Studies on the Metabolism of the Protozoa

THE MOLECULAR STRUCTURE OF A STARCH-TYPE POLYSACCHARIDE FROM *POLYTOMA UVELLA*

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1. *Polytoma uvella*, when grown in an acetate-containing medium, synthesizes a starch-type polysaccharide. 2. The starch differs from normal plant starches in having a lower iodine affinity, and in being relatively insoluble; this latter property makes fractionation difficult. 3. The starch contains about 16% of amylose, and on fractionation yields a branched amylopectin component that is similar in structure to a typical plant starch amylopectin.

Although the synthesis of starch-type polysaccharides is a characteristic feature of the higher land plants, it is also found in the lower Orders of the Plant Kingdom including some unicellular organisms. The latter include various phytoflagellates that are members of the Chlorophyceae, e.g. *Polytomella coeca* (Bourne, Stacey & Wilkinson, 1950), *Dunaliella bioculata* (Eddy, Fleming & Manners, 1958) and *Nitella translucens* (Anderson & King, 1961). In view of the small but significant differences that have been observed in the properties of starch from various land plants (Whelan, 1958), it was decided to examine the starch from another phytoflagellate, and to compare the properties with the starches isolated from above-mentioned species. The organism chosen, *Polytoma uvella*, is a fresh-water-inhabiting non-photosynthetic member of the Chlorophyceae. Various workers, including Pringsheim (1937), have qualitatively observed the synthesis of starch when *Polytoma uvella* was grown in an acetate medium, but no chemical study of this carbohydrate has been reported. The related species *Polytoma obtusum* was shown by Brechot (1937) to contain a polysaccharide that stained blue with iodine, and was degraded by amylases to reducing sugars. As grown in culture, the cells of *Polytoma uvella* are ovoid, and about $10\mu \times 15\mu$ in size; there are two equal flagellae approximately the same length as the body. In bulk, the cells have an orange colour due to carotenoid pigments (Links, Verloop & Havinga, 1960). The structure and biology of this organism is described by Hollande (1942).

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MATERIALS AND METHODS

Materials. *Polytoma uvella* was cultivated in a medium containing 0.2% of sodium acetate, 0.1% of Oxoid yeast extract and 0.1% of Oxoid peptone, pH 6.0–6.5. For bulk growth, batches of 36 2l. conical flasks were used, each containing 400 ml. of medium; incubation was for 6 days at 24° in the dark. A total of 14 batches, representing approx. 200l. of medium, was grown; the cultures were harvested by centrifugation, and the cells washed and stored in methanol. The latter extracted the carotenoid pigments, leaving a pure-white preparation of flagellates.

Methods. The analytical methods were those used by Manners & Ryley (1955), Archibald, Hirst, Manners & Ryley (1960) and Archibald, Cunningham, Manners, Stark & Ryley (1963).

RESULTS

The polysaccharide was isolated from the cells by the following general method. A methanolic suspension of the cells (approx. one-quarter of the total material in 25 ml. of methanol) was disrupted in an MSE ultrasonic disintegrator (18000–20000 c./sec., power output 60 w, for 6 min.) and the cell residue collected by centrifugation. This was deproteinized by digestion with amylase-free trypsin at pH 7.6 as described by Clarke & Stone (1960), and extracted three times at 0° with aq. 90% saturated urea solution. The residue was washed successively with distilled water, 50% (v/v) ethanol, methanol and ether to give 3.0 g. of a pure-white powder, which by microscopic examination was free from whole cells and other cell debris.

Properties of the polysaccharide. Acid hydrolysis gave D-glucose (identified with D-glucose oxidase)

in 96% yield (by cuprimetric titration), and no other reducing sugars. The polysaccharide was insoluble in water, but dissolved slowly in dilute alkali; it had $[\alpha]_D +149^\circ$ (c 0.9 in N-sodium hydroxide). A neutralized solution stained blue with iodine, and was partly degraded to maltose by β -amylase, showing it to be a starch-type polysaccharide.

The polysaccharide had a 'blue value' of 0.244 determined by the method of Bourne, Haworth, Macey & Peat (1948); samples of potato, oat and maize starches had 'blue values' 0.42, 0.40 and 0.43 respectively. From the relation between 'blue value' and amylose content (Anderson & King, 1961), the protozoal starch had an amylose content of about 16%. By potentiometric titration (Bates, French & Rundle, 1943), the iodine affinity of the starch was 3.1 g. of iodine bound/100 g. of polysaccharide. Since purified amyloses have an iodine affinity of 19.5%, this indicated that the starch contains 16% of amylose. Under similar conditions, potato, oat and maize starches had iodine affinities of 3.9, 4.0 and 4.4%.

Fractionation of the starch. Standard methods for this procedure involve complete dissolution of the starch followed by selective precipitation of the amylose component with a polar organic compound. Attempts to disperse the starch in hot water followed by the addition of butan-1-ol or thymol under conditions that were satisfactory for the fractionation of potato or cereal starches gave yields of insoluble complexes that were much greater than that expected from the amylose content. From 518 mg. of starch, 440 mg. of a butan-1-ol complex was obtained, and from 388 mg. of starch, 287 mg. of a thymol complex. These complexes had abnormally low iodine affinities as determined by potentiometric titration, and fractionation has not occurred. Attempts to modify the starch granules by pre-treatment with liquid ammonia (Hodge, Montgomery & Hilbert, 1948) or with dimethylsulphoxide (Killion & Foster, 1960) as applied to other starches were not effective.

The starch was finally dissolved in hot alkali, even though this treatment is known to degrade the amylose component (Gilbert, 1958); the following fractionations were carried out in an atmosphere of nitrogen, to minimize such degradation. A paste of starch (0.5 g.) in warm methanol was stirred with 5N-potassium hydroxide (50 ml.) for 4 hr. at 20°, and for 5 min. at 40°, and then cooled. After neutralization to pH 7 with sulphuric acid, the temperature was raised to 70°, and the solution saturated with butan-1-ol. The precipitate that formed on cooling was collected by centrifugation, dissolved in water and reprecipitated with butan-1-ol; it was washed with ethanol and ether, and dried to give amylose I (60 mg.), the iodine complex of

which had 'blue value' 0.57 and $\lambda_{\max}$ 630 m μ . The supernatant solution was extracted with ether, dialysed for 48 hr. at 20° against tap water and freeze-dried. The product, amylopectin I (300 mg.), had 'blue value' 0.20 and $\lambda_{\max}$ 570 m μ , showing that a separation of polysaccharides of different iodine-staining power had been effected.

A second portion of starch (0.5 g.) was heated at 40° for 2 hr. in 2N-sodium hydroxide (40 ml.) in an atmosphere of nitrogen and for 0.5 hr. at 60°. The solution was cooled, neutralized (pH 7), heated to 70° and thymol (700 mg.) added. After 3 days under nitrogen at 20°, the solution was centrifuged, and the amylose-thymol complex collected, dissolved in water and reprecipitated three times with butan-1-ol to give amylose II (70 mg.). This fraction had 'blue value' 0.60, $\lambda_{\max}$ 635 m μ , iodine affinity 7.1% and β -amylolysis limit 95%. The supernatant solution from the thymol precipitation was dialysed against tap water for 48 hr. and freeze-dried to give amylopectin II (290 mg.). This fraction had 'blue value' 0.12, $\lambda_{\max}$ 545 m μ and β -amylolysis limit 58%. The average chain length determined (a) by oxidation with 0.1 M-sodium metaperiodate at 20° (Manners & Wright, 1961), (b) by α -amylolysis of the β -dextrin, as described by Manners & Wright (1962), was 24 and 23 glucose residues respectively. The average exterior and interior chain lengths are therefore 16 and 6-7 respectively. By similar means, oat amylopectin had chain lengths of 20 by both methods, and maize amylopectin 23 and 21 by methods (a) and (b) respectively.

DISCUSSION

The study of protozoal polysaccharides presents difficulties arising from their isolation from relatively small quantities of cells of low polysaccharide content, and their subsequent purification. In the present work, cell disruption by ultrasonic vibrations followed by enzymic digestion of proteinaceous material has yielded a polysaccharide of high purity, which had the properties of a two-component starch.

As with starches isolated from other members of the Chlorophyceae (Bourne *et al.* 1950; Eddy *et al.* 1958; Anderson & King, 1961), the iodine affinity of *Polytoma uvella* starch (which is a measure of the amylose content) was significantly lower than that of land-plant starches, and of the starch from the flagellate *Chilomonas paramecium* (Class Cryptophyceae) (Archibald *et al.* 1960). A further difference from plant starches was the insolubility of the protozoal starch granules under conditions in which potato and cereal starches were completely dispersed. The starches from *Polytomella coeca* and *Chilomonas paramecium* were also relatively insoluble (Bourne *et al.* 1950; Archibald *et al.* 1960).

Table 1. *Properties of potato, protozoal and algal starches*

Property	Potato starch	<i>Polytoma uvella</i>	<i>Polytomella coeca</i> *	<i>Chilomonas paramecium</i> †	<i>Nitella translucens</i> ‡	<i>Dunaliella bioculata</i> §
Whole starch:						
[α] _D in N-NaOH	+159°	+149°	+160°	+157°	+163°	+169°
Iodine affinity ¶	3.9	3.1	—	5.0	2.3	2.5
'Blue value'	0.42	0.24	0.36	0.54	0.16	—
Amylose content (%)	20	15	13–16	45	12	12–14
Amylose component:						
'Blue value'	1.50	0.60	1.13	0.98	—	—
β -Amylolysis limit (%)	99	95	89	95	—	93
Amylopectin component:						
$\lambda_{\max}$ of iodine complex (m μ)	550	545	—	540	—	—
'Blue value'	0.14	0.12	0.11	0.13	—	—
Average chain length	22	23–24	—	22	19	15–16
β -Amylolysis limit (%)	57	58	48	60	56	60
Exterior chain length	15	16	—	15–16	13	12
Interior chain length	6	6–7	—	5–6	6	2–3

* Bourne *et al.* (1950).† Archibald *et al.* (1960).

‡ Anderson & King (1961).

§ Eddy *et al.* (1958).|| [α]_D in water.

¶ Expressed as g. of iodine bound/100g. of starch.

The *Polytoma uvella* starch could be fractionated only after dissolution in warm alkali. This probably caused some degradation of the amylose component, the iodine affinity of which was abnormally low, but the presence of essentially unbranched chains of α -(1 $\rightarrow$ 4)-linked glucose residues is shown by the high β -amylolysis limit. As shown in Table 1, the properties of the amylopectin component were very similar to those of amylopectins from other biological sources.

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