Studies on the Metabolism of the Protozoa

THE MOLECULAR STRUCTURE OF THE RESERVE POLYSACCHARIDE FROM *ASTASIA OCELLATA*

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1. The flagellate *Astasia ocellata* synthesizes as reserve carbohydrate a water-insoluble polysaccharide (paramylon) containing chains of β -(1 $\rightarrow$ 3)-linked D-glucose residues. 2. The average chain length, determined by methylation analysis, is about 43, and the minimum degree of polymerization from periodate oxidation data is 50–55. Most of the molecules are therefore essentially linear.

A characteristic feature of euglenoid flagellates is that they synthesize granules of paramylon (paramylum) as reserve carbohydrate. We now report the results of a structural study of this polysaccharide from *Astasia ocellata*; a preliminary account of this work has been published (Manners, Ryley & Stark, 1964). *Astasia ocellata* is a freshwater flagellate (Class Euglenineae), being a non-photosynthetic colourless counterpart of *Euglena quartana*, and closely related to *Khawkinsella halli* (Jahn & McKibben, 1937). Organisms grown in culture are elongate, varying in size in the range 30–50 μ by 13–18 μ . Locomotion is by means of a single flagellum, which may be up to 1.5 times the body length. On microscopic examination, the most obvious cell structures are ovoid or polygonal granules of paramylon 1.6–2.7 μ in size. For convenience, the polysaccharide is referred to below as 'astasian', and the borohydride-reduced polysaccharide as 'astasitol'.

MATERIALS AND METHODS

Materials

Astasia ocellata was grown in a medium containing 0.1% of sodium acetate, 0.1% of Oxoid Lab Lemco, 0.2% of Difco yeast extract, 0.2% of Difco Bacto tryptone and 0.001% of CaCl_2 . Since growth and acetate utilization resulted in a gradual increase in pH to a maximum about pH 8, the initial pH of the medium was lowered to 5.5 to allow maximal growth over the tolerated pH range. Extremely poor growth was obtained when Oxoid yeast extract and tryptone were substituted for the Difco products. For bulk growth, batches of 25 l. conical flasks were used, each containing 500 ml. of medium, and incu-

bated at 24° in the dark for 6–7 days. The cells were harvested by centrifugation and washed and stored in methanol.

Isolation of paramylon (astasian). The method of extraction was essentially that of Clarke & Stone (1960). The cells from 128 l. of medium were washed with ethanol, suspended in water and disrupted by ultrasonic vibrations (MSE ultrasonic disintegrator, 18000–20000 cye./sec., power output 60 W, for 6 min.). The cell debris, after being washed with ethanol and ether and then dried, weighed 16 g. This material was incubated with 0.4 g. of trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) in 400 ml. of 0.1 M-sodium phosphate buffer, pH 7.6, at 37° for 40 hr., the mixture centrifuged, and the residue extracted three times with saturated urea solution and washed thoroughly with water. The off-white residue was stirred in 0.1 M-NaCl solution and centrifuged at 400g for 5 min. followed by 5 min. at 1800g. Two layers were formed, a highly compact white lower layer and a thin loosely packed brown upper layer of denatured protein, which was carefully removed. This process was repeated, and then the white material was suspended in water (1500 ml.) and shaken vigorously with toluene (150 ml.) for 24 hr. The mixture was centrifuged slowly at 160g and the toluene, toluene-protein gel and water were removed. This procedure was repeated five times, to give 8.8 g. of a white solid, corresponding to 55% of the dried cell material.

Methods

Analytical methods were as described by Archibald, Cunningham, Manners, Stark & Ryley (1963). Descending chromatograms were carried out at room temperature on Whatman no. 1 paper with the following solvent systems: *A*, ethyl acetate-pyridine-water (10:4:3, by vol.); *B*, butanol-ethanol-water (40:11:19, by vol.); *C*, ethyl methyl ketone-water-aq. ammonia (sp.gr. 0.88) (200:17:1, by vol.). Paper-chromatographic mobilities of unsubstituted sugars are expressed as R_{Gluc} values, relative to D-glucose, and of methylated sugars as R_{F} values. Methylation was carried out by the method of Kuhn & Trischmann (1963)

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as modified by Dr D. A. Rees (personal communication) with dimethyl sulphate, dimethylformamide and $\text{Ba(OH)}_2 \cdot 8\text{H}_2\text{O}$, followed by the procedure of Kuhn, Trischmann & Low (1955) with dimethylformamide, methyl iodide and Ag_2O . The methylated sugars in an acid hydrolysate were separated by cellulose column chromatography with ethyl methyl ketone-water (10:1, v/v) as solvent. Infrared spectra were measured in Nujol with a Perkin-Elmer Infracord spectrophotometer. The *Rhizopus arrhizus* enzyme preparation was that used by Cunningham & Manners (1964).

RESULTS

Analysis of astasian. The polysaccharide granules were iodophobic, and insoluble in both boiling water and 2*N*-sulphuric acid at 100°; they dissolved after gentle agitation in *N*-sodium hydroxide to give a solution with $[\alpha]_D^{17} + 17^\circ$ (c 1.02); neutralization of this alkaline solution gave an insoluble gelatinous precipitate. Hydrolysis of granules (10mg.) at 100° with 1ml. of 90% (w/w) formic acid for 2hr. and then with 2ml. of 2*N*-sulphuric acid for 3hr., followed by neutralization, gave a solution containing D-glucose (as shown by paper chromatography in solvent *A* before and after treatment with D-glucose oxidase); other monosaccharides, mannitol and sorbitol were absent. The glucose content was 95.7%. Paper chromatography in solvents *A* and *B* of a partial acid hydrolysate showed the presence of glucose, laminaribiose, laminaritriose and laminaritetraose. The infrared spectrum of astasian showed an absorption band at 890cm^{-1} characteristic of β -linked polysaccharides and was identical with the spectrum of paramylon from *Peranema trichophorum* (Archibald *et al.* 1963).

Incubation of a suspension of astasian (8mg.) with β -glucosidase (10mg. of the *Rhizopus arrhizus* preparation in 2ml. of 0.1*M*-sodium citrate buffer, pH 4.8) resulted in the liberation of sugars with the R_{glc} values of glucose, laminaribiose and laminaritriose. Enzymic hydrolysates of samples of paramylon from *Euglena gracilis* and of chryso-laminarin (Beattie, Hirst & Percival, 1961) contained the same three sugars, and in all hydrolyses the laminarisaccharides were hydrolysed to glucose on prolonged incubation.

When polysaccharide (70.1mg.) was oxidized with 10ml. of 0.015*M*-sodium metaperiodate at 27° for 72hr., the reduction of periodate amounted to 0.14mol.prop./glucose residue. In parallel experiments, the reduction of periodate by laminarin and by paramylon from *Peranema trichophorum* was 0.30 and 0.03mol.prop./glucose residue (Archibald *et al.* 1963). The apparent degree of polymerization of astasian was calculated from the amount of formaldehyde released on oxidation of 104mg. with 25ml. of 0.03*M*-sodium metaperiodate at 20°. After

oxidation for 11, 13, 15, 17 and 19 days, the yield of formaldehyde was 0.0172, 0.0176, 0.0181, 0.0182 and 0.0193mol.prop./glucose residue. Assuming that 1mol. of formaldehyde arises from the reducing group of each polysaccharide molecule (Archibald *et al.* 1963), the last two results correspond to a degree of polymerization of 52–55 glucose residues.

The methylation of astasian (3g.) under nitrogen by the method of Kuhn & Trischmann (1963) was attempted, but the yield of methylated polysaccharide (0.4g.) was too low to enable a complete analysis to be carried out. However, methanolysis of a few milligrams of methylated astasian followed by gas-liquid-chromatographic analysis showed the presence of the methyl glycosides of 2,4,6-tri-*O*-methyl-D-glucose as the major products, and the glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose as minor products. Paper-chromatographic analysis of an acid hydrolysate (with solvent *C*) also showed the presence of these two methylated sugars. Since (1→3)-linked glucans are very labile to alkali, and may be degraded from the reducing end group during methylation under alkaline conditions, astasian was converted into the corresponding alcohol (astasitol), which should be more stable to alkali.

Preparation of astasitol. Preliminary experiments showed that the conditions for the effective reduction of astasian had to be carefully controlled. Astasian (3.3g.) was dissolved in dimethyl sulphoxide (400ml.) and a solution containing 1g. of potassium borohydride in 30ml. of water added slowly with vigorous stirring. The mixture remained as a clear solution for 24hr. but thereafter became opaque, viscous and gel-like. The gel was stirred with ethanol (1vol.) and a voluminous precipitate collected by centrifugation and washed thoroughly with ethanol and ether (to avoid caking) before being dried. The product was dissolved in 300ml. of dimethyl sulphoxide with warming and 1g. of potassium borohydride in 20ml. of water was added. The mixture was treated as above and the reduction repeated. After the third reduction the gel was freed as far as possible from dimethyl sulphoxide by centrifugation, suspended in 200ml. of water and 2g. of potassium borohydride was added with stirring. The gel-like slurry was stirred for 12hr. and 5*N*-sodium hydroxide added very slowly, in an atmosphere of nitrogen, until the solution was about 0.1*N*, and clear. The solution was stirred for 48hr., neutralized to pH 7 with acetic acid and 1vol. of ethanol was added. The precipitate was collected by centrifugation, washed thoroughly with ethanol, butanol and ether and then dried to give 3.05g. of astasitol.

Examination of astasitol. The product had similar solubility properties to astasian and pretreatment with alkali was required to effect solution.

Astasitol (45.0mg.) was dissolved in 8ml. of *N*-sodium hydroxide solution at 18°. Portions (0.2ml.) were hydrolysed successively with 1ml. of 90% formic acid and 2ml. of 2*N*-sulphuric acid and neutralized; the glucose content of the solution was 5.84mg./ml., corresponding to a polyglucose content of 93.5% in the astasitol. The alkaline solution (7ml.) was neutralized and oxidized with 2ml. of 0.3*M*-sodium metaperiodate in a total volume of 15ml. The formaldehyde content of 1ml. samples after 13, 16 and 19 days corresponded to 0.0364, 0.0352 and 0.0394 mol. prop./glucose residue, equivalent to a minimum degree of polymerization of 50–55 residues.

Methylation analysis. Astasitol (2.75g.) was dissolved in dimethyl sulphoxide (200ml.) at 60° by stirring for 3hr. in an atmosphere of nitrogen. The solution was cooled to 2° and dimethylformamide (200ml.) and barium hydroxide octahydrate (200g.) were added. The mixture was stirred vigorously at 2° for 30min. to produce a thick white slurry, and dimethyl sulphate (36ml.) added. Subsequent additions of 36ml. of dimethyl sulphate were made 1, 1.5 and 2hr. later, the mixture being allowed to warm to room temperature 30min. after the last addition. After about 30–40min. the mixture began to heat up (to 40–50°), producing a clear solution. The apparatus was sealed with a calcium chloride tube, and gentle stirring continued for 3 days at room temperature. Ammonia solution (sp.gr. 0.88; 60ml.) was then added to neutralize excess of dimethyl sulphate, and the mixture extracted successively with 300ml. and then four times with 100ml. of hot chloroform. The combined chloroform extracts were washed with water and concentrated under vacuum to yield 1.70g. of partly methylated astasitol. This was dissolved in 20ml. of dimethylformamide, and 20ml. of methyl iodide and 6g. of silver oxide were added. The mixture was stirred in the dark at 18° for 24hr. and the silver oxide was collected by filtration and washed thoroughly with hot chloroform. The filtrate and concentrated chloroform extracts were methylated two additional times with methyl iodide and silver oxide at 18° for 24hr. and twice by refluxing with methyl iodide over silver oxide for 48hr. The product (yield 0.95g.) had methoxy content 43.9%.

Methylated astasitol (0.90g.) was hydrolysed with 20ml. of 90% formic acid at 100° for 1hr. followed by 20ml. of 2*N*-sulphuric acid for 2hr. The neutralized (barium carbonate) hydrolysate was applied to a cellulose column (45cm. × 3.5cm.); elution was carried out with ethyl methyl ketone–water (10:1, v/v) at the rate of 11ml./hr. and 5ml. fractions were collected and analysed by paper chromatography with solvent *C*.

Fractions 36–40 contained tetra-*O*-methylglucose

(R_F 0.81); fraction 42 contained this sugar and a trace of tri-*O*-methylglucose (R_F 0.65); fractions 44–46 contained the latter and a trace of another sugar (R_F 0.73); fractions 48–68 contained only the tri-*O*-methylglucose with R_F 0.65; fractions 72–154 probably contained di-*O*-methyl sugars.

Fractions 36–40 were evaporated to dryness to yield 13mg. of a syrup, which crystallized over a period of 6 days. After three recrystallizations from light petroleum (b.p. 40–60°) the m.p. and mixed m.p. with an authentic sample of 2,3,4,6-tetra-*O*-methyl-D-glucose was 84–85°. The crystals ($[\alpha]_D + 85^\circ$ in water) had the same R_F value in solvents *B* and *C* as an authentic sample of 2,3,4,6-tetra-*O*-methyl-D-glucose and also gave an identical X-ray powder photograph.

Fractions 48–68 crystallized on evaporation; the yield was 700mg. The crystals had the same R_F value in solvents *B* and *C* as an authentic sample of 2,4,6-tri-*O*-methyl-D-glucose and gave an identical X-ray powder photograph. After three recrystallizations from ethyl acetate the crystals had m.p. and mixed m.p. 123–124°.

Fractions 43–47 on evaporation yielded 2mg. of a syrup. The major component had the R_F of 2,4,6-tri-*O*-methyl-D-glucose, and the minor component (approx. 10%) the R_F of the 2,3,4-tri-*O*-methyl isomer.

Fractions 72–154 on evaporation yielded 14mg. of a syrup. Gas-liquid chromatography of a methanolsate showed the presence of the α - and β -isomers of methyl 2,4-di-*O*-methyl-D-glucoside together with two other peaks that differed from the α - and β -isomers of the glycosides of the 2,3- or 3,4-di-*O*-methyl-D-glucose but could be due to the glycosides of the 4,6-isomer.

The ratio of the methylated sugars was estimated by quantitative paper chromatography, with authentic samples of 2,3,4,6-tetra-, 2,4,6-tri- and 4,6-di-*O*-methyl-D-glucose for the calibration. The molar proportions of tetramethyl:trimethyl:dimethyl sugar were 2.22:95.34:2.44 in one analysis and 2.40:94.74:2.86 in a second experiment. These results indicate an average chain length of about 43. In parallel experiments with hydrolysates of methylated laminaritol (borohydride-reduced laminarin) and methylated astasian, the chain length of both was about 20–25 glucose residues, confirming that severe degradation of astasian (but not of laminaritol) occurred during methylation.

DISCUSSION

Previous studies (Archibald *et al.* 1963) have shown that the flagellates *Ochromonas malhamensis* and *Peranema trichophorum* synthesize reserve carbohydrates containing a high proportion of β -(1→3)-glucosidic linkages. The present results

show that *Astasia ocellata* has a similar synthetic activity.

The new polysaccharide 'astasian' may amount to 55% of the dry weight of the cells, and is composed entirely of D-glucose residues. The β -configuration of the linkages is shown by the low specific rotation, infrared spectrum and susceptibility to a β -glucosidase from *Rhizopus arrhizus*, the pattern of hydrolysis being similar to that of laminarin, chrysolaminarin and paramylon from *Euglena gracilis* (see Table 1).

Since, for the first time, gram quantities of a protozoal β -glucan were available, a methylation analysis was planned since this would give unequivocal evidence of the type of glycosidic linkage. However, preliminary experiments showed that astasian was seriously degraded during the alkaline conditions of methylation, a feature that is now known to be particularly marked with (1 $\rightarrow$ 3)-glucans (Whistler & BeMiller, 1958), and may involve both the fragmentation of the molecule and stepwise degradation from the reducing end group with the formation of D-glucometasaccharinates. This type of degradation can normally be minimized by reduction of terminal 3-substituted glucose residues to sorbitol residues, but owing to the insoluble nature of astasian a single treatment with potassium borohydride reduced only about 25% of the available reducing groups. To overcome this difficulty, the careful addition of potassium borohydride to a solution of astasian in dimethyl sulphoxide was used, and astasitol was eventually recovered in 86% yield.

Methylation analysis of astasitol gave a mixture of 95% of 2,4,6-tri-O-methyl-D-glucose, about 2.3% of 2,3,4,6-tetra-O-methyl-D-glucose and 2.6% of di-O-methylglucose. These results show the pres-

ence of chains containing on average about 43 (1 $\rightarrow$ 3)-linked glucose residues. Since the minimum degree of polymerization, by two methods of periodate oxidation analysis, was about 50–55, the molecules are substantially linear.

It is now evident that the solubility of essentially linear β -(1 $\rightarrow$ 3)-glucans is inversely related to the degree of polymerization. A glucan of degree of polymerization 35 is soluble in both water and dilute alkali, whereas glucans of degree of polymerization 80–85 are insoluble in water and dissolve only with difficulty in alkali (Archibald *et al.* 1963; Johnson *et al.* 1963). The solubility properties and degree of polymerization of astasian are intermediate between the above values.

Most β -(1 $\rightarrow$ 3)-glucans (e.g. laminarin, chrysolaminarin, leucosin, yeast glucan) contain minor structural features. These include (1 $\rightarrow$ 6)-inter-chain or (1 $\rightarrow$ 6)-inter-residue linkages. Although the hydrolysate of methylated astasitol contained some di-O-methylglucose and a trace of 2,3,4-tri-O-methylglucose, we consider that these are probably not structurally significant (or represent not more than one anomalous linkage per three molecules) in view of the known difficulty in the methylation of β -(1 $\rightarrow$ 3)-glucans (Perlin & Taber, 1963) and the fact that the observed methoxyl content was 43.9% instead of the theoretical 45.6%. The molecular structure of astasian is therefore very similar to that of paramylon from *Peranema trichophorum* except for the significant difference in degree of polymerization (see Table 1).

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Table 1. *Properties of β -(1 $\rightarrow$ 3)-glucans*

Property	Laminarin	Chrysolaminarin	Leucosin from <i>Ochromonas malhamensis</i>	Paramylon from <i>Astasia ocellata</i> (astasian)	Paramylon from <i>Peranema trichophorum</i>	Paramylon from <i>Euglena gracilis</i>
$[\alpha]_D$ (in water)	-9°	-6°†	+10°	—	—	—
$[\alpha]_D$ (in NaOH)	+9°	—	—	+17°	+16°	+28°‡
Hydrolysis to laminari-saccharides by <i>Rhizopus</i> preparation	+	+	+	+	+	+
Infrared spectrum absorption peak (cm. ⁻¹)	890	890	890	890	890	890
Reduction of periodate (mol.prop.)	0.30	0.30†	0.17	0.14	0.03	0.02*
Average chain length	19*	12†	—	43	—	—
Degree of polymerization	24*	21†	36	50–55	80	150‡

* See Annan, Hirst & Manners (1965).

† See Beattie *et al.* (1961).

‡ See Clarke & Stone (1960).

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