Studies on the Metabolism of the Protozoa

2. THE GLYCOGEN OF THE CILIATE *TETRAHYMENA PYRIFORMIS* (*GLAUCOMA PIRIFORMIS*)

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A number of Protozoa synthesize intracellular carbohydrate, described as glycogen on account of the brown colour given with iodine; this polysaccharide may be diffusely distributed throughout the cell, or localized in 'glycogen vacuoles'. There do not seem to have been any investigations of the chemical nature of protozoal glycogen, nor of its physiological function. Recently, however, an opportunity for such an investigation arose from the observation, briefly reported in the preceding paper of this series (Ryley, 1951), that the protozoan *Glaucoma piriformis* may have an intracellular glycogen content as high as 22%. This protozoan will be referred to in the present study as *Tetrahymena pyriformis* in accordance with the new nomenclature advocated by Corliss (1952). The protozoal glycogen has been isolated in pure form and subjected to a detailed analysis.

MATERIALS AND METHODS

Ciliate preparations. Tetrahymena pyriformis was cultivated in a peptone medium as described in the following paper (Ryley, 1952). It was found that the peptone used in the preparation of the medium contained 0·44% of a KOH-resistant, ethanol-precipitable, glycogen-like material. Cells were harvested after 6 or 12 days growth, using a small angle centrifuge, or, in the case of larger preparations, the Sharples supercentrifuge.

Analytical methods. Total nitrogen was determined by digestion with H_2SO_4, using SeO_2 as catalyst. After digestion, ammonia was distilled *in vacuo* in the Parnas-Heller apparatus (Parnas & Heller, 1924), and estimated colorimetrically with Nessler's reagent. Phosphorus was estimated by the method of Fiske & Subbarow (1925), after digestion of a sample with 60% $HClO_4$ and a little HNO_3. Glycogen was estimated by the method of Good, Kramer & Somogyi (1933). Reducing sugar was estimated iodometrically by the method of Somogyi (1945), while glucose was determined as the loss in reducing value of a sample after treatment with glucose oxidase and catalase (Mann, 1946). The glycogen end-group assay was carried out by the method of KIO_4 oxidation developed by Halsall, Hirst & Jones (1947), as modified by Bell & Manners (1952). β-Amylolysis of glycogen was effected by treating the glycogen with crystalline sweet potato β-amylase, kindly supplied by Prof. C. S. Hanes, F.R.S., as described by Bell & Manners (1952). Molecular weight determinations were made by the light-scattering method, as described by Harrap & Manners (1952). We are indebted to Dr B. S. Harrap for the measurement of the turbidity of the glycogen.

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RESULTS

Glycogen content of the cells

After growth in a peptone medium of low carbohydrate content, the cells stained a uniform brown colour with iodine. Growth was accompanied by the production of free ammonia; after 6 days' growth, the ammonia content of the medium, as determined by steam distillation *in vacuo*, had increased from 42 to 88 μg./ml., there being a corresponding slight increase in the pH. Cells were harvested after 6 and after 12 days' growth at 30°, washed in 0·45% saline and then water, and made to a thick suspension in water; glycogen was estimated in duplicate 1 ml. samples of this suspension. Such analyses gave cell glycogen contents of 16, 14 and 17% for three 6-day cultures, and 23 and 22% for two 12-day cultures (on a dry weight basis).

Isolation and purification of glycogen

Five large-scale glycogen preparations were carried out, using in all a total of 130 l. of 12-day culture. The cells were harvested, treated with 2 vol. 30% (w/v) KOH at 100° for 30 min., cooled, centrifuged to remove a little insoluble material, and the crude glycogen precipitated with 1·1 vol. absolute ethanol. The precipitate was removed on the centrifuge, washed with 60%, 80% and absolute ethanol, and ether, and dried *in vacuo* over H_2SO_4. In this way, a total of 4·8 g. crude glycogen were obtained. This product contained 0·52% N and 0·32% P, and was 66% pure on a basis of glucose liberated by acid hydrolysis. Further purification was achieved by precipitation with 80% (w/v) acetic acid (Bell & Young, 1934). Crude glycogen (4·8 g.) was dissolved in 130 ml. water; a brown, gelatinous, insoluble residue was removed on the centrifuge. Glacial acetic acid (520 ml.) was added to the supernatant, and the precipitate centrifuged off. It was dissolved in 61 ml. water, a little insoluble material was removed, and the glycogen precipitated by adding 244 ml. glacial acetic acid. This precipitation with acetic acid was repeated twice more, and the purified glycogen washed twice in 200 ml. of 80%, 90%

Table 1. *A comparison of the properties of* Tetrahymena, *rabbit-liver and rabbit-muscle glycogens*

(Data for rabbit-liver and rabbit-muscle glycogens taken from Bell (1948), Bell & Manners (1952) and Harrap & Manners (1952).)

Property	Source of glycogen		
	T. pyriformis	Rabbit liver	Rabbit muscle
$[\alpha]_D$ (water)	+195°	+198°	+196°
Iodine coloration	Yellow-brown	Red-brown	Purple-brown
Unit chain length:			
(*a*) KIO_4 method	13	13	13
(*b*) Methylation method	—	12	13
Conversion to maltose by β-amylase (%)	44	43	45
Molecular weight (light scattering)	$9{\cdot}8 \times 10^6$	$6{\cdot}8 \times 10^6$	$2{\cdot}8 \times 10^6$

and absolute ethanol and in ether. After a preliminary drying at 37°, the glycogen was dried *in vacuo* over P_2O_5 for 3 hr. at 100°. This gave 3·12 g. of purified glycogen.

Characterization of purified glycogen

The purified glycogen was a white powder, freely soluble in water, forming an opalescent solution, which gave a yellow-brown colour with dilute iodine. The iodine-staining power of the glycogen was quickly destroyed on treatment with salivary α-amylase, this being accompanied by the liberation of reducing sugar. The purified, dry glycogen had $[\alpha]_D^{18°} + 195°$ in water (2 dm. tube, *c*, 0·3); N, 0·095; P, 0·069; ash 0·46 %; reducing value 0·22 % that of an equivalent weight of maltose. In Table 1, the properties of this purified glycogen are summarized, and compared with those of rabbit-liver and rabbit-muscle glycogens.

Glucose content. Hydrolysis of the glycogen for 2 hr. in 0·6 N-hydrochloric acid, followed by determination of the reducing value, indicated that the purified glycogen was 97 % pure, assuming it to be a polymer of glucose. That glucose was the only sugar liberated by acid hydrolysis was demonstrated by incubating samples of the hydrolysate with phosphate buffer (pH 5·6), and a well washed suspension of baker's yeast, or a mixture of glucose oxidase and catalase. Incubations were carried out in small conical flasks, which were shaken for 3 hr. at 37°; the yeast cells were then centrifuged off. The resulting solutions were non-reducing, indicating that all the sugar initially present was fermentable by yeast, and oxidizable by glucose oxidase.

Acid hydrolysis. The behaviour of *Tetrahymena* glycogen on acid hydrolysis was compared with that of rabbit-liver glycogen and starch. Both glycogens were completely hydrolysed by 0·6 N-hydrochloric acid at 100° in 60 min., while during the same time starch was 92 % hydrolysed. In 0·1 N-hydrochloric acid, both glycogens were hydrolysed at an identical rate (50 % in 150 min.) while starch was hydrolysed somewhat faster (50 % in 90 min.) (Fig. 1).

Unit chain length. On complete oxidation with potassium periodate 211·3 mg. glycogen gave 4·6 mg. formic acid. This corresponds to a unit chain length of 13 glucose residues.

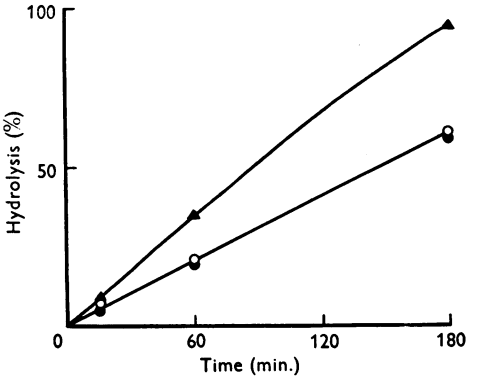

Fig. 1. Hydrolysis of starch, rabbit-liver glycogen and *Tetrahymena* glycogen in 0·1 N-HCl at 100°; ▲—▲, starch; ●—●, *Tetrahymena* glycogen; ○—○, rabbit-liver glycogen.

β-Amylolysis. Glycogen from *Tetrahymena* (43·6 mg.), on treatment with crystalline β-amylase, was converted to maltose as follows: 38 % in 30 min., 42 % in 135 min., 44 % in 20 hr., and 44 % in 47 hr.

Molecular weight. Light-scattering determinations indicated a molecular weight of $9{\cdot}8 \times 10^6$.

DISCUSSION

Most free-living Protozoa synthesize some type of intracellular carbohydrate, and it is interesting to compare the different ways in which these carbohydrate reserves arise. In a great number of cases, intracellular carbohydrate takes the form of starch granules, the production of which is associated with the presence of plastids in the cell. This starch may have a photosynthetic origin, or may be elaborated from simple carbon compounds present in the

growth medium, usually short-chain fatty acids. Polysaccharide formation in the flagellate *Polytomella cœca* has been studied in some detail. Rabinovich (1938) observed that starch formation by this flagellate, grown in a synthetic medium containing asparagine as nitrogen source, and acetate as carbon source, was associated with a sub-cuticular, reticulate leucoplast. Lwoff, Ionesco & Gutmann (1950) found that the organism, although it could not utilize free sugars, contained a phosphorylase system which would convert glucose-1-phosphate to polysaccharide. They prepared a quantity of this protozoal polysaccharide, which was examined chemically by Bourne, Stacey & Wilkinson (1950). It was found that the polysaccharide, which stained purple with iodine, was a starch-like substance, having an amylopectin content of about 85%. Oxford (1951) observed that a number of large holotrichous ciliates, found in the sheep rumen, were able to ferment simple sugars present in the food of the host, with the formation of intracellular carbohydrate granules; this carbohydrate was composed entirely of glucose, and stained a brownish purple colour with iodine.

With *Tetrahymena pyriformis*, growth of the ciliate and the formation of intracellular glycogen are accompanied by the liberation of free ammonia in the medium. In view of the low carbohydrate content of the growth medium, it seems possible that intracellular carbohydrate may arise from the products of protein hydrolysis and oxidation. This glycogen forms an energy store, utilizable under conditions of unfavourable oxygen tension (Ryley, 1952). From the table, it can be seen that the glycogen synthesized by *T. pyriformis* is very similar to mammalian glycogens, except that it has a rather higher molecular weight.

SUMMARY

1. A glucose-containing polysaccharide has been isolated from the ciliate *Tetrahymena pyriformis* (*Glaucoma piriformis*), grown in a peptone medium without added carbohydrate.

2. The purified polysaccharide shows all the properties of a typical animal glycogen. It has a molecular weight of $9{\cdot}8 \times 10^6$, $[\alpha]_D^{18°} + 195°$, and a unit chain length of 13 glucose residues.

3. Hydrolysis of the glycogen by crystalline sweet potato β-amylase gives 44% conversion to maltose.

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