Metabolism of the Reserve Polysaccharide of *Streptococcus mitis*

PROPERTIES OF A TRANSGLUCOSYLASE

By GWEN J. WALKER

Institute of Dental Research, Sydney, New South Wales, Australia

(Received 22 April 1966)

1. A transglucosylase has been separated from cell extracts of *Streptococcus mitis*, and has been partially purified by chromatography on DEAE-cellulose. 2. The transglucosylase was present in the six strains of *Streptococcus mitis* that were examined, and the activity of the enzyme was the same whether the cells had grown on glucose or on maltose. Four of the strains could store intracellular iodophilic polysaccharide when grown on high concentrations of glucose or maltose (1%), but none of the strains stored polysaccharide during growth on 0.1% glucose. The activity of transglucosylase in cell extracts was the same whether or not the cells had stored polysaccharide. 3. The transglucosylase degrades amylose in the presence of a suitable acceptor, transferring one or more glucosyl residues from the non-reducing end of the donor to the non-reducing end of the acceptor. With [^{14}C]glucose as acceptor the maltodextrins produced were labelled in the reducing glucose unit only. 4. The enzyme can synthesize higher maltodextrins from maltose and maltotriose. Maltotetraose is disproportionated to give products of sufficient chain length to give a stain with iodine. 5. The action pattern of *S. mitis* during the degradation of synthetic amylose was shown to be intermediate between the single-chain and multi-chain mechanism.

Gibbons & Kapsimalis (1963) have described a strain of *Streptococcus mitis* that could store intracellular iodophilic polysaccharide when the cells were provided with excess of glucose. Catabolism of this polysaccharide could provide *S. mitis* with energy in a utilizable form (Gibbons, 1964a). Gibbons & Socransky (1962) have suggested that some oral bacteria may contribute to the caries process in humans by storing polysaccharides within the cell, and then metabolizing this reserve carbohydrate to lactic acid when the exogenous sugar supply was no longer available. Polysaccharide-storing organisms might thus be responsible for maintaining carious plaque at a low resting pH. Gibbons (1964b) found that 54% of the organisms cultivated from plaque from caries-active individuals were capable of storing large quantities of polysaccharide, whereas only 29% of the organisms from caries-inactive plaque had this ability. The organisms present in high dilutions of plaque were characterized by Gibbons, Socransky, de Araujo & van Houte (1964). Streptococci were the single most numerous group, but *Streptococcus salivarius* was absent since none of the streptococci formed mucoid colonies when grown on sucrose. Krasse (1954) also found that *S. salivarius* comprised only 1% of the streptococci in plaque; the other strepto-

cocci consisted mainly of *S. mitis*. Howell, Rizzo & Paul (1965) reported that streptococci were the predominant organisms in human dental plaques, but none of the strains had all the characteristics of *S. mitis* or of *S. salivarius*.

Weiss, King, Kestenbaum & Donohue (1965) have shown that maltose is incorporated into a polysaccharide, similar to that formed from glucose, by *S. mitis*. Maltose was utilized at the same rate as glucose.

No studies of the enzymic mechanisms for the synthesis and degradation of the reserve polysaccharide of *S. mitis* have been reported. In the present paper the isolation and properties of a cell-bound transglucosylase are described. This enzyme can synthesize oligosaccharides from maltose by transferring α -(1 $\rightarrow$ 4)-glucosidic linkages. Amylose and amylopectin are degraded in the presence of a suitable acceptor. Some experiments that define the action pattern of the enzyme are described.

MATERIALS AND METHODS

Carbohydrates. Waxy-maize starch was prepared by the method of Schoch (1957) from hand-sorted single-cross Tapicorn seed kindly given by the Bear Hybrid Corn Co., Decatur, Ill., U.S.A. Waxy-maize β -dextrin was prepared from the starch by the action of sweet-potato

β -amylase (crystalline). Amylose was prepared from potato starch by the method of Hobson, Pirt, Whelan & Peat (1951). [^{14}C]Glucose and [^{14}C]maltose were purchased from The Radiochemical Centre, Amersham, Bucks. Maltotriose was prepared as described by Peat, Whelan & Kroll (1956). Maltotetraose, maltopentaose, maltohexaose and maltoheptaose were isolated by paper chromatography from the products of the reaction between potato amylose and crystalline α -amylase from *Bacillus subtilis*. Isomaltose and panose were prepared by the action of the transglucosylase of *Aspergillus oryzae* on maltose. All other carbohydrates were obtained from commercial sources and were purified by paper chromatography when necessary.

Enzymes. Hexokinase (crystalline) was purchased from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, W. Germany. Glucose oxidase (pure) and horseradish peroxidase were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Potato phosphorylase was prepared by the method of Baum & Gilbert (1953) as described by Whelan (1955). Human salivary α -amylase was isolated by the method of Fischer & Stein (1961). The purification was taken as far as the dialysis stage. Sweet-potato β -amylase (crystalline) was purchased from the Worthington Biochemical Corp., Freehold, N.J., U.S.A. Pullulanase was prepared from *Aerobacter aerogenes* as described by Bender & Wallenfels (1961).

Organisms. Strain S3 was obtained from Dr R. J. Gibbons, and strain 439 came from the Department of Bacteriology, University of Melbourne. Strains FW213, FW225, FW251 and RB1633 were from Dr G. Colman, Wright-Fleming Institute of Microbiology. The results of biochemical tests on all strains were in agreement with the characteristics listed by Murray (1957) for *S. mitis*.

Preparation of cell extract and cell-free filtrate. The organisms were grown in the medium described by Gibbons & Kapsimalis (1963), which contained 2% trypticase, 0.4% K_2HPO_4 , 0.1% KH_2PO_4 , 0.2% NaCl and 1% glucose. The inoculated medium was incubated anaerobically ($\text{N}_2 + \text{CO}_2$; 95:5) at 39° for 18 hr. The cells were centrifuged at 2° for 10 min. at 2000g, and the residue was washed twice with 0.067M-phosphate buffer, pH 7.1, then resuspended in the same buffer (25 ml. for each litre of broth). The cells were disrupted for 15 min. in a Raytheon model DF101 magnetostriiction oscillator. The residue was removed by centrifuging for 15 min. at 27000g, and the enzymes in the

supernatant solution were dialysed for 16 hr. against 0.01M-phosphate buffer, pH 6.4.

Chromatography on DEAE-cellulose. A portion (25 ml.) of the cell extract was applied to a column (1 cm. $\times$ 25 cm.) of DEAE-cellulose (Brown Co., Berlin, N.H., U.S.A.) previously washed with 0.01M-phosphate buffer, pH 6.4. The column was eluted with increasing concentrations of phosphate buffer, the gradient being linear between 0.01 and 0.40M. Seventy fractions (7 ml. each) were collected at a flow rate 50 ml./hr. The fractions containing peak activity of transglucosylase, which were eluted with 0.30–0.35M buffer, were pooled and used where possible without dialysis. The enzyme could be stored at -20° without loss in activity over 12 months.

Determination of transglucosylase activity. The digest (1.0 ml.) contained amylose (1 mg.), glucose (2.5 mg.) and enzyme (0.1 ml. in 0.3M-phosphate buffer, pH 6.4). A control digest was incubated without glucose. After 15 min. at 35° portions (0.1 ml.) from the digest and control were stained with iodine [0.1 ml. of a solution that contained iodine (2 g./l.) and KI (20 g./l.)] and diluted to 5 ml. The extinction at 650 m μ was read in 1 cm. cells in a Unicam spectrophotometer. The value obtained in the digest was subtracted from the control to which no acceptor had been added, and the result was a measure of the transferring ability of the enzyme. A linear relationship between enzyme concentration and decrease in iodine stain was obtained when the latter did not exceed 0.2.

Paper chromatography. Maltodextrins were separated on Whatman no. 3MM paper during irrigation with ethyl acetate-pyridine-water (10:4:3, by vol.) for 48 hr. The papers were dipped in AgNO_3 -NaOH (Trevelyan, Procter & Harrison, 1950). [^{14}C]Maltodextrins were located by radioautography. Carbohydrates eluted from paper chromatograms with water were determined by the primary cysteine- H_2SO_4 method of Dische, Shettles & Osnos (1949). Glucose was separated from sorbitol in butan-2-one-acetic acid-water saturated with boric acid (9:1:1, by vol.) as described by Rees & Reynolds (1958), and the dipping reagents contained pentaerythritol (Frahn & Mills, 1959).

Determination of carbohydrate storage in the cells. (a) Rhamnose/hexose ratios. Cells from 1 l. of medium were harvested as described above and washed five times with ice-cold buffer and resuspended in water (15 ml.). A portion (0.5 ml.) was diluted to 5 ml. with water, and portions

Table 1. *Carbohydrate storage in Streptococcus mitis strains*

Experimental details are described in the Materials and Methods section.

Strain	Source	Rhamnose/hexose ratio in cells grown in medium containing			Glucose content (%) of acid-hydrolysed cells. From medium containing		
		(a)	(b)	Ratio (b)/(a)	(a)	(b)	Ratio (a)/(b)
		1% glucose	0.1% glucose		1% glucose	0.1% glucose	
S3	Human oral plaque	0.13	1.97	15.2	37.1	2.6	14.3
FW213	Throat swab	0.17	2.50	14.7	28.6	2.3	12.4
FW225	Sputum	0.16	0.89	5.5	16.7	2.6	6.4
439	—	0.40	1.98	4.8	10.1	2.2	4.6
RB1633	Submandibular abscess	1.27	1.20	0.95	2.8	2.8	1.0
FW251	Urine	2.23	0.34	0.17	1.3	2.3	0.6

(0.1–0.5 ml.) were tested for hexose content, expressed as glucose, by the method of Dische *et al.* (1949), and for rhamnose content by the method of Dische & Shettles (1948) as modified by Gibbons (1955). Rhamnose/hexose ratios were calculated for cells that had grown on high (1%) and low (0.1%) concentrations of glucose. The value for the 0.1% cells divided by that for the 1% cells was a measure of polysaccharide storage by the cells.

(b) Enzymic method. The remainder of the cell suspension (14.5 ml.) obtained under (a) above was freeze-dried. Portions (8–10 mg.) were hydrolysed with 1.5 N-H₂SO₄ (1 ml.) for 2 hr. at 100°, neutralized with NaOH, and diluted to 10 ml. Portions (0.1–1.0 ml.) were incubated with glucose-oxidase reagent (Huggett & Nixon, 1957) as modified by Dahlquist (1961). The ratio of glucose in the 1% cells to that in the 0.1% cells was similar to that obtained from the rhamnose/hexose determinations under (a) (see Table 1).

RESULTS

S. mitis strain 439 was grown in media where glucose was replaced by maltose (1%) and starch (1%). Growth on starch was poor, being less than 10% of that on glucose, and the carbohydrate was not utilized. Maltose supported the growth of the bacteria and was utilized as extensively as glucose. Glucose and a series of maltodextrins were detected in the broth by paper chromatography. Oligosaccharides up to maltohexaose were present in similar amounts to maltose; spots corresponding to higher maltodextrins were slight. When the cell-free filtrates were incubated with amylose in standard activity digests there was virtually no change in iodine stain over 48 hr. This indicated the absence of amylase and transglucosylase in the medium.

Storage of polysaccharide in S. mitis strains. The cells were grown on nutrient agar containing 1% glucose or 1% maltose, and after 2 days the colonies were stained with iodine. Strains S3 and FW213 were strongly iodophilic, strains 439 and FW225 also stained well, but strains RB1633 and FW251 gave a very feeble stain. A more accurate estimation of polysaccharide storage by these organisms was obtained by determining the amount of glucose released on acid hydrolysis of the cells. When the cells were grown on 0.1% glucose, the percentage of glucose in the whole cells was 2.5% for each of the six strains. No glucose remained in the medium, and it was concluded that the organisms contained little or no reserve polysaccharide when grown under these conditions. After growth on 1% glucose the amount of glucose released on acid hydrolysis varied considerably between the strains. The glucose content of strains RB1633 and FW251 remained the same whether they were grown on media containing 0.1% or 1% glucose, indicating the inability of these cells to store polysaccharide. Strains 439 and FW225 yielded five and six times

as much glucose as the controls grown on 0.1% glucose. The ratio for strains S3 and FW213 was 14 and 12 respectively. These results are shown in Table 1, where there are also included data for the rhamnose/hexose ratios of the whole-cell suspensions. Comparisons of these ratios for the cells grown on media containing 1.0% and 0.1% glucose agree well with those obtained by the more specific enzymic method of glucose determination. Thus the measurement of rhamnose and hexose in the cell suspension is a rapid and accurate test for the storage of polysaccharide.

Transglucosylase activity of S. mitis extracts. Cell extracts were prepared from all strains, and the results of transglucosylase activity and nitrogen content are shown in Table 2. All the extracts showed transglucosylase activity, strains S3 and RB1633 being the most active, and strain FW251 having the lowest specific activity. With the exception of strain RB1633, which stored no polysaccharide but had high transglucosylase activity, there was a relation between transglucosylase activity and ability to store polysaccharide. Strain RB1633, unlike the other five strains, was devoid of α -(1→6)-glucosidase (G. J. Walker, unpublished work).

The transglucosylase activity of strain FW213 was determined after growth for 1 day and 2 days. The activity per milligram of nitrogen fell from 13.8 at 24 hr. to 8.7 at 48 hr.

In a further experiment strain S3 was grown for 24 hr. in media containing 1.0% and 0.1% glucose. The cell count per ml. of the washed suspensions was 12.5×10^9 and 12.0×10^9 respectively. The cells from the 1% glucose broth were twice the size of those grown on 0.1% glucose, and the turbidity ($E_{570\text{ m}\mu}$) of the former was over seven times that of the cells grown on 0.1% glucose. The results for transglucosylase activity of the cell extracts (Table 3) indicated that this enzyme was present

Table 2. *Transglucosylase activity of Streptococcus mitis*

Activities per 0.1 ml. of cell extract are shown. The nitrogen content of the dialysed extracts was determined by the micro-Kjeldahl method.

Strain	Trans-glucosylase activity Fall in I ₂ stain	N (mg./ml. of extract)	Trans-glucosylase activity/ mg. of N
S3	1.0	0.659	15.2
FW213	0.63	0.465	13.6
FW225	0.32	0.279	11.5
439	0.55	0.487	11.3
RB1633	0.85	0.460	18.5
FW251	0.074	0.169	4.4

Table 3. *Transglucosylase activity of extracts of strain S3 grown in media containing 1% and 0.1% glucose*

The cell extracts (0.02 ml.) were incubated in the standard activity digests.

Time (hr.)	Fall in iodine stain	
	Glucose in medium	
	(0.1%)	(1.0%)
0.25	0.100	0.097
0.50	0.158	0.168
1.50	0.271	0.275

Table 4. *Relative transglucosylase activity in cells grown on glucose (1%) and on maltose (1%)*

The dialysed cell extracts (0.02 ml.) were added to standard transglucosylase-activity digests.

Medium..... Growth time (days)... Time (hr.)	Fall in iodine stain		
	Glucose	Maltose	
		1	2
0.25	0.072	0.108	0.066
0.50	0.131	0.147	0.095
1.50	0.291	0.265	0.251

in equal amount irrespective of the polysaccharide content of the cells.

The amylomaltase of *Escherichia coli* is produced only when the organism is grown on a medium containing maltose (Monod & Torriani, 1950). Accordingly the transglucosylase activity of *S. mitis* S3 cell extracts was compared for cells that had grown on glucose (1%) and on maltose (1%) for 1 and 2 days. The results (Table 4) showed an enzymic activity in the three extracts. The amount of reserve carbohydrate in the 1-day cells was also similar, being 27% and 24% of the dry matter for cells grown on glucose and maltose respectively.

Strain RB1633 was also grown on maltose (1%), and examined for transglucosylase activity of the cell extract and for carbohydrate storage by the cells. The transglucosylase activity (0.78/0.1 ml. of cell extract) was similar to the value 0.85 obtained for cells grown on glucose (1%). The rhamnose/hexose ratio of the whole cells was 1.4 compared with 1.3 for those grown on glucose (Table 1). Thus growth on maltose has not enabled this strain to store polysaccharide.

Effect of various conditions on transglucosylase activity. The fractions from the DEAE-cellulose chromatography showing maximum activity were pooled and used in the experiments which follow. The enzyme had no hydrolytic activity towards

Fig. 1. Effect of pH on transglucosylase activity. The activity digests were incubated at 35° for 0.5 hr. (○) and 2 hr. (●).

Fig. 2. Effect of temperature on transglucosylase activity. The digests were incubated at pH 6.4 for 0.5 hr. (●) and 1 hr. (○).

maltose, maltodextrins or amylose. The dialysed enzyme was incubated with amylose and glucose at various pH values. The pH optimum was 7.5 (Fig. 1). The effect of temperature on the reaction was studied between 20° and 50°. The optimum temperature for a 30 min. reaction was 46° (Fig. 2). A temperature of 35° was chosen for reactions with transglucosylase.

Activities of various carbohydrates as acceptors. The amount of glucose in the activity digest was varied from 0.6 to 6 mg., and the accepting ability of methyl α -D-glucoside, isomaltose and panose was compared with that of glucose (Fig. 3). Maximum

Fig. 3. Determination of the accepting efficiency of glucose (○), methyl α -D-glucoside (●), isomaltose (△) and panose (▲). The transglucosylase-activity digests (1 ml.), containing various weights of the sugars, were incubated for 1 hr. at 35°.

accepting activity was achieved with 2.5 mg. of glucose, and methyl α -D-glucoside, isomaltose and panose were 30, 9 and 7% as efficient as glucose. The accepting efficiency of maltose was tested under conditions unsuitable for the disproportionation of maltose (see below). The activity digests contained transglucosylase from strain RB1633 (0.1 ml.) and glucose or maltose (2.5 mg.). The fall in iodine stain with maltose as acceptor was 50% of that in the glucose digest. The dextrans produced in the reaction with methyl α -D-glucoside as acceptor did not react with the benzylamine reagent of Bayly & Bourne (1953) and no glucose was produced during the transferring reaction. The fall in iodine stain of amylose was entirely due to the transfer of glucose residues to methyl α -D-glucoside, showing that the reducing group of the acceptor need not be free.

The products of the reaction with amylose and isomaltose and panose were compared with those obtained in similar experiments with the transglucosylase of *S. bovis* (Walker, 1965). The tetrasaccharide and pentasaccharide produced with panose as acceptor had exactly the same R_F values as those prepared with the *S. bovis* enzyme. Further proof of the identity of the dextrans was given by the result of pullulanase action on the tetrasaccharide. Maltose was the only product, thus indicating that glucosyl residues could be transferred only to the non-reducing end of panose to give a 6²- α -maltozymaltose. The trisaccharide and tetrasaccharide formed by transferring glucosyl

residues to isomaltose were found to have identical R_F values to isopanose and 6- α -maltotriosylglucose respectively. The tetrasaccharide was hydrolysed by β -amylase to give maltose and isomaltose. Isomaltose and panose could not act as donors in the reaction with transglucosylase.

Transfer of glucose residues from amylose to [¹⁴C]glucose. A digest containing amylose (25 mg.), [¹⁴C]glucose (25 mg., 0.025 mc) and transglucosylase (1 ml.) in 6.25 ml. was incubated under toluene at 35°. When the iodine stain of the amylose had fallen to 2% of the original (22 hr.), the digest was heated to inactivate the enzyme, deionized with Bio-Deminrolit (Permutit Co., London, W.4), and concentrated to dryness. The maltodextrins were separated by chromatography on paper, and eluted with water. The position of the label in maltose and maltotriose was determined as follows. The oligosaccharides (350 μ g.) were reduced with sodium borohydride (7 mg.) in 0.3 ml. for 4 hr. at room temperature. The solution was then made 0.5N with respect to sulphuric acid, and the maltitol and maltotriitol were hydrolysed at 100° for 2.5 and 4 hr. respectively. After deionization and removal of boric acid as methyl borate, glucose and sorbitol were separated by paper chromatography and eluted with water. The radioactivity was measured in an Ekco liquid-scintillation counter N644A used with an N530G scaler (Ekco Electronics Ltd., Southend-on-Sea, Essex). In both cases 98% of the total radioactivity appeared in sorbitol, showing that maltose and maltotriose were labelled exclusively at the reducing glucose residue.

Action of transglucosylase on maltose and maltotriose. The enzyme (0.4 ml.) was incubated with maltose and maltotriose (5 mg.) in 0.8 ml. for 24 hr. Examination of the products by paper chromatography revealed that maltose had been disproportionated mainly to glucose, maltotriose, maltotetraose, maltopentaose and maltohexaose, and there was a trace of maltoheptaose. The products from maltotriose were glucose, maltose and higher dextrans up to malto-octaose. There were also smaller amounts of products with very low R_F values.

The relative rate of action of *S. mitis* transglucosylase on maltose and maltotriose was followed by measuring the release of glucose with glucose oxidase. The results (Fig. 4) suggested that the enzyme had a more rapid action on maltotriose. The initial rate of glucose release was faster, and equilibrium was achieved more rapidly. When the enzyme acted on maltose each transfer must result in the release of glucose, whereas with maltotriose glucose was liberated only when two glucosyl units were transferred. Evidence from paper chromatography indicated that the transglucosylase could

Fig. 4. Action of transglucosylase on maltose (●), maltotriose (○) and on both maltose and maltotriose (Δ). The digests (1 ml.) contained purified transglucosylase (0.25 ml.) from strain RB1633, and the substrate concentration was 0.01 M. Portions (0.1 ml.) were withdrawn for the determination of glucose with glucose oxidase. The addition of more enzyme at 24 hr. produced no further release of glucose.

transfer glucosyl and maltosyl residues with equal ease since glucose, maltose, maltotetraose and maltopentaose appeared in approximately equal amounts among the early reaction products.

Further evidence of the lower affinity of the enzyme for maltose was obtained when lower concentrations of enzyme were incubated with maltose and maltotriose. No glucose could be detected in the maltose digest after 2 hr. of incubation with the lowest concentration of enzyme whereas a linear release of glucose occurred in a similar digest containing maltotriose (Fig. 5). After 4 hr. glucose was released in the maltose digest at an ever-increasing rate, showing that the initial lag period was not due to poisoning of the enzyme. The effect of adding small amounts of maltotriose to the maltose digests was tested (Fig. 6). The smallest concentration of added maltotriose (1 mM) decreased the lag period to a considerable extent. After 24 hr. incubation the maltose digest contained 335 μg. of glucose; the addition of 1 mM-maltotriose raised this figure to 614 μg., although glucose

Fig. 5. Effect of enzyme concentration on the disproportionation of maltose by transglucosylase. The digests (1 ml.) contained maltose (10 mM) and 0.1 ml. (○), 0.2 ml. (▲) or 0.3 ml. (□) of purified transglucosylase from strain RB1633. A linear release of glucose occurred when the enzyme (0.1 ml.) was incubated with 0.01 M-maltotriose (●).

Fig. 6. Action of transglucosylase on maltose in the presence of maltotriose. Various concentrations of maltotriose, 10 mM (□), 5 mM (●), 2 mM (○), 1 mM (▲) and none (Δ), were present in digests (1 ml.) containing maltose (10 mM) and transglucosylase (0.1 ml. of strain RB1633). Controls containing the above quantities of maltotriose, but no maltose, were treated similarly. The values for glucose released in the controls were subtracted from those in the digests to give the results shown for glucose released from the maltose component of the digests.

released from 1 mM-maltotriose alone was only 51 μg. Thus it appeared that maltotriose had a significant activating effect on the reaction with maltose. Higher concentrations of maltotriose (10 mM) were required before the lag period was entirely removed, and under these conditions glucose release from maltose became almost linear with time, and was 80% of the rate of glucose release from maltotriose.

In the presence of higher concentrations of

Table 5. *Relative action of transglucosylase on maltose and maltotriose*

The digests (1ml.) contained maltose or maltotriose (10mM), purified transglucosylase from strain RB1633 (0.05 or 0.1ml.), and glucose-oxidase reagent [0.5ml. containing glucose oxidase (0.17mg.), horseradish peroxidase (17μg.) and 1% *o*-dianisidine (0.017ml.) dissolved in 0.5M-tris buffer, pH7.0]. The extinction of the digests was read at intervals during the incubation at 35°.

Time (min.)	Vol. of enzyme (ml.).....	Glucose released (μg.)			
		From maltose		From maltotriose	
		0.05	0.10	0.05	0.10
15		0.29	0.98	2.33	5.51
30		—	—	4.80	13.00
60		0.39	1.55	12.30	30.80
90		0.88	1.85	22.30	48.60
120		0.94	1.85	34.90	—

enzyme, the amount of glucose released in digests containing both maltose and maltotriose was close to the sum of glucose released when maltose and maltotriose were incubated separately with transglucosylase (Fig. 4).

The effect of including the glucose-oxidase reagent in the 1ml. digests containing maltose or maltotriose was investigated. It was expected that the removal of glucose might activate the reaction of the low concentration of transglucosylase with maltose. Oxidation of the chromogen occurred in the maltotriose digest, but there was virtually no reaction with maltose during 2hr. (Table 5).

In these experiments the substrate was not in excess. Increasing the concentration of maltose up to 0.075M had no effect on the lag period obtained when the concentration of transglucosylase was low. The results obtained when higher concentrations of transglucosylase were incubated with various substrate concentrations are shown in Fig. 7(a), and Lineweaver-Burk plots of $1/v$ vs. $1/S$ are given in Fig. 7(b). The K_m values for maltose and maltotriose were approximately 0.1 and 0.05M respectively.

Action of transglucosylase on [14 C]maltose. Transglucosylase (3.5ml.) was incubated with [14 C]-maltose (24.3mg., 0.024mc) in 7ml. After 24hr. at 35° more enzyme (1ml.) was added, and after incubation for a further 24hr. a test with glucose oxidase indicated that the equilibrium concentration of glucose had been reached. The products of the reaction were separated by paper chromatography and eluted with water. The radioactivity and yield of the maltodextrins are shown in Table 6. The molar proportion of maltose to glucose at equilibrium was calculated to be 0.59 (by wt.) and 0.57 (by counts). This is similar to the value of 0.6 reported by Monod & Torriani (1950) and 0.52 reported by Wiesmeyer & Cohn (1960) for the amylomaltase of *Escherichia coli*.

Fig. 7. (a) Effect of substrate concentration on the action of transglucosylase on maltose (●) and on maltotriose (○). The digests (1.5ml.) contained purified enzyme (0.1ml.) from strain 439. After 30 min. at 35° portions (0.3ml.) were boiled and incubated with glucose oxidase for 1hr. (b) Lineweaver-Burk plots of the results shown in (a).

Synthesis of iodine-staining polysaccharides. Although the action of transglucosylase on maltose and maltotriose allowed the synthesis of oligosaccharides of D.P. no greater than 6 and 8 respectively, incubation of the enzyme with maltotetraose and maltodextrins of higher chain length gave products that stained with iodine (Fig. 8).

Fig. 8. Synthesis of iodine-staining polysaccharides from maltotriose ($\square$), maltotetraose ($\circ$), maltopentaose ($\triangle$), maltohexaose ($\blacktriangle$) and maltoheptaose ($\diamond$). The digests contained substrate (0.01M) and *S. mitis* strain 439 transglucosylase (0.5 ml.). The effect of removing glucose was studied in a maltose digest ($\bullet$) and a maltotriose digest ($\blacksquare$) as above that also contained ATP (3 mg.), magnesium chloride (10 mM) and hexokinase (0.05 ml. of a 1:10 dilution). The iodine stain of portions (0.05 ml.) of the digests in 0.4 ml. was read in 1 cm. micro-cells.

Fig. 9. Relative action of *S. mitis* transglucosylase on amylose ($\circ$), amylopectin ($\triangle$) and amylopectin β -dextrin ($\square$) in the presence of glucose. Controls without glucose have filled-in symbols.

When the glucose released during the action on maltose and maltotriose was converted into glucose 6-phosphate, iodine-staining polysaccharide was produced. At 50 hr. (Fig. 8) $\lambda_{\max}$ was 520 and 550 $m\mu$ respectively; thus chains of average D.P. 22 and

Table 6. *Equilibrium concentration of the products of transglucosylase action on [14 C]maltose*

Experimental conditions are given in the text.

Product	Wt. (mg.)	Radioactivity (counts/100 sec.)
Glucose	4.62	20 622
Maltose	5.16	22 260
Maltotriose	3.52	15 923
Maltotetraose	2.32	9 560
Maltopentaose	1.49	6 809
Maltohexaose + higher dextrans	2.58	11 216

35 had been synthesized (compare Bailey & Whelan, 1961).

Relative action of transglucosylase on amylose, β -dextrin and amylopectin. Digests in which amylopectin and β -dextrin replaced amylose as donor were incubated with transglucosylase. The results (Fig. 9) showed that the enzyme could react with the branched substrates, although at a slower rate than with amylose. This suggested that the enzyme was either contaminated with a debranching enzyme, or was capable of transferring branched fragments. The breaking of the α -(1 $\rightarrow$ 6)-glucosidic linkages could not be attributed to α -(1 $\rightarrow$ 6)-glucosidase since the same results were obtained with strain S3 as with strain RB1633. The latter strain contained no α -(1 $\rightarrow$ 6)-glucosidase.

Action pattern of transglucosylase on synthetic amylose. The transglucosylase has been shown to transfer one or more glucosyl residues from the non-reducing end of the donor molecule. When the enzyme reacts with a polysaccharide there is a possibility of more than one attack on a single chain before dissociation of the complex. If such multiple attacks occurred to a marked extent, the degradation of a single chain would be largely completed before the enzyme proceeded to the next chain. The alternative mechanism would be a multi-chain action in which glucosyl units were transferred in random fashion from all chains. A decision between these two possibilities was made for potato phosphorylase (Whelan & Bailey, 1954) and for β -amylase (Bailey & Whelan, 1957) by using as substrates synthetic amyloses of known average chain length. A large increase in the iodine-staining capacity of amylose occurs as the chain length increases from 18 to 70, and this is accompanied by a linear increase in $\lambda_{\max}$ from 490 $m\mu$ to 605 $m\mu$. Bailey & Whelan (1961) have described the exact relationship between the iodine stain and chain length of synthetic amyloses. On degradation of synthetic amylose having a chain length less than 70 by an exclusively single-chain mechanism there would be no change in $\lambda_{\max}$ of the iodine-stained

products; a multi-chain attack would result in a fall in average chain length and a concomitant fall in $\lambda_{\max}$.

In the present work a synthetic amylose of D.P. 40 was prepared by incubating potato phosphorylase (10ml., 2 units) with maltohexaose (6.7mg.), 8.3% ammonium molybdate (0.5ml.), 0.1M-glucose 1-phosphate (3.5ml.) and 0.05M-citrate buffer (11ml.) at 35°. The reaction was

stopped after 2½ hr. when determinations of inorganic phosphate and $\lambda_{\max}$ of the iodine-stained polymer indicated a chain length 40, and the amylose was dialysed overnight. The polysaccharide was then incubated with transglucosylase and glucose, and the progress of the reaction was measured by iodine-staining, the absorption curves being plotted in the region 500–660m μ . At the same time the reaction of three other enzymes on

Fig. 10. Degradation of synthetic amylose (D.P. 40). Light-absorption curves of iodine-stained products obtained during the reactions. The digests (2ml.) contained amylose (2mg.) and (a) purified transglucosylase of *S. mitis* strain 439 (0.2ml.) and glucose (5mg.); (b) β -amylase (0.5 unit), tris-maleate buffer, pH 5.2 (10mm), serum albumin (0.05%) and glutathione (0.5mm); (c) potato phosphorylase (0.1 unit), 0.5M-phosphate buffer, pH 6 (0.15ml.); (d) salivary α -amylase (0.2ml. of a 1:1000 dilution) and 0.2M-phosphate buffer, pH 6.9 (0.2ml.). All incubations were at 35°.

the same substrate was studied. The slope of the straight line joining the peaks of the curves was an indication of the action pattern of the enzyme (Bailey & Whelan, 1957). The values of the slopes (Fig. 10) obtained with phosphorylase, β -amylase and transglucosylase were 40, 25 and 10 respectively. Salivary α -amylase, an enzyme that fragments amylose by random attack, gave a slope of 60. Since a single-chain type of degradation

would give zero slope, the results indicated a greater proportion of single-chain attack for transglucosylase than for β -amylase. Bailey & French (1957) calculated that β -amylase makes on the average four enzymic attacks per encounter with a polymer.

Attempts were made to provide conditions whereby the transglucosylase might show a higher proportion of multi-chain action. In one experiment, the concentration of glucose was decimated, thus giving more opportunity for the enzyme-substrate complex to dissociate before a further transfer to the glucose acceptor could occur. In another experiment, the reaction was carried out at 2°, since Bailey & Whelan (1957) found that the proportion of multi-chain action of β -amylase was increased at temperatures removed from the optimum. In neither case was there an increase in the value of the slope.

Action of transglucosylase on terminally labelled 47-unit amylose. [¹⁴C]Maltohexaose (0.5 mg.), prepared by the action of transglucosylase on [¹⁴C]-maltose (see above), was incubated with 0.1 M-glucose 1-phosphate (0.3 ml.), potato phosphorylase (0.9 ml.), 0.2 M-citrate buffer, pH 6.0 (0.3 ml.) and 8.3% ammonium molybdate (0.05 ml.) in 2.5 ml. After 2 hr. at 35° λ_{max} of the iodine-stained product was 570 m μ , corresponding to D.P. 47 (Bailey & Whelan, 1961). The digest was heated at 100° for 2 min. to inactivate the enzyme, and the mixture was dialysed overnight at room temperature. The synthetic amylose (1.25 mg.) was incubated with glucose (3.75 mg.) and transglucosylase (0.15 ml.) in 3.3 ml., and at intervals portions (0.05 ml.) were stained with iodine (0.05 ml.) in 1 ml., and other portions (0.5 ml.) were boiled, deionized, concentrated and applied to paper chromatograms. The dextrins were located by radioautography, eluted with water, and their radioactivity was determined in the liquid-scintillation counter. The results (Fig. 11) show that release of labelled maltodextrins occurred from the beginning of the reaction. At

Fig. 11. Radioactivity of maltodextrins released during the degradation of terminally labelled synthetic amylose by transglucosylase. At 20 hr. the incorporation of radioactivity into maltose (●), maltotriose (Δ), maltotetraose (○) and maltopentaose (□) was 8255, 7290, 4158 and 3493 counts/100sec. respectively.

Table 7. *Degradation of synthetic amylose with transglucosylase*

Terminally labelled synthetic amylose was incubated with transglycosylase and glucose as described in the text. The maltodextrins were separated by paper chromatography and determined by the method of Dische *et al.* (1949).

Time (hr.)	Iodine stain	λ_{max} (m μ)	Yield of maltodextrins (μ g.)		
			Maltose	Maltotriose	Maltotetraose
0	0.92	570	—	—	—
0.83	0.74	570	—	—	—
1.7	0.56	570	—	—	—
2.75	0.39	—	14.8	10.6	2.5
3.75	0.29	565	24.6	15.8	7.1
8	—	—	46.0	28.5	11.4
20	0	—	119.4	54.1	22.0

2½ hr. when the iodine stain was down to 50% of the original (Table 7), the label in the dextrans was 25% of the total amount released at 20 hr. Single-chain attack would have released more labelled maltodextrans, and multi-chain attack would have released far less at this stage. The results therefore indicated that the action pattern of transglucosylase was intermediate between single- and multi-chain types of degradation.

DISCUSSION

The mechanism of action of *S. mitis* transglucosylase was similar to that of D-enzyme (Walker & Whelan, 1957), amyloamylase (Wiesmeyer & Cohn, 1960) and *S. bovis* transglucosylase (Walker, 1965) in one respect. All four enzymes transferred glucosyl residues from the non-reducing end of the donor to the non-reducing end of the acceptor. The ability of *S. mitis* transglucosylase to transfer glucosyl residues to methyl α -D-glucoside showed that the reducing end of the acceptor was not required to be free. The maltodextrans produced in the reaction between amylose and [14 C]glucose were labelled only at the reducing end.

Both *S. mitis* transglucosylase and amyloamylase were able to transfer glucosyl residues from maltose, whereas maltotriose is the smallest donor substrate for D-enzyme and *S. bovis* transglucosylase. Nevertheless *S. mitis* transglucosylase, unlike amyloamylase, could disproportionate the linkages of maltotriose more rapidly than maltose. Wiesmeyer (1962) reported that maltose alone could function as glucosyl donor in the forward reaction with amyloamylase, although maltosaccharides could serve in the back reaction. Another difference between *S. mitis* transglucosylase and amyloamylase is that the latter is an inducible enzyme. No amyloamylase is produced when *E. coli* cells are grown on glucose. The transglucosylase of *S. mitis* on the other hand must be considered a constitutive enzyme. It was present in all the strains investigated, and its activity was of the same order whether the cells were grown on glucose or on maltose. Neither was the presence of the enzyme dependent on the storage of reserve polysaccharide by the cell, for the two strains that did not synthesize polysaccharide possessed transglucosylase activity, and the strains that were highly active in synthesizing polysaccharide also showed activity when they were grown under unsuitable conditions for the storage of polysaccharide.

No explanation has been found for the lag period that occurred when low concentrations of transglucosylase were incubated with maltose. It should be noted that the lag period was largely removed when the enzyme concentration was increased threefold (Fig. 5); an increase in substrate concen-

tration produced no such effect. It was established that maltotriose activated the reaction with maltose to an extent that greatly exceeded the additive effect observed when higher concentrations of transglucosylase acted on an equimolar mixture of maltose and maltotriose.

The use of synthetic amyloses as substrates indicated that the action pattern of transglucosylase was largely single-chain. It could not be decided whether the enzyme preferentially transferred one, two or more glucosyl residues from the donor molecule. The transglucosylase of rabbit muscle prefers to move a chain of three maltosidically linked glucose residues from the donor to the acceptor molecule, and maltohexaose is the smallest linear donor for the reaction (Brown & Illingworth, 1962). The fact that the reaction of *S. mitis* transglucosylase with maltose was slower than with maltotriose did not imply that the enzyme would transfer single glucosyl residues with difficulty from substrates other than maltose. Indeed, both glucosyl and maltosyl residues were transferred during the disproportionation of maltotriose. That transfer of single glucosyl residues from polysaccharides occurred to a significant extent was indicated by maltose being the oligosaccharide produced in highest yield during all stages of the degradation of synthetic amylose (Table 7).

The ability of the transglucosylase to transfer glucosyl residues from amylose to isomaltose and panose has provided a rapid method for the preparation of the 6- α -maltodextrinyl glucose and 6 β - α -maltodextrinyl maltose series of sugars. These branched dextrans make useful substrates for determining the specificity of enzymes that hydrolyse α -(1 $\rightarrow$ 6)-glucosidic linkages (cf. Abdullah *et al.* 1966). The enzyme in this case has the same specificity as the transglucosylase of *S. bovis* (Walker, 1965); these enzymes make transfers only to the non-reducing end of isomaltose and panose. This is in contrast with the transglucosylase of *B. macerans*, which will couple to the middle and non-reducing end of panose (Summer & French, 1956). Glycogen synthetase from rabbit skeletal muscle will not transfer glucosyl residues from UDP-glucose to the side chains of branched dextrans whether the length of the chains is one, two or three glucose units (Brown, Illingworth & Kornfeld, 1965).

The transglucosylase activity in *S. mitis* cell extracts was extremely high compared with the activities of the other enzymes that reacted with amylose. When cell extracts were incubated with amylose as controls for transglucosylase-activity digests, the fall in iodine stain was less than 5% of that which occurred when glucose was present. This action was due to phosphorylase and a branching enzyme (G. J. Walker, unpublished work).

Although the major enzyme involved in the degradation of α -(1 $\rightarrow$ 4)-glucosidic linkages in the storage polysaccharide of *S. mitis* is likely to be phosphorylase yet the transglucosylase could supplement this action in the vicinity of branch linkages by transferring glucosyl residues that are inaccessible to phosphorylase. Such transferases have been found in muscle (Walker & Whelan, 1960; Brown & Illingworth, 1962) and have been implicated in glycogen degradation because of their ability to expose glucose stubs that are then susceptible to the action of amylo-(1 $\rightarrow$ 6)-glucosidase (Abdullah & Whelan, 1963; Brown, Illingworth & Cori, 1963).

The transglucosylase was able to synthesize maltodextrins of sufficiently high chain length to stain with iodine from maltotetraose, and also from maltose when glucose was removed, but the role of this enzyme in the synthesis of reserve polysaccharide cannot be defined at present. Its function could be to provide suitable primers for the action of branching enzyme or glycogen synthetase. The latter enzyme has been demonstrated in several species of bacteria (Greenberg & Preiss, 1964).

This work was supported by a grant from the National Health and Medical Research Council of Australia. The assistance of Mr P. Power in part of the work is gratefully acknowledged.

REFERENCES

- Abdullah, M., Catley, B. J., Lee, E. Y. C., Robyt, J., Wallenfels, K. & Whelan, W. J. (1966). *Cereal Chem.* **43**, 111.
- Abdullah, M. & Whelan, W. J. (1963). *Nature, Lond.*, **197**, 979.
- Bailey, J. M. & French, D. (1957). *J. biol. Chem.* **226**, 1.
- Bailey, J. M. & Whelan, W. J. (1957). *Biochem. J.* **67**, 540.
- Bailey, J. M. & Whelan, W. J. (1961). *J. biol. Chem.* **236**, 969.
- Baum, H. & Gilbert, G. A. (1953). *Nature, Lond.*, **171**, 983.
- Bayly, R. J. & Bourne, E. J. (1953). *Nature, Lond.*, **171**, 385.
- Bender, H. S. & Wallenfels, K. (1961). *Biochem. Z.* **334**, 79.
- Brown, D. H. & Illingworth, B. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1783.
- Brown, D. H., Illingworth, B. & Cori, C. F. (1963). *Nature, Lond.*, **197**, 980.
- Brown, D. H., Illingworth, B. & Kornfeld, R. (1965). *Biochemistry*, **4**, 486.
- Dahlquist, A. (1961). *Biochem. J.* **80**, 547.
- Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.
- Dische, Z., Shettles, L. B. & Osnos, M. (1949). *Arch. Biochem.* **22**, 169.
- Fischer, E. H. & Stein, E. A. (1961). *Biochem. Prep.* **8**, 27.
- Frahn, J. L. & Mills, J. A. (1959). *Aust. J. Chem.* **12**, 65.
- Gibbons, M. N. (1955). *Analyst*, **80**, 268.
- Gibbons, R. J. (1964a). *J. Bact.* **87**, 1512.
- Gibbons, R. J. (1964b). *J. dent. Res.* **43**, 1021.
- Gibbons, R. J. & Kapsimalis, B. (1963). *Arch. oral Biol.* **8**, 319.
- Gibbons, R. J. & Socransky, S. S. (1962). *Arch. oral Biol.* **7**, 73.
- Gibbons, R. J., Socransky, S. S., de Araujo, W. C. & van Houte, J. (1964). *Arch. oral Biol.* **9**, 365.
- Greenberg, E. & Preiss, J. (1964). *J. biol. Chem.* **239**, rc4314.
- Hobson, P. N., Pirt, S. J., Whelan, W. J. & Peat, S. (1951). *J. chem. Soc.* p. 801.
- Howell, A., Rizzo, A. & Paul, F. (1965). *Arch. oral Biol.* **10**, 307.
- Huggett, A. St G. & Nixon, D. A. (1957). *Lancet*, **273**, 368.
- Krasse, B. (1954). *Odont. Revy*, **5**, 203.
- Monod, J. & Torriani, A. (1950). *Ann. Inst. Pasteur*, **78**, 65.
- Murray, E. G. D. (1957). In *Bergey's Manual of Determinative Bacteriology*, 7th ed., p. 519. Baltimore: Williams and Wilkins Co.
- Peat, S., Whelan, W. J. & Kroll, G. W. F. (1956). *J. chem. Soc.* p. 53.
- Rees, W. R. & Reynolds, T. R. (1958). *Nature, Lond.*, **181**, 767.
- Schoch, T. (1957). In *Methods in Enzymology*, vol. 3, p. 5. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Summer, R. & French, D. (1956). *J. biol. Chem.* **222**, 469.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- Walker, G. J. (1965). *Biochem. J.* **94**, 299.
- Walker, G. J. & Whelan, W. J. (1957). *Biochem. J.* **67**, 548.
- Walker, G. J. & Whelan, W. J. (1960). *Biochem. J.* **76**, 264.
- Weiss, S., King, W. J., Kestenbaum, R. C. & Donohue, J. J. (1965). *Ann. N.Y. Acad. Sci.* **131**, 839.
- Whelan, W. J. (1955). In *Methods in Enzymology*, vol. 1, p. 194. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Whelan, W. J. & Bailey, J. M. (1954). *Biochem. J.* **58**, 560.
- Wiesmeyer, H. (1962). In *Methods in Enzymology*, vol. 5, p. 141. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Wiesmeyer, H. & Cohn, M. (1960). *Biochim. biophys. Acta*, **39**, 427.