115. *Studies on the Metabolism of the Protozoa. Part VIII.* The Molecular Structure of a Starch-type Polysaccharide from* Chilomonas paramecium.

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Chilomonas paramecium when grown in an acetate-containing medium synthesizes a starch-type polysaccharide. This contains *ca.* 45% of an essentially linear amylose, which has a lower degree of polymerization and lower iodine-binding power than potato amylose. The amylopectin component has an average chain length of 22 glucose residues, and is similar in many properties to a typical plant amylopectin.

Although the formation of starch is normally considered to be characteristic of the higher terrestrial plants, the presence of water-insoluble iodophilic granules has been noted in many other organisms. These include bacteria, *e.g.*, *Streptococcus pyogenes* [1] and *Corynebacterium diphtheriae*,[2] flagellated protozoa, *e.g.*, *Polytomella coeca*,[3] and fresh-water algæ, *e.g.*, *Dunaliella bioculata*.[4] We now describe a chemical and enzymic investigation of the starch-type polysaccharide synthesized by the flagellate *Chilomonas paramecium* (class, Cryptophyceae; order, Cryptomonadina).

Chilomonas paramecium is a free-living, motile organism (length 18—30 μ, breadth 9—12 μ, with two anterior flagellæ 10 μ in length); when it is grown on a synthetic medium, starch is slowly synthesized (rate 22 μg. per 10^6 cells per hr. at 25°).[5] Hutchens and his co-workers [5] showed that hot water extracted material giving a deep blue stain with iodine ($\lambda_{max.}$ 625 mμ), whilst a suspension of the insoluble residue gave a reddish-purple colour. These appear to be amylose and amylopectin-type polysaccharides respectively. Extraction of the cells with 0·5N-sodium hydroxide at 100° destroyed the cell structure; the resulting solution gave a purple-blue colour with iodine ($\lambda_{max.}$ 550 mμ). No fractionation of the starch was attempted.

A pure culture of the flagellate was grown on an acetate-containing medium (120 l.), and starch (yield, 2·3 g.) was extracted from the harvested cells by the chloral hydrate method.[6] The starch, originally present as iodophilic polygonal granules (diameter 2—5 μ), was obtained as a white amorphous powder, insoluble in water, but soluble in dilute sodium hydroxide solution ($[\alpha]_D$ +157° in N-NaOH). A dilute neutralized solution gave a blue stain with iodine [blue value [7] (B.V.) 0·54; $\lambda_{max.}$ 590 mμ]. An acid hydrolysate contained glucose as the sole carbohydrate (paper chromatography and treatment with glucose oxidase) and failed to react with an acid-resorcinol [8] (ketose) reagent. By cuprimetric titration, the glucose content was 78%; † the starch sample also contained inorganic material (10%) and protein (6%) which in view of the conditions of growth of the organism, and extraction of the polysaccharide, were considered to have no structural significance. Potentiometric titration of the iodine complex,[9] kindly performed by Dr. A. W. Arbuckle, indicated an iodine affinity of 5·0%; on the assumption that the amylose component has an iodine affinity of 10·6% (p. 557), this corresponds to an

* Part VII, Ryley, *Biochem. J.*, 1956, **62**, 215.
† All analytical figures of starch fractions are based on the observed glucose contents.

[1] Crowley and Jevons, *J. Gen. Microbiol.*, 1955, **13**, 226.
[2] Carlson and Hehre, *J. Biol. Chem.*, 1949, **177**, 281.
[3] Bourne, Stacey, and Wilkinson, *J.*, 1950, 2694.
[4] Eddy, Fleming, and Manners, *J.*, 1958, 2827.
[5] Hutchens, Podolsky, and Morales, *J. Cell. Comp. Physiol.*, 1948, **32**, 117.
[6] Meyer and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 875.
[7] Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924.
[8] Bell, "Modern Methods of Plant Analysis," Springer-Verlag, Berlin, 1955, Vol. II, p. 21.
[9] Anderson and Greenwood, *J.*, 1955, 3016.

amylose content of 47%. Incubation with barley β-amylase (which also contains Z-enzyme [10]) gave 75% conversion into maltose. The above evidence indicates the presence of a two-component starch, since the corresponding β-amylolysis limits of normal amylose and amylopectin are *ca.* 100 and 60% respectively.

The starch was then treated with 0·1N-sodium hydroxide at 100° for 10 min., in an atmosphere of nitrogen. After centrifugation, the supernatant solution was neutralized and treated with acetone to give alkali-soluble polysaccharide (fraction I, yield 56%).

TABLE 1. *Properties of* Chilomonas paramecium *starch fractions.*

Fraction	Original starch	I	Ia	Ib	II
Glucose content (%)	78	88	97	82	95
Iodine affinity (%)	5·0	6·1	10·6	0·09	1·60
B.V.	0·54	0·65	0·98	0·13	0·20
Iodine complex, $\lambda_{max.}$ (mμ)	590	620	645	540	520
β-Amylolysis limit (%)	75	81	95	60	64
Amylose content calc. from					
(*a*) iodine affinity	47	58	—	—	15
(*b*) B.V.	48	62	—	—	8
(*c*) β-amylolysis limit	43	60	—	—	11

Acetone was also added to a suspension of alkali-insoluble material, causing coagulation and giving a precipitate (fraction II, yield 29%). Analysis of the two fractions (see Table 1) indicated partial separation into components showing marked differences in iodine-binding power (cf. ref. 11).

β-Amylolysis of fraction II resulted in 64% conversion into maltose. On prolonged oxidation with sodium metaperiodate at 2°,[12] 1·07 moles of periodate were reduced per glucose residue, showing the apparent absence of 1,2- or 1,3-glucosidic linkages. The production of formic acid corresponded to an average chain length ($\overline{CL}$) of 25 glucose residues, a value similar to that of many amylopectins.

Since fraction I had many of the properties of a plant starch of high amylose content (cf. refs. 13 and 14), a portion was fractionated by the thymol method, and the resulting amylose complex purified by three precipitations with butanol.

The amylose (Fraction Ia) had a glucose content of 97%, B.V. 0·98, $\lambda_{max.}$ 645 mμ, and iodine affinity 10·6%. Incubation with purified soya-bean β-amylase gave 90% conversion into maltose; in presence of Z-enzyme, the β-amylolysis limit was 95%. The protozoal amylose thus contains few, if any, anomalous linkages. An estimate of the degree of polymerisation ($\overline{DP}$) was made viscometrically, the observed limiting viscosity number indicating $\overline{DP}$ 300—350. Fraction Ia therefore consists of essentially unbranched chains of α-1,4-linked D-glucose residues, but differs markedly in iodine-binding power and $\overline{DP}$ from highly purified potato amylose (*e.g.*, iodine affinity 19·5%, $\overline{DP}$ 3200 [10]). However, the $\overline{DP}$ is of the same order of magnitude as that reported for amylose preparations from apple,[15] maize,[16] malted barley,[17] and wheat starches.[18]

Since the amylopectin (fraction Ib; yield 80 mg.) had a glucose content of only 82% (it was isolated by freeze-drying, and contained inorganic material), analysis was confined to the interaction with iodine and enzymic degradation. Fraction Ib had an iodine affinity of 0·09%, showing the absence of amylose-type material, and B.V. 0·13. The

[10] Cowie, Fleming, Greenwood, and Manners, *J.*, 1957, 4430.
[11] Baum and Gilbert, *J. Colloid Sci.*, 1956, **11**, 428.
[12] Manners and Archibald, *J.*, 1957, 2205.
[13] Potter, Silveira, McCready, and Owens, *J. Amer. Chem. Soc.*, 1953, **75**, 1335.
[14] Wolff, Hofreiter, Watson, Deatherage, and MacMasters, *ibid.*, 1955, **77**, 1654.
[15] Potter, Hassid, and Joslyn, *ibid.*, 1949, **71**, 4075.
[16] Nussenbaum and Hassid, *J. Biol. Chem.*, 1951, **190**, 673.
[17] Aspinall, Hirst, and McArthur, *J.*, 1955, 3075.
[18] Potter, personal communication.

β-amylolysis limit was 60 and 82%, before and after treatment with yeast isoamylase. The outermost inter-chain linkages are therefore of the α-1,6-type.

The present study has shown that *Chilomonas paramecium* synthesizes a starch of small granular size which can be fractionated into essentially linear (amylose) and branched (amylopectin) components. Chloral hydrate extraction of the cells yields a starch-type polysaccharide which, however, differs from potato starch by virtue of its relative insolubility in hot water (compare *P. coeca* starch [3]), of its significantly higher iodine binding power and β-amylolysis limit, and of the properties of the amylose component (the corresponding figures for potato starch are B.V. 0·4, iodine affinity 4·0%, and β-amylolysis limit 60—70%) (see Table 2). On the assumption that fraction Ia represents the pure amylose, the amylose content of the original starch is 45 ± 5%, *i.e.*, higher than that of most plant starches (20—30%) * and markedly different from *P. coeca* starch (13—16%).[3] The low iodine-binding power of the amylose may be correlated with the

TABLE 2. *Properties of protozoal and potato starches.*

Property	*Chilomonas paramecium*	*Polytomella coeca* [3]	*Holotrich ciliates* [22]	Potato starch [24]
Whole starch				
$[\alpha]_D$ in N-NaOH	+157°	+160°	+171°	+159° [a]
Amylose content (%)	45	13—16	0	20
B.V.	0·54	0·36	0·05	0·4
Amylose component				
B.V.	0·98	1·13	—	1·4—1·5
β-Amylolysis limit (%)				
(*a*) β-amylase alone	90	—	—	77 [10]
(*b*) β-amylase + Z-enzyme	95	89	—	99 [10]
Amylopectin component				
$\overline{CL}$	22	—	22	22
Iodine complex, $\lambda_{max.}$ (mμ)	540	—	540 [b]	545
B.V.	0·13	0·11	0·05	0·06—0·13
β-Amylolysis limit (%)				
(*a*) β-amylase alone	60	48	63 [c]	53, 61 [c]
(*b*) after pretreatment with isoamylase	82	—	80 [c]	80, 77 [c]

[a] Determined by Mr. A. Wright. [b] Determined by Dr. A. M. Liddle.
[c] Data from Gunja, Manners, and Khin Maung, *Biochem. J.*, in preparation.

low $\overline{DP}$; thus, there is evidence that the $\lambda_{max.}$ of amylose-type chains is directly related to the $\overline{DP}$,[19] provided that $\overline{DP} < 500$. In view of recent reports [20] on the lability of amylose to oxygen and alkali, the question of degradation during isolation must be considered. In our experiments, degradation during anaerobic fractionation is unlikely; nevertheless, the possibility of inadvertent degradation during the original extraction remains, and it is being examined. It must be noted, however, that the chloral hydrate method has been successfully used for the extraction of other protozoal starches.[3,21,22]

The *Chilomonas* amylopectin appears to be similar to most plant amylopectins. Fraction II contains *ca.* 11% of amylose; with allowance for this, the production of formic acid on periodate oxidation corresponds to a $\overline{CL}$ of 22. This is identical with that for potato amylopectin.[23] Further, the β-amylolysis limits of fraction Ib are similar to those of two samples of potato amylopectin (Table 2). The average lengths of the

* However, the amylose content of the starch from certain varieties of pea [13] and maize [14] is much higher, *e.g.*, wrinkled pea (var. Laxton's progress) starch has an amylose content of 43% (unpublished work).

[19] Kerr, Cleveland, and Katzbeck, *J. Amer. Chem. Soc.*, 1951, **73**, 3916.
[20] For reviews, see Gilbert, *Stärke*, 1958, **5**, 95; Whistler and BeMiller, *Adv. Carbohydrate Chem.*, 1958, **13**, 310.
[21] Forsyth, Hirst, and Oxford, *J.*, 1953, 2030.
[22] Forsyth and Hirst, *J.*, 1953, 2132; see also Mould and Thomas, *Biochem. J.*, 1958, **69**, 327.
[23] Gunja and Manners, *Chem. and Ind.*, 1959, 1017.

exterior and interior chains (15—16 and 5—6 respectively) agree closely with those found for other amylopectins.[24]

The present results provide further information on the biochemical relations between the various groups of protozoa. Ciliates, *e.g.*, *Cycloposthium*,[21] holotrichs,[22] and *Tetrahymena pyriformis*,[25] and certain parasitic flagellates (the so-called animal-like group), *e.g.*, *Trichomonas foetus* [26] *and Trichomonas gallinae*,[26] synthesize amylopectin or glycogen-type polysaccharides. In contrast, the plant-like flagellates store either laminarin-type polysaccharides, *e.g.*, *Ochromonas malhamensis* [27] and *Euglena* [28] or starches, *e. g.*, *Polytoma* [29] and *P. coeca*.[3] *Chilomonas paramecium* can now be added to the latter group.

Experimental

The analytical methods employed have been described in Parts II [25] and VI [26] of this Series; the enzyme preparations were those reported in refs. 4 and 10.

Flagellate Preparation.—A pure culture of *Chilomonas paramecium* was maintained at 24°, with sub-inoculations at intervals of one week, in a sterile medium adjusted to pH 6·0—6·5, containing 0·1% (w/v) of sodium acetate and 0·1% (w/v) of " Oxoid " brand Lab-lemco. In large-scale cultures (20 two-litre flasks each containing 1·5 l. of medium) the flagellate was grown at 28° for 5 days, and the cells, which contained polygonal iodophilic granules (2—5 μ diam.), were harvested by gentle centrifugation.

Isolation and Properties of the Starch.—The cells were extracted twice with 33% chloral hydrate solution (100 ml.) at 80° for 1 hr.[6] Acetone (2 vol.) was added to the cooled centrifuged extract, giving an impure starch contaminated with cell debris. The pooled yield from four 30 l. preparations was 2·74 g. This product was purified by a further extraction with chloral hydrate, precipitated, washed with acetone, and air-dried at 37° (yield 2·25 g.). Before further examination, contaminating chloral hydrate was removed by extraction (Soxhlet) with methanol.

The starch was a white amorphous powder, insoluble in water but soluble in dilute sodium hydroxide solution. An acid hydrolysate contained glucose (paper chromatography and glucose oxidase treatment) and on treatment with acid-resorcinol [8] gave a reaction of 0·011 unit per mg. Under identical conditions, fructose, maltose, and " AnalaR " soluble starch gave values of 3·00, 0·016, and 0·014 unit per mg. respectively. The following properties of the starch were noted: $[\alpha]_D$ +157° (*c* 0·54 in N-NaOH); Found: glucose, 78·4%; ash 9·7%; protein-N, 0·94% (determined by the colorimetric biuret method of Robinson and Hogden [30]). A dilute solution was stained deep blue with iodine (B.V. 0·54), showing maximum absorption at 590 mμ. On potentiometric titration, the iodine complex gave a typical starch curve, extrapolation indicating an iodine affinity of 5·0%. Incubation with barley β-amylase [10] at pH 4·6 and 35° (with 54 units of β-amylose per mg. of starch) gave 75% conversion into maltose after 24 hr. The reducing power of the digest did not increase on further incubation.

Extraction of the Starch with Alkali.—The starch (1·5 g.) was stirred in 0·1N-sodium hydroxide (100 ml.) at 100° for 10 min. in an atmosphere of nitrogen. After cooling and neutralization by dilute sulphuric acid (phenolphthalein), an insoluble gelatinous residue was collected by centrifugation. Acetone precipitation of the solution gave fraction I (840 mg.) (Found: glucose, 88·0%; ash, 5·9%; B.V., 0·65; $\lambda_{max.}$ of iodine complex, 620 mμ; iodine affinity, 6·1%; β-amylolysis limit, 81%). Treatment of the residue with acetone gave fraction II (440 mg.) (Found: glucose, 94·5%; ash, 2·7%; B.V., 0·196; $\lambda_{max.}$ of iodine complex, 520 mμ; iodine affinity, 1·60%; β-amylolysis limit, 64%).

Periodate Oxidation of Fraction II.—Fraction II (160 mg.) was moistened with alcohol and shaken overnight with 2N-sodium hydroxide (10 ml.). The solution was then neutralized with dilute sulphuric acid (Methyl Red) and diluted with water to 25 ml. 20 ml. of this solution (equivalent to 121·2 mg. of glucosan) were oxidized in the dark at 2° with 8% sodium metaperiodate solution (3 ml.) and water (to 25 ml.). A solution of periodate in water was also

[24] Manners, *Quart. Revs.*, 1955, **9**, 84; Whelan, " Encyclopedia of Plant Physiology," Springer-Verlag, Berlin, 1958, Vol. VI, p. 154.
[25] Manners and Ryley, *Biochem. J.*, 1952, **52**, 480.
[26] *Idem, ibid.*, 1955, **59**, 369.
[27] Archibald, Manners, and Ryley, *Chem. and Ind.*, 1958, 1516.
[28] Kreger and Meeuse, *Biochim. Biophys. Acta*, 1952, **9**, 699.
[29] Brechot, *Compt. rend.*, 1937, **126**, 555.
[30] Robinson and Hogden, *J. Biol. Chem.*, 1940, **135**, 727.

prepared. Samples (3 ml. or 2 ml.) were analysed at intervals for the production of formic acid and the reduction of periodate:

Time of oxidation (days)	7	10	12
Total formic acid prodn. (mg.)	1·17	1·40	1·37
Reduction of periodate (mole/glucose residue)	1·04	1·07	1·07

The production of formic acid indicates a CL value of 25 glucose residues; the theoretical periodate reduction is 1·04 mol. if only 1,4- and 1,6-glucosidic linkages are assumed to be present.

Thymol Fractionation of Fraction I.—Fraction I (0·5 g.) was suspended in water (20 ml.) and added to vigorously stirred boiling water (65 ml.), in an atmosphere of nitrogen. The solution was heated at 98° for 20 min., then allowed to cool, and a small insoluble residue was removed by centrifugation. The clear solution was heated to 60°, powdered thymol (1·5 g.) added, and the mixture stirred at 60° for 30 min. and then kept at room temperature for 3 days. The thymol–amylose complex was collected by centrifugation and directly dispersed in boiling water (50 ml.) under nitrogen, and redistilled butanol (5 ml.) was added. The mixture was stirred at 95° for 30 min., then allowed to cool slowly to room temperature, and the butanol–amylose complex removed by centrifugation. The amylose was purified by two further precipitations with butanol, and isolated by treatment of the butanol complex with butanol. This gave fraction Ia (129 mg.) [Found: glucose, 97·0%; B.V., 0·98; $\lambda_{max.}$ of iodine complex, 645 mμ; iodine affinity, 10·6%; limiting viscosity number (in 0·2M-potassium hydroxide), 45·4]. By the approximate relation $-\overline{DP} = 7{\cdot}4[\eta]$,[31] this corresponds to $\overline{DP}$ 335.

The supernatant solution from the thymol precipitation was freeze-dried, to give fraction Ib. This was extracted (Soxhlet) with methanol to remove thymol, redissolved in water, and freeze-dried (yield 80 mg.) (Found: glucose, 82%; B.V., 0·125; iodine affinity, 0·09%).

Enzymic Degradation of Fractions Ia *and* Ib.—Fraction Ia (18·5 mg.) was dissolved in 0·2M-acetate buffer (pH 4·6; 5 ml.), and purified soya-bean β-amylase solution [10] (0·1 ml.; 1000 units) and water (to 25 ml.) were added. After incubation at 35° for 24 hr., the β-amylolysis limit was 90%. On being treated with barley β-amylase containing Z-enzyme [10] (50 β-amylase units/mg.), fraction Ia (10·4 mg.) in a final volume of 25 ml. gave 95% conversion into maltose.

Fraction Ib (9·8 mg.) was dissolved in 0·2N-sodium hydroxide (2 ml.), then neutralized, and 0·2M-acetate buffer (pH 5·8; 5 ml.) added, followed by aqueous yeast isoamylase (5 mg./ml.; 2 ml.). After incubation at 20° for 18 hr., the isoamylase was inactivated by heat, and β-amylase solution (500 units; 1 ml.) and water (to 25 ml.) were added. The maltose content after 36 hours' incubation at 35° indicated a β-amylolysis limit of 82%. In a control experiment without isoamylase, the β-amylolysis limit was 60%.

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[31] Cowie and Greenwood, *J.*, 1957, 2862.