360 g.) were stirred with 33% aqueous chloral hydrate solution (20 c.c.) at 80° for 3¾ hours. The insoluble residue, which was collected on the centrifuge, was re-extracted for 2 hours with 30 c.c. of the chloral hydrate solution. The combined extracts were injected in a fine stream into acetone (100 c.c.), and the flocculent precipitate was dried by trituration with acetone and ether, the ether being finally removed in a vacuum-desiccator over phosphoric oxide, to give fraction C1. The residue was washed repeatedly with acetone and ether and dried as above (fraction C2). This contained acid-hydrolysable polysaccharide equivalent to 22·6% of the weight of the dried bacteria.

Extraction of the Bacteria by Autoclaving.—The dried bacteria (1·415 g.) were autoclaved with barium carbonate (0·6 g.) and water (180 c.c.) at 20 lb./sq. in. for 3½ hours. The residue, containing barium carbonate, was removed on the centrifuge and, to the extract, acetone (2 volumes) was added. The resulting precipitate was removed on a centrifuge and was extracted twice by being stirred with 50 c.c. of 1% sodium acetate solution for 30 minutes at room temperature. To the combined extracts two volumes of acetone were added, and the precipitate was compacted on the centrifuge and dried by trituration with acetone and ether as above (fraction A1). The residue was also hardened in acetone and dried to give fraction A2.

The residue from the autoclaving was similarly extracted with sodium acetate to give fraction A3 and a residue (fraction A4). These fractions contained small amounts (9% and 5%) of polysaccharide soluble in 0·1N-sodium hydroxide and giving a purple iodine stain.

Autoclaving of Potato Starch.—Potato starch was autoclaved under the conditions described above. The blue values of the starch before and after autoclaving were 0·474 and 0·471 respectively. Thus this treatment causes no degradation of the polysaccharide.

Characterisation of Fraction A1 *as a Polyglucose.*—A portion (0·0141 g.) of fraction A1 was hydrolysed for 5 hours with 2N-sulphuric acid (2 c.c.) in a stoppered tube heated in a boiling water-bath. After neutralisation with barium carbonate and centrifugation, the precipitate was washed with water (2 c.c.), the washings were added to the original supernatant liquid, and the combined solutions evaporated under reduced pressure at *ca.* 40° to a syrup. Spots of the syrup were placed on a strip of Whatman No. 1 filter paper which was developed downwards with ethyl methyl ketone saturated with water (Partridge, *Biochem. J.*, 1948, **42**, 238) for 51½ hours at room temperature together with a reference mixture of glucose, fructose, and arabinose. On treatment with aniline hydrogen phthalate reagent (Partridge, *Nature*, 1949, **164**, 443) glucose, together with very small traces of fructose and a pentose with an R_F value corresponding to ribose, was found in the acid hydrolysate. The acid-hydrolysable material is thus largely a polyglucose.

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UNIVERSITY COLLEGE OF NORTH WALES,
BANGOR, N. WALES.

THE ROWETT RESEARCH INSTITUTE,
BUCKSBURN, ABERDEENSHIRE.

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