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Studies on Carbohydrate-Metabolizing Enzymes

2. TRANS- α -GLUCOSYLATION BY EXTRACTS OF *TETRAHYMENA PYRIFORMIS**

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In continuation of studies on transglucosylation reactions (Duncan & Manners, 1958; Anderson & Manners, 1959; Duncan, Manners & Thompson, 1959) we now describe an investigation of the metabolism of maltose by cell-free extracts of the ciliate *Tetrahymena pyriformis*. The presence of a maltase in such extracts has been noted by Ryley (1952).

* The paper by Anderson & Manners (1959) is regarded as Part 1.

Since maltase activity may be regarded as a trans- α -glucosylation reaction in which water provides the acceptor substrate:

where $\text{H}\cdot\text{OR}$ is the glucosyl acceptor (Gottschalk, 1958), it follows that, if oligosaccharide synthesis takes place in concentrated solutions of maltose,

the nature of the products provides information on the specificity of the maltase for the acceptor H $\cdot$ OR. This has been obtained in the present study by chromatographic separation and characterization of the oligosaccharides synthesized from 18% maltose solution. In addition, the ability of other sugars to function as acceptors has been examined with various maltose-monosaccharide mixtures.

MATERIALS AND METHODS

Analytical methods. The methods used have been described by Duncan & Manners (1958) and Anderson & Manners (1959). An ethyl acetate-pyridine-water (10:4:3, by vol.) mixture was used as solvent for paper chromatography.

Organism. A pure culture of *Tetrahymena pyriformis* (GL) was kindly supplied by Dr J. F. Ryley and was maintained at room temperature in a sterile medium, adjusted to pH 7.3, containing 1% (w/v) of Oxoid brand bacteriological peptone and 0.4% (w/v) of sodium chloride. Sub-inoculations were made at intervals of 2 weeks (Ryley, 1952).

For large-scale culture, the organism was grown in the dark at 30° in 2 l. conical flasks containing 400 ml. of medium. Maximum culture density was reached after 6 days. The contents of twenty-two such flasks were combined and the cells collected by using a Sharples super-centrifuge. The cells were then dispersed in 150 ml. 0.1 M-sodium citrate buffer (pH 6.0) and freeze-dried to give 7.0 g. of yellow-brown powder. Microscopic examination of an aqueous suspension of this material showed that the cells had disintegrated. Part of the freeze-dried powder (0.35 g.) was suspended in 10 ml. of water and kept, with frequent stirring, at room temperature for 1 hr. Centrifuging at 3500 g yielded a yellow, opalescent solution which was free from cells and cell particles.

A further portion of freeze-dried cells (approx. 7 g.) was extracted with 200 ml. of water and centrifuged, and the extract was then freeze-dried (yield, 4.8 g.). This material was stored at 2° over calcium chloride; the maltase activity was not appreciably diminished after 1 year. However, the following experiments were carried out within 4 weeks of preparation of the extract.

The possibility of reducing sugars' being present in this extract, or arising by autolysis of soluble polysaccharide, was examined by paper-chromatographic analysis of a 4% (w/v) aqueous solution during incubation at 35° for 5 days. A faint trace of glucose was detected initially; this did not increase.

EXPERIMENTAL AND RESULTS

Carbohydrase activity of extract

Digests containing carbohydrate (2%, w/v) and extract (2%, w/v) were incubated at 35° for 24 hr. The following carbohydrates were hydrolysed: maltose, isomaltose, panose, maltotriose, starch, cellobiose, sucrose, methyl α -D-glucoside, α -D-glucosyl phosphate. Lactose was not attacked. In view of the considerable α -glucosidase activity, the action was investigated with a concentrated solution of maltose (a) alone, (b) in the presence of possible α -glucosyl acceptors.

Action on concentrated solution of maltose

A digest containing maltose (20%, w/v) and extract (1%, w/v) was incubated at 35° in the presence of toluene. A number of oligosaccharides were produced, as shown in Table 1.

Preparation and isolation of oligosaccharides

Thrice-recrystallized and chromatographically-pure maltose (30.0 g.), 150 ml. of 0.1 M-sodium citrate buffer (pH 6.0) and 20 ml. of enzyme extract were incubated, with toluene, at 35° for 10 days. The enzyme extract was prepared from 3.8 g. of freeze-dried cells, which were dispersed in 60 ml. of water at room temperature for 2 hr. and then centrifuged (3500 g at 2°). Paper-chromatographic analysis at intervals gave the same results as those shown in Table 1. The digest was then heated for 20 min. at 98°, and filtered.

The oligosaccharides were separated by chromatography on a charcoal-Celite column (160 $\times$ 7.5 cm.) and elution with increasing concentrations of aqueous ethanol. Fractions (1–2 l.) were collected, concentrated to approx. 5 ml., freed from traces of charcoal and Celite and examined by paper chromatography. Fractions containing like components were combined, and the sugars isolated in the usual manner by crystallization from ethanol or by freeze-drying. The approximate yields are given in Table 2. All the fractions were homogeneous by paper chromatography and electrophoresis, and on acid hydrolysis gave only glucose.

Characterization of oligosaccharides

Fraction 1. The data in Table 2 and the specific rotation in water (+52°) characterize this sugar as D-glucose.

Fraction 2. Partial and total acid hydrolysis gave glucose; emulsin had no action. This disaccharide is therefore isomaltose.

Table 1. *Oligosaccharides synthesized from maltose by cell-free extracts of Tetrahymena pyriformis*

+, + +, + + + and *t* indicate intense, strong, moderate and weak spots, respectively. Glucose, maltose, isomaltose, maltotriose and panose had R_G values of 1.00, 0.62, 0.47, 0.39, 0.28, respectively.

Time of incubation (days)	R_G values					
	1.00	0.62	0.47	0.39	0.28	Others
2	+	+	+	+	+	<i>t</i>
4	+	+	<i>t</i>	+	+	<i>t</i>
6	+	+	+	+	+	+
8	+	+	+	+	+	+
10 and 15	+	+	+	+	+	+

Fraction 3. This was identified as maltose by specific rotation ($+132^\circ$), partial acid hydrolysis and the data in Table 2.

Fraction 4. This trisaccharide had the same R_g as authentic panose and $[\alpha]_D + 148^\circ$ in water (c. 2.3); cf. $[\alpha]_D + 148^\circ$ reported for panose by Duncan & Manners (1958). A partial acid hydrolysate contained glucose, maltose and isomaltose; the corresponding aldonic acid, prepared by hypiodite oxidation, gave glucose and isomaltose. On periodate oxidation, 0.94 mol.prop. of formaldehyde was released. The theoretical value is 1.00 mol.prop.

Fraction 5. This trisaccharide was characterized as maltotriose by R_g value, $[\alpha]_D + 161^\circ$ in water (c. 2.7) [cf. $[\alpha]_D + 160$ and $+164^\circ$ quoted by Whelan, Bailey & Roberts (1953) and Duncan & Manners (1958), respectively] and slow hydrolysis to glucose and maltose by salivary α -amylase. Partial acid hydrolysates of the sugar and the corresponding aldonic acid both contained glucose and maltose.

Fraction 6. This sugar was a tetrasaccharide with R_g 0.18 and $[\alpha]_D + 172^\circ$ in water (c. 0.4); cf. values of R_g 0.17 and $[\alpha]_D + 177^\circ$, reported by Duncan & Manners (1958). Partial hydrolysis with acid gave glucose, maltose, isomaltose, panose and maltotriose. The corresponding aldonic acid gave the same products on partial hydrolysis, except that maltotriose was absent. This finding eliminates 6¹- and 6²- α -glucosylmaltotriose [see Crook & Stone (1957) for definition of nomenclature] as possible structures. The characterization as the 6³ isomer is confirmed by the periodate oxidation (Table 2).

Fraction 7 (with Miss Zeenat H. Gunja). This tetrasaccharide had $[\alpha]_D + 180^\circ$ in water (c. 0.9); cf. $[\alpha]_D + 177^\circ$, quoted by Whelan *et al.* (1953) for maltotetraose. Partial hydrolysis with acid gave glucose, maltose and maltotriose. α - and β -Amylolytic gave a mixture of glucose and maltose, and maltose, respectively.

Trans- α -glucosylation to other sugars

Digests containing maltose (5%) and a pentose, D-galactose, or D-fructose (5%) and enzyme extract

(1%) were prepared and incubated at 35° . After 3–5 days' incubation the presence of new components in addition to isomaltose, maltotriose and panose was detected by paper chromatography. The new sugars had R_g values of 0.64 (D-xylose acceptor), 0.69 (D-lyxose acceptor), 0.64 (D-ribose acceptor), 0.72 (L-arabinose acceptor), 0.32 (D-galactose acceptor). The new sugars formed in the digests containing pentoses gave pink stains with aniline oxalate and are presumably α -glucosylpentose disaccharides. Fructose did not function as an acceptor substrate.

Other transglucosylation reactions

Various carbohydrates (20% solution) and extract (2%) were incubated for 5 days at 35° . The following results were obtained by paper chromatography: (a) isomaltose: products had R_g 1.0, 0.47, 0.19 (? isomaltotriose); a trace of higher oligosaccharides was also present; (b) methyl α -D-glucoside: the main product had R_g 1.0; a trace of higher oligosaccharides was produced; (c) D-glucose, (d) D-xylose, (e) α -D-glucosyl phosphate: no synthesis of oligosaccharides.

DISCUSSION

The ability of maltase preparations from a variety of biological sources to catalyse the synthesis of oligosaccharides from maltose has been reported by many workers (see Duncan & Manners, 1958).

The present results (Tables 1 and 2) show that cell-free extracts of the ciliate *Tetrahymena pyriformis* have a similar activity. On incubation with 18% maltose solution, the main products are panose and maltotriose; smaller quantities of isomaltose, 6³- α -glucosylmaltotriose and maltotetraose are also formed. Since the extracts are unable to polymerize glucose [in contrast with certain fungal preparations (Peat, Whelan & Hinson, 1955)], it follows that the oligosaccharides are formed by transglucosylation in which the OH groups at both C-4 and C-6 of the non-reducing residue of maltose and maltotriose can function as acceptors for α -glucosyl

Table 2. *Properties of the oligosaccharides*

The degree of polymerization was determined from reducing-power measurements before and after acid hydrolysis. The periodate oxidation value is expressed as moles of formaldehyde per mole of oligosaccharide liberated on oxidation at pH 8. With fractions 5 and 6 a small amount of sugars with R_g 0.31 and 0.15 was also eluted with 8% (v/v) ethanol.

Fraction no.	Eluent	Yield (g.)	R_g	Degree of polymerization	Periodate oxidation value	Identity
1	Water	—	1.00	1.00	1.01	Glucose
2	Water	0.41	0.47	1.90	0.08	Isomaltose
3	Ethanol (2–6%, v/v)	5.96	0.62	2.10	1.70	Maltose
4	Ethanol (6%, v/v)	2.94	0.28	3.20	0.94	Panose
5	Ethanol (6–8%, v/v)	3.29	0.41	2.94	2.80	Maltotriose
6	Ethanol (8%, v/v)	0.58	0.18	3.70	1.98	6 ³ - α -Glucosylmaltotriose
7	Ethanol (10–12%, v/v)	0.35	0.26	4.08	—	Maltotetraose

radicals. Panose does not appear to be a favourable acceptor, since 6³- α -isomaltosylmaltose and related sugars are not produced. The ability to transfer to C-4 differentiates the protozoal enzyme system from various fungal maltases which can only synthesize (1 $\rightarrow$ 6)-linked oligosaccharides (e.g. Pazur & French, 1952).

The acceptor specificity of the protozoal maltase system is generally similar to that observed with extracts of *C. rupestris* (Duncan & Manners, 1958) except that the latter is unable to use monosaccharides as acceptor substrates. Since a number of pentoses and also D-galactose can accept α -glucosyl radicals with the *T. pyriformis* system, it is probable that most of the isomaltose is formed by the interaction of a glucosyl-enzyme complex with the primary alcohol group of a glucose molecule rather than by the hydrolysis of the (1 $\rightarrow$ 4)-linkage of panose. This view is supported by the fact that isomaltose synthesis and the formation of higher oligosaccharides ($R_g < 0.28$) occur simultaneously, showing that the equilibrium between hydrolysis (i.e. water as glycosyl acceptor) and trans- α -glucosylation to maltotriose favours the latter.

Isomaltose itself can function as a glucosyl donor, but the product appears to be isomaltotriose rather than panose or a maltosaccharide. One possible explanation for this is that the maltase and isomaltase activities of the *T. pyriformis* extracts are due to different enzymes. The non-identity of these activities in extracts of yeast and intestinal mucosa (for example, Larnier & Gillespie, 1956) has already been established.

SUMMARY

1. Cell-free extracts of the ciliate *Tetrahymena pyriformis* show hydrolytic activity towards

maltose, isomaltose, cellobiose, sucrose, methyl α -D-glucoside, α -D-glucosyl phosphate and starch.

2. In concentrated solution, oligosaccharides are synthesized from maltose and from isomaltose, but not from glucose.

3. Oligosaccharide synthesis from maltose results in the formation of isomaltose, maltotriose, panose, 6³- α -glucosylmaltotriose and maltotetraose.

4. The acceptor specificity for α -glucosyl radicals by the protozoal enzyme system and other maltases is discussed.

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Enzyme Systems in Marine Algae

3. TRANS- β -GLUCOSYLATION BY EXTRACTS OF *CLADOPHORA RUPESTRIS* AND *ULVA LACTUCA**

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During a study of the hydrolysis of cellodextrin by an extract of *Cladophora rupestris*, Duncan, Manners & Ross (1956) noted that the products of enzyme action, as examined by paper chromatography, included laminaribiose. Since the original

substrate did not contain β -1:3-glucosidic linkages, it seemed possible that this sugar had arisen from cellobiose by trans- β -glucosylation involving a double-displacement mechanism:

Cellobiose + enzyme-H $\rightarrow$ glucose + glucosyl-enzyme

Glucosyl-enzyme + H $\cdot$ OR $\rightarrow$ enzyme-H + glucosyl $\cdot$ OR

where RO $\cdot$ H is an acceptor substrate, in this case D-glucose acting at its C-3 hydroxyl group.

* Part 2. Duncan & Manners (1958).

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