517. *The Composition of the Polysaccharide synthesised by* Polytomella cœca.

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Polytomella cœca synthesises a starch from ethyl alcohol or acetic acid as the source of carbon. The starch is best separated from proteins and other cellular components with chloral hydrate solution. It contains 13—16% of amylose and 84—87% of amylopectin. *Polytomella cœca* may well be a convenient organism for the production of an enzyme capable of synthesising branched polysaccharides of the amylopectin–glycogen class.

In a recent communication (*Biochim. Biophys. Acta*, 1950, **4**, 270) Lwoff, Ionesco, and Gutmann reported that *Polytomella cœca*, a flagellate not containing chlorophyll, synthesises during its growth a relatively large amount of a granular starch, which gives a purple stain with iodine. They observed that, whereas the organism grows rapidly on a synthetic medium containing ammonium sulphate as the nitrogen source and acetic acid or ethyl alcohol as the carbon source, it is unable to utilise sugars such as glucose, maltose, sucrose, and trehalose. The French workers obtained evidence that the immediate precursor of the starch is glucose-1 phosphate and that this ester is converted into the polysaccharide by the agency of phosphorylase.

Since it is now generally agreed that phosphorylases isolated from plant and animal sources convert the Cori ester into linear polyglucoses of the amylose type, and that a supplementary enzyme is necessary for the synthesis of branched structures of the amylopectin–glycogen class (for summaries see Cori, Swanson, and Cori, *Fed. Proc.*, 1945, **4**, 234; Bernfeld and Meutémédian, *Helv. Chim. Acta*, 1948, **31**, 1724, 1735; Barker, Bourne, Wilkinson, and Peat, *J.*, 1950, 93), it is of interest to know whether the starch of *Polytomella cœca* contains amylopectin and hence

whether the organism might be expected to yield an enzymic branching factor. Through the courtesy of Dr. Lwoff we have been able to examine his culture of *Polytomella cœca* and the starch which it synthesises. We now furnish evidence that this starch is composed of both amylose and amylopectin and is indeed richer in the latter component than are most natural starches.

On microscopical examination it can be seen that individual *Polytomella* organisms contain starch grains, which are much smaller than those of potato starch but which, nevertheless, can be identified easily under the microscope. The organism can be ruptured very readily by mechanical means, even by centrifuging at high speeds, with release of the starch grains. The sediment consists mainly of starch, together with small amounts of minerals, proteins, and other cellular debris. A sample of starch prepared in this way from alcohol and sodium acetate as the carbon source was kindly supplied by Dr. Lwoff; this will be referred to below as " untreated starch."

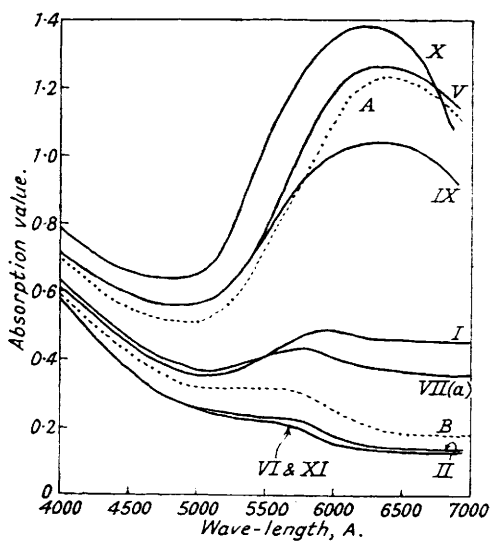
When a portion of the untreated starch was boiled with water and centrifuged, three distinct layers were obtained, namely, a clear solution at the top, a turbid viscous solution in the centre, and a light brown solid at the bottom. The addition of alcohol to the separated aqueous layers precipitated fractions I (45·4%) and II (22·0%), respectively. The light brown solid was sub-divided by extraction with hot alkali into a soluble portion (fraction III; 14·1%) and an insoluble portion (fraction IV; 5·0%). Neither of these alkali-treated fractions was soluble in water and so they could not be submitted to the usual diagnostic tests for amylose and amylopectin; they contained the whole of the protein present in the untreated starch.

TABLE I.

Characteristics of starch fractions I—VI.

Fraction.	I.	II.	III.	IV.	V.	VI.
Yield (%) from untreated starch	45·4	22·0	14·1	5·0	12·3	30·6
Ash (%)	0·4	1·6	2·5	13·9	0·0	1·3
Protein tests	−ve	−ve	+ve	+ve	−ve	−ve
Conversion (%) into glucose by acid	98	94	90	72	99	98
B.V.	0·46	0·14	—	—	1·18	0·13
Limiting conversion (%) into maltose with β-amylase	68	—	—	—	89	52

Light-absorption curves of starch–iodine solutions.



A = Potato amylose
B = Potato amylopectin
} *Typical samples prepared by the thymol method* (*cf. Bourne, Donnison, Haworth, and Peat,* loc. cit.).

In its blue value and in its limiting conversion into maltose with β-amylase (see Table I and Fig.), fraction I closely resembled potato starch, whereas fraction II was similar to potato amylopectin and to waxy-maize starch (cf. Barker, Bourne, and Peat, *J.*, 1949, 1712). Thus

it is reasonable to suppose that the whole of the amylose component of the untreated starch had been isolated in fraction I, the fraction most readily dispersed in boiling water. By selective precipitation with thymol, fraction I was separated into its linear (V) and branched (VI) constituents (cf. Bourne, Donnison, Haworth, and Peat, *J.*, 1948, 1687). The amylose component represented 27·0% of fraction I, corresponding to 12·3% of the untreated starch.

TABLE II.

Characteristics of starch fractions VII(*a*)—XI.

Fraction.	VII(*a*).	VIII.	IX.	X.	XI.
Yield (%) from untreated starch	88·9	7·3	16·9	14·1	65·4
Ash (%)	0·8	17·4	1·4	—	0·0
Protein tests	−ve	+ve	−ve	−ve	−ve
Conversion (%) into glucose by acid	94	55	93	—	92
B.V.	0·36	—	0·96	1·13	0·11
Limiting conversion (%) into maltose with β-amylase	54	—	71	89	48
$[\alpha]_D^{15}$ in 0·5N-sodium hydroxide	+160°	—	—	—	—

A more convenient and more efficient method for the purification of the untreated starch proved to be that of exhaustive extraction with an aqueous solution of chloral hydrate (cf. Meyer and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 875). A control experiment revealed that this treatment had a negligible effect upon the blue value of potato starch. Approximately 90% (fraction VII) of the untreated *Polytomella cœca* starch was recovered from the chloral hydrate solution; it was free from protein and contained only a trace of mineral matter. The material which was insoluble in the chloral hydrate solution (fraction VIII) was rich in both proteins and minerals. Thus fraction VII can be regarded as virtually pure *Polytomella cœca* starch. Its physical nature was such that it did not disperse in boiling water and exhibited a strong tendency to form swollen lumps in alkali. It was rendered readily soluble in dilute alkali by slowly adding, with vigorous stirring, water and then alkali to a hot alcoholic suspension and precipitating the starch [fraction VII(*a*)] by adding alcohol to the resulting solution.

Chromatographic analysis of a hydrolysate of fraction VII(*a*) by the filter-paper method revealed one aldose spot and no ketose spots; the aldose spot corresponded to a reference glucose spot. The blue value and the β-amylolysis limit of fraction VII(*a*) indicated that it contained somewhat less amylose than does potato starch (Table II and Fig.). Confirmation of this was afforded when the polysaccharide was subjected to selective precipitation with thymol, for the precipitated fraction (IX) represented only 19%. The material not precipitated by thymol (fraction XI) had the characteristics of amylopectin. Retreatment of fraction IX with thymol yielded an amylose [fraction X; 15·8% of fraction VII(*a*)] having B.V. 1·13 and giving 89% of maltose with β-amylase. From this result and that quoted above it can be seen that the untreated *Polytomella cœca* starch contained approximately 90% of pure starch, of which about 13—16% was amylose and the rest amylopectin.

In view of these observations *Polytomella cœca* should be a fruitful source of a branching factor responsible for the synthesis of amylopectin. Preliminary indications that this is so have been obtained already. Furthermore, the simplicity of the carbon source, the rapid growth of mass cultures, and the ease with which the starch can be isolated commend *Polytomella cœca* for the synthesis of ^{14}C-labelled amylose and amylopectin and of the carbohydrates which can be derived therefrom.

EXPERIMENTAL.

Analysis of Starch Fractions.—All polysaccharide samples were dried for several hours at 60° in a vacuum over phosphoric oxide before being analysed by the following methods: (a) *Ash content.* The polysaccharide (12—15 mg.) was weighed into a platinum boat and heated to constant weight in a small muffle furnace. (b) *Protein tests.* In order to detect the presence of proteinaceous impurities, each starch fraction was submitted to the sodium fusion test for nitrogen and to Millon's mercuric nitrite test for phenolic amino-acids. (c) *Hydrolysis by aqueous acid.* The amount of glucose formed when the polysaccharide was heated in a boiling water-bath with 7% sulphuric acid for 6 hours was determined by cuprimetric titration, as described by Bourne, Donnison, Haworth, and Peat (*loc. cit.*). (d) *β-Amylolysis.* The limiting conversion into maltose effected by soya-bean β-amylase at pH 4·8 was determined by the method of Bourne, Donnison, Haworth, and Peat (*loc. cit.*). (e) *Iodine stains.* Two expressions, absorption value (A.V.) and blue value (B.V.), are used in this paper to describe the intensities of the iodine stains of polysaccharides; they were defined by Bourne, Haworth, Macey, and Peat (*J.*, 1948, 924). Since the B.V. is determined under standard conditions (polysaccharide, 1 mg./100 c.c.; iodine, 2 mg./100 c.c.; potassium iodide, 20 mg./100 c.c.) and at λ 6800 A., it is characteristic of the polysaccharide under examination. On the other hand, the A.V. is a convenient term for an absorption measurement made under any given set of conditions.

Untreated Starch from Polytomella cœca.—The untreated starch employed in these investigations had been isolated from a mass culture of *Polytomella cœca*. Details of the growth of such a mass culture, in which alcohol and sodium acetate serve as the carbon source, were given by Lwoff, Ionesco, and Gutmann (*loc. cit.*). This starch, which contained protein (4·1%) and mineral matter (2·3% ash), was only partially dispersed in boiling water.

Extraction of the Untreated Starch with Boiling Water and with Alkali.—A suspension of the finely-ground untreated starch (dry weight, 1·925 g.) in alcohol (20 c.c.) was stirred into boiling water (200 c.c.). The paste was boiled and stirred for 35 minutes, cooled, and centrifuged; three distinct layers were formed: between the bottom layer of light-brown solid and the clear aqueous upper solution was a turbid viscous layer.

The top layer was mixed with alcohol (1·5 vols.) and the precipitated polysaccharide was collected. It was ground thoroughly with alcohol and then with ether, and finally dried at 60° in a vacuum over phosphoric oxide, giving fraction I (0·873 g.; 45·4%). In the same way, fraction II (0·422 g.; 22·0%) was recovered from the central viscous layer.

The light brown residue was extracted, at 50°, with three portions (20 c.c. each) of 0·25N-sodium hydroxide. The extracts were combined, neutralised to phenolphthalein with hydrochloric acid, and dialysed in Cellophane against distilled water. The polysaccharide was precipitated from solution with alcohol (1·5 vols.), and washed and dried as above, to give fraction III (0·272 g.; 14·1%).

The alkali-insoluble material was washed with a small volume of water, ground with alcohol and then with ether, and dried as above, to give fraction IV (0·096 g.; 5·0%).

Extraction of the Untreated Starch with Chloral Hydrate Solution.—The method employed was essentially that reported by Meyer and Bernfeld (*loc. cit.*). The untreated starch (dry weight, 2·00 g.) was stirred at 80° for 1 hour with a 33% solution (100 c.c.) of chloral hydrate in water. The insoluble residue, which was collected in the centrifuge, was stirred with two fresh portions (20 c.c. each) of warm chloral hydrate solution.

The combined extracts were filtered through sintered glass and injected in a fine stream into acetone (250 c.c.). The flocculent polysaccharide was collected and hardened by trituration with acetone. The last traces of chloral hydrate were removed by extraction in a Soxhlet apparatus with acetone for 2 hours and then with ether for 1 hour. The purified product (fraction VII; 1·778 g.; 88·9%) was dried as above.

The material insoluble in chloral hydrate, when washed repeatedly with acetone and then with ether and dried as above, constituted fraction VIII (0·146 g.; 7·3%).

Reprecipitation of Fraction VII.—As obtained above, fraction VII consisted of hard granules, which, owing to their insolubility in water and their tendency to form gelatinous lumps in alkali, were unsuitable for quantitative measurements. After the following treatment, it was recovered in a form which was readily soluble in dilute alkali.

Fraction VII, suspended in a small volume of alcohol, was heated in a boiling water-bath and stirred at high speed while water (150 c.c. per 1 g. polysaccharide) was added. The stirring and heating were continued for 20 minutes. At this stage most of the polysaccharide had dissolved, the rest remaining as a very fine suspension. The addition of a few drops of 10% sodium hydroxide solution caused rapid completion of the solution process. After 5 minutes, the solution was allowed to cool and was then neutralised with hydrochloric acid. The polysaccharide was precipitated with alcohol (1·5 vols.), triturated with alcohol and then with ether, and dried as above to give fraction VII(*a*), having $[\alpha]_D^{15}$ + 160° (*c*, 0·19 in 0·5N-sodium hydroxide).

Extraction of Potato Starch with Chloral Hydrate Solution.—Potato starch (B.V. 0·42; 2·00 g.) dissolved completely when treated with chloral hydrate solution (100 c.c.), as described above. The polysaccharide was precipitated from the viscous solution with acetone, purified, and dried. The product had B.V. 0·41. Thus the effect of this treatment on the B.V. of either amylose or amylopectin is negligible.

Characterisation of Fraction VII(a) *as a Polyglucose.*—A portion (40 mg.) of fraction VII(*a*) was hydrolysed for 5 hours with 2N-sulphuric acid (1·0 c.c.) in a sealed tube which was heated in a water-bath at 100°. The hydrolysate and water washings (1·0 c.c.) were transferred to a small centrifuge tube, neutralised with barium carbonate, and centrifuged. The supernatant liquid, in which the concentration of sugars was approximately 2%, was analysed chromatographically on filter paper by the method of Partridge (*Nature*, 1946, **158**, 270). Five reference solutions were employed, namely, glucose (0·1%), galactose (0·1%), fructose (0·1%), sucrose (0·1%), and a mixture of these four sugars (0·1% each).

Spots of the hydrolysate and of each of the five reference solutions were applied along a line drawn 10 cm. from the top edge of each of two sheets (A and B; 57 × 23 cm.) of Whatman No. 1 filter-paper. A pad of cotton-wool was fastened to the lower edge of each sheet to absorb the solvent as it reached the bottom, and thus to enable the sugars to travel a greater distance (cf. Miettinen and Virtanen, *Acta Chem. Scand.*, 1949, **3**, 459). The sheets were irrigated for 72 hours at 15—18° with the butanol phase of a mixture of *n*-butanol (400 c.c.), ethanol (100 c.c.), ammonia (10 c.c.; *d* 0·880), and water (490 c.c.). After being dried in a current of warm air, sheet A was sprayed with aniline hydrogen phthalate reagent (Partridge, *Nature*, 1949, **164**, 443), while sheet B was sprayed with an acid resorcinol reagent.

Sheet B showed no spots other than the reference spots of sucrose and fructose, indicating that no ketose constituted more than 5% of the monosaccharide units in fraction VII(*a*). Sheet A showed a small but distinct separation of the reference aldoses, glucose and galactose; the hydrolysate of fraction VII(*a*) gave only one spot, and this corresponded to the reference glucose spot. Thus it is probable that no aldose, other than glucose, constituted more than 5% of the monosaccharide units in fraction VII(*a*).

Treatment of the Polysaccharides with Thymol.—A solution of the polysaccharide in 0·5N-sodium hydroxide (2 c.c. per 100 mg.) was diluted with water (35 c.c. per 100 mg.) and neutralised with 0·5N-sulphuric acid. Powdered thymol (0·2 g. per 100 mg. of polysaccharide) was added, and the mixture kept at 30° for 36 hours.

The precipitated polysaccharide–thymol complex was removed by the centrifuge and washed twice with saturated thymol-water, the washings being added to the original supernatant liquid. The amylose complex was washed thoroughly with absolute alcohol and then with ether, and the residual polysaccharide was dried to constant weight in a vacuum over phosphoric oxide. The soluble polysaccharide was precipitated from the aqueous solution with alcohol, washed, and dried.

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