700. *Immunopolysaccharides. Part VII.*[1] *The Transglucosylase Action of* Betacoccus arabinosaceous *Dextransucrase.*

By R. W. Bailey, S. A. Barker, E. J. Bourne, and M. Stacey.

Dextransucrase isolated from maltose–sucrose cultures of *B. arabinosaceous* contained only small amounts of dextran and the slight activity it exhibited with sucrose alone was increased almost fivefold by the addition of maltose. The isolation and characterisation of various oligosaccharides from sucrose–maltose and sucrose–methyl α-D-glucoside cultures is described.

It was envisaged (Bailey, Barker, Bourne, and Stacey[1]) that growth of *Betacoccus arabinosaceous* on a medium containing maltose as well as sucrose would provide a source much depleted of dextran and might render possible the isolation of dextran-free dextransucrase. Although dextransucrase isolated under these conditions could not be completely freed from dextran, such preparations contained only one-tenth of the dextran present in the dextransucrase isolated from cultures containing sucrose alone.[1] The enzyme was isolated by fractionation with ethanol and dialysed to remove contaminating oligosaccharides and residual alcohol since the latter, if retained, would render the enzyme inactive (Table 2).

This dextransucrase exhibited several marked differences in properties from the heavily dextran-complexed dextransucrase.[1] It was much more unstable to heat; more than half its activity was lost after 3 days at 25° (Table 3). Its optimum temperature and pH, *ca.* 25° and 5·0 respectively, differed little however from those of dextransucrase isolated from sucrose cultures.

The major difference was that a dextransucrase with low dextran content exhibited only slight activity when incubated with sucrose alone. The addition of maltose, however, increased the rate of fructose production by values of up to 300—400%. In the presence of other sugars, particularly isomaltose, methyl α-D-glucoside, and D-glucose, the rates of fructose production were proportionately higher than those with a dextransucrase of high dextran content.[1] The inhibition of the reaction by fructose was similarly enhanced (Table 7). The synthesis of additional oligosaccharides was observed when the added sugar was maltose, isomaltose, methyl α-D-glucoside, D-glucose, cellobiose, or lactose.

[1] Part VI, Bailey, Barker, Bourne, and Stacey, preceding paper.

These oligosaccharides were more readily available by actual growth of the organism in the presence of these added sugars. Fractionation of a maltose–sucrose culture after growth for 48 hr. at 25° gave crystalline panose having the correct physical constants, and the homologous tetra- and penta-saccharides produced by the transfer of one and two glucose units respectively in α-1 : 6-linkage to the non-reducing end of panose. These structures of the tetra- and penta-saccharide were suggested by examination of the products of complete and partial hydrolysis. Periodate oxidation of the alcohols of the tetra- and penta-saccharide confirmed these assignments. The tetrasaccharide alcohol consumed 9·02 mols. of periodate and produced 3·83 mols. of formic acid and 1·91 mols. of formaldehyde per mol. of alcohol. The theoretical figures are 9, 4, and 2 respectively. The pentasaccharide alcohol consumed 11·2 mols. of periodate and produced 4·75 mols. of formic acid and 1·85 mols. of formaldehyde per mol. of alcohol. The theoretical figures are 11, 5, and 2 respectively.

Fractionation of a methyl α-D-glucoside–sucrose culture after growth at 25° for 5 days gave chromatographically pure fractions of a suspected methyl α-bioside and a methyl α-trioside. Both were non-reducing, showed the correct methoxyl contents, and on complete hydrolysis gave glucose as the only sugar. Paper-chromatographic examination of their partial hydrolysates showed isomaltose among the products from the methyl α-bioside, and isomaltotriose among those from the methyl α-trioside. This suggested that the compounds were methyl α-isomaltoside and methyl α-isomaltotrioside, respectively. These structures were confirmed by periodate oxidation. The methyl α-bioside consumed 3·94 mols. of periodate and produced 2·1 mols. of formic acid per mol. of glycoside. The theoretical figures are 4 and 2 respectively. The methyl α-trioside consumed 5·96 mols. of periodate and produced 2·64 mols. of formic acid per mol. of glycoside. The theoretical figures are 6 and 3 respectively. Similar glycosides have been recently isolated [2] by the action of *Leuconostoc mesenteroides* dextransucrase on sucrose–methyl α-glucoside mixtures.

The synthesis of such oligosaccharides confirmed the view that the principal, but probably not the only (cf. ref. 1), function of the dextransucrase was the transfer of glucose residues in α-1 : 6-linkage to the non-reducing end-group of the sugar acceptor. This view was also supported by the detection of isomaltose, isomaltotriose, etc., in a sucrose–glucose culture.

Experimental

Standard Method of Isolation of Dextransucrase with Low Dextran Content.—An aqueous medium (140 c.c.) containing yeast extract (1%), $Na_2NH_4PO_4$ (0·5%), KH_2PO_4 (0·1%), $MgSO_4$, $7H_2O$ (0·05%), sucrose (2%), and maltose (10%) was adjusted with sodium hydroxide to pH 7·0, steam-sterilised at 15 lb./sq. in. for 30 min., and readjusted to pH 7·0 under sterile conditions. After inoculation with *Betacoccus arabinosaceous* (Birmingham strain), it was incubated at 25° for 30—36 hr. until the pH of the culture was 5·90.

The bacterial cells were removed by centrifuging and ethanol (47 c.c.; final concentration 25% v/v) was added to the culture fluid while the temperature was kept at 0° in this and subsequent operations. After 1 hr., the suspension was centrifuged and the precipitate, which contained 6% of the dextransucrase originally present in the culture, when estimated by the standard method in the presence of 100 mg. of maltose, was discarded. A further precipitate, containing 25% of the original dextransucrase activity, was likewise discarded after centrifuging the suspension obtained by addition of further ethanol (13 c.c.; final concentration 30% v/v).

The supernatant liquid was cooled to −20° and more ethanol (80 c.c.; final concentration 50% v/v) was slowly added with stirring. The suspension was kept at −20° for 1 hr. and then centrifuged at −18° for 20 min. (2000 r.p.m.). The heavy precipitate was dissolved in 0·05M-citrate buffer (pH 5·0; 40 c.c.) and dialysed for 48 hr. against 0·05M-citrate buffer (pH 5·0; 2 × 2 l.) at 0° in dialysis tubing that had been thoroughly washed with distilled water and 0·05M-citrate buffer (pH 5·0). The solution was then freeze-dried to a powder (0·702 g.) which showed only slight dextransucrase activity in the absence of a primer molecule but which, in

[2] Jones, Jeanes, Stringer, and Tsuchiya, *J. Amer. Chem. Soc.*, 1956, **78**, 2499.

the presence of maltose (100 mg.), exhibited a 373% increase in the rate of fructose production from sucrose and then displayed 19% of the original dextransucrase activity in the culture fluid.

Standard Method for the Determination of Dextransucrase Activity.—The standard method described [1] for the estimation of dextransucrase with high dextran content was also used for the assay of preparations isolated from sucrose–maltose cultures except that, owing to the low activity of the latter enzyme in the absence of a primer molecule, an additional digest was incubated containing maltose (100 mg.), enzyme (2 c.c.), and sucrose (300 mg.) in 0·05M-acetate buffer (pH 5·0; 3 c.c.). In determinations of the activity of the centrifuged culture fluid (no maltose added) the amount of fructose it contained already was allowed for in calculating the dextransucrase activity.

Development of the Standard Method of Isolation of Dextransucrase with Low Dextran Content.—Preliminary experiments showed a high yield of oligosaccharides (see below) and very little dextran when the bacterium was cultured in a medium containing 50% of maltose and 10% of sucrose. Excessive wastage of maltose was avoided by using 10% of maltose and 2% of sucrose in the standard medium.

(a) *Time of growth.* A standard medium was inoculated and incubated at 25°. Aliquot parts were withdrawn at intervals for the measurement of pH and dextransucrase content of the centrifuged culture fluid (Table 1). The culture did not become viscous.

TABLE 1. *Effect of time of growth.*

Time (hr.)	0	16	23	38	48
pH of culture	7·0	6·6	6·45	5·05	4·2
Dextransucrase activity (units/c.c.)	—	0·9	1·5	5·5	4·7

(b) *Fractionation with ethanol.* Culture fluid (90 c.c.) containing 6 units/c.c. was cooled to 0° and ethanol (48 c.c.; final concentration 35% v/v) was added slowly with stirring. After 1 hr. at 0° the suspension was centrifuged at 0° and the precipitate dissolved in 0·05M-citrate buffer (pH 5·0; 50 c.c.), to give fraction A. The alcoholic supernatant solution was cooled to −20° and ethanol (42 c.c.) added slowly with stirring. After 30 min. at −20° the suspension was centrifuged at −18° and the brown precipitate obtained dissolved in 0·05M-citrate buffer (pH 5·0; 50 c.c.), to give fraction B. A portion of this solution was dialysed at 0° for 24 hr. against 0·05M-citrate buffer (pH 5·0; 2 × 2 l.), to give fraction C. After freeze-drying of part of this solution, the brown powder obtained was redissolved to give fraction D. The activities are in Table 2.

TABLE 2. *Activity of enzyme fractions.*

Fraction	A	B	C	D
Dextransucrase recovered (%)	37	0	12·5	12·5
Maltose priming power (%)	+100	0	+400	+400

Stability of Dextransucrase.—Purified enzyme (75 mg.) prepared by the standard method was dissolved in 0·02M-acetate buffer (pH 5·0; 12 c.c.) and stored in a tightly stoppered flask immersed in a thermostat at 25°. At intervals, the activity of aliquot portions (2 c.c.) was determined by the standard method in the presence of maltose (100 mg.) after incubation for 20 hr. (see Table 3).

TABLE 3. *Stability of dextransucrase.*

Time (hr.)	0	24	48	72	96
Activity (units/2 c.c.)	4·5	3·18	2·62	2·12	1·81

Carbohydrate Content of Dextransucrase.—A solution of enzyme (20 mg./c.c.) was submitted to paper partition chromatography with the organic phase of a mixture of butanol (40%), ethanol (10%), water (49%), and ammonia (1%). Spraying with alkaline silver nitrate [3] disclosed no sugars. Part (1 c.c.) of the enzyme solution was hydrolysed by 2N-sulphuric acid at 100° for 4 hr. After neutralisation with barium carbonate, filtration, and chromatography, a faint spot corresponding to glucose could be detected when 0·1 ml. of the filtrate was applied. Quantitative estimates (Shaffer and Hartmann [4]) of the glucose produced after hydrolysis of two batches of enzyme prepared by the standard method were 7·26% and 7·84% respectively.

Stability of Fructose in the Presence of the Enzyme.—Fructose (10 mg.) in 0·05M-acetate

[3] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.
[4] Shaffer and Hartmann, *J. Biol. Chem.*, 1921, **45**, 377.

buffer (pH 5·0; 2 c.c.) was incubated with enzyme (1 c.c.) for 20 hr. at 25°. Reducing power corresponding to 10·02 mg. of fructose could then be detected.

Effect of pH *on Enzyme Activity.*—Portions of freeze-dried dextransucrase with low dextran content were dissolved in water and the solutions adjusted to pH 4, 5, and 6 severally. Aliquot parts (2 c.c.) of each solution containing 20 mg. of enzyme, were mixed with sucrose (300 mg.) and maltose (100 mg.) dissolved in 0·05M-acetate buffer (10 c.c.) of the same pH, and the enzyme activity was measured after 20 hours' incubation at 25° (see Table 4).

TABLE 4. *Effect of* pH *on enzyme activity.*

pH	4·0	5·0	6·0
Apparent activity (units/20 mg. of enzyme)	0·0	6·75	3·36

Effect of Temperature on Enzyme Activity.—The activity of portions (20 mg.) of the enzyme was measured by the standard method after incubation at various temperatures for 20 hr. in the presence of sucrose (300 mg.) and maltose (100 mg.) dissolved in 0·05M-acetate buffer (pH 5·0; 7 c.c.) (see Table 5).

TABLE 5. *Effect of temperature on enzyme activity.*

Temp.	19°	25°	29°	36°
Apparent activity (units/20 mg. of enzyme)	4·62	6·75	4·62	0·90

Effect of Added Maltose on Enzyme Activity.—The activity of portions (20 mg.) of freeze-dried enzyme was measured by the standard method after incubation for 20 hr. at 25° in the presence of various amounts of maltose (see Table 6).

TABLE 6. *Effect of added maltose on enzyme activity.*

Maltose (mg.)	0	12·5	25	50	75	100	200
Fructose produced (mg.)	15·5	31·0	37·2	52·5	57·0	67·5	72·5
Increase in fructose production (%)	—	100	140	238	268	335	368

Effect of Added Sugars on Enzyme Activity.—The activity of dextransucrase (20 mg. portions) was measured by the standard method in the presence of a series of sugars (100 mg. each) which were added to the digests as their solutions in 0·05M-acetate buffer (2 c.c.; pH 5·0). Control digests to determine the effect of the enzyme on the added sugar alone were also prepared and incubated (20 hr.). Suitable aliquot portions were submitted to alkaline oxidation, and the fructose was estimated in the usual way (see Table 7).

TABLE 7. *Effect of added sugars on enzyme activity.*

Added sugar	Change in fructose yield (%)	Added sugar	Change in fructose yield (%)
Isomaltose	+351	Melibiose	+20·0
Maltose	+335	αα-Trehalose	+12·9
Methyl α-D-glucoside	+100	D-Galactose	+ 2·8
D-Glucose	+ 32	α-D-Glucose 1-phosphate	0·0
Cellobiose	+ 18·8	Fructose	−36·0
Lactose	+ 15·9		

Chromatographic examination of the digests revealed the presence of additional oligosaccharides where the added sugars were maltose, isomaltose, methyl α-D-glucoside, D-glucose cellobiose, and lactose, severally.

Oligosaccharide Synthesis by Growing Cultures of Betacoccus arabinosaceous *in the Presence of Added Sugars.*—(a) *Maltose–sucrose medium.* The same medium (40 c.c.) that was used for the production of low dextran content dextransucrase was inoculated with *Betacoccus arabinosaceous*, and incubated for 48 hr. at 25°. The culture (pH 4·26) was adjusted to pH 7·0 (with N-sodium hydroxide), additional sucrose (4 g.) added, and the culture incubated for a further 48 hr. The culture was neutralised, centrifuged, and heated for 10 min. at 90°, and the mixture separated on a charcoal–Celite column (l. 40 cm.; diam. 4 cm.) by elution with aqueous ethanol (Whistler and Durso [5]). Fructose (3·0 g.) [containing some glucose and an unknown ketose-containing disaccharide ($R_{glucose}$ 0·56)] and maltose (3·6 g.) were eluted with water (1·5 l.) and 5% aqueous ethanol (2 l.), respectively. Pure trisaccharide I (1·24 g.) and a mixed fraction

[5] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.

(2·07 g.), containing trisaccharide I together with small amounts of maltose and tetrasaccharide II, were eluted with 5% aqueous ethanol (5 l.) and 10% aqueous ethanol (1 l.). Crude tetrasaccharide II (1·25 g.) and pentasaccharide III (0·92 g.) were removed with 10% aqueous ethanol (2 l.) and 15% aqueous ethanol (3 l.), respectively. A higher oligosaccharide fraction (1·65 g.) was obtained with 30% aqueous ethanol (1 l.). Refractionation of the crude tetra- and penta-saccharide fractions on a charcoal column gave tetrasaccharide II (0·4 g.) and pentasaccharide III (0·29 g.), both chromatographically pure.

(b) *Characterisation of the oligosaccharides.* Trisaccharide I, after two crystallisations from methanol–water (4 : 1 v/v), had m. p. 222—224°, undepressed on admixture with panose. It showed $[\alpha]_D^{21}$ +155·9° ⟶ +153·9° (18 hr.) (*c* 0·974 in H_2O) (Found: C, 42·9; H, 6·2. Calc. for $C_{18}H_{32}O_{16}$: C, 42·9; H, 6·4%). Its infrared spectrum in the region 1027—715 cm.$^{-1}$ and mobility as a benzylamine complex [7] on a paper chromatogram irrigated with butanol–ethanol–water–ammonia (40 : 12 : 20 : 1) were identical with those of panose.

Tetrasaccharide II moved as a single component $R_{glucose}$ 0·16 (cf. panose, $R_{glucose}$ 0·29) when separated as a benzylamine complex.[7] Paper ionophoresis of the benzylamine complex in formic acid [8] confirmed that it was a tetraose (mobility 0·476). The sugar showed $[\alpha]_D^{19}$ +164·1° (*c* 1·06 in H_2O) and its reducing power (Shaffer–Hartmann method [4]) was equivalent to 24·6% of that of glucose (cf. theor., 27·0%). Complete hydrolysis of the tetrasaccharide (5·3 mg.) with 1·5N-sulphuric acid for 4 hr. at 100° gave glucose (identified on a paper chromatogram) equivalent to a 94·2% conversion. Paper chromatographic analysis of a partial hydrolysate (N-sulphuric acid for 1 hr. at 90°) of the tetrasaccharide showed components with R_F values identical with those of glucose, isomaltose, maltose, panose, isomaltotriose, and tetrasaccharide II. A portion of the tetrasaccharide (0·1 g.) was reduced with an aqueous solution of sodium borohydride (0·1 g. in 50 c.c.) at room temperature for 2 hr. The tetrasaccharide alcohol was recovered as a freeze-dried powder {0·077 g.; $[\alpha]_D^{19}$ +120° (*c* 0·86 in H_2O)} after use of Amberlite IR-120 to remove sodium ions and distillation with methanol to remove boric acid. Treatment of the alcohol of the tetrasaccharide (41·4 mg.) in water (50 c.c.) with 0·25M-sodium periodate (5 c.c.) in the dark at room temperature gave the following results: The periodate consumption, expressed in mol. per mol. of alcohol, was: 3·85 (0·25 hr), 6·92 (1 hr.), 7·73 (2 hr.), 9·02 (4 hr.), 9·02 (6 hr.). After 6 hr., 3·83 mols. of formic acid and 1·91 mols. of formaldehyde were produced per mol. of alcohol.

Pentasaccharide III had $R_{Glucose}$ 0·10 when separated as benzylamine complex and showed $[\alpha]_D^{19}$ +167° (*c* 0·736 in H_2O), and a reducing value equivalent to 13·5% that of glucose (theor., 21·7%). Complete hydrolysis of the pentasaccharide (as above) gave glucose (identified on a paper chromatogram) equivalent to a 95·5% conversion. Paper chromatography of a partial hydrolysate (prepared as for the tetrasaccharide) of the pentasaccharide showed components with R_F values identical with those of glucose, isomaltose, maltose, panose, isomaltotriose, tetrasaccharides, and the original pentasaccharide. Reduction of the pentasaccharide (77 mg.) with sodium borohydride (as above) gave the corresponding alcohol (48 mg.), $[\alpha]_D^{19}$ +150° (*c* 0·96 in H_2O). On periodate oxidation (as above), the mols. of periodate consumed per mol. of alcohol were: 3·5 (0·25 hr.), 6·0 (1 hr.), 10·25 (3 hr.), 10·83 (5 hr.), 11·20 (6 hr.), 11·20 (10 hr.). After 10 hr., 4·75 mols. of formic acid and 1·85 mols. of formaldehyde were produced per mol. of alcohol.

(c) *Methyl α-D-glucoside–sucrose medium.* Standard medium (50 c.c.), in which the maltose was replaced by methyl α-D-glucoside (12·5 g.), was inoculated with *Betacoccus arabinosaceous* and incubated at 25° for 5 days. The culture was neutralised with N-sodium hydroxide, then heated at 90° for 10 min., and the mixture separated on a charcoal–Celite column (l. 30 cm.; diam. 3 cm.). Elution with water (3·5 l.) removed glucose, fructose, and some methyl α-D-glucoside. The remainder of the methyl α-D-glucoside was removed with aqueous ethanol (1%; 2 l.), and the suspected methyl α-bioside IV (0·884 g.) with 3% aqueous ethanol (1 l.) and 4% aqueous ethanol (2 l.). A mixed fraction (0·1 g.) containing suspected methyl α-bioside and methyl α-trioside was eluted with 6% aqueous ethanol (1 l.). Chromatographically pure methyl α-trioside V (0·506 g.) was obtained on washing with 10% aqueous ethanol (2 l.) and 12·5% aqueous ethanol (1 l.).

(d) *Characterisation of oligosaccharides* IV *and* V. The methyl α-bioside was non-reducing

[6] Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171.
[7] Bayly and Bourne, *Nature*, 1953, **171**, 385.
[8] Barker, Bourne, Grant, and Stacey, *ibid.*, 1956, **177**, 1125.

to Shaffer–Hartmann reagent,[4] had an $R_{Glucose}$ value of 0·68 (cf. isomaltose, 0·37) in the organic phase of a mixture of butanol–ethanol–water–ammonia (40 : 10 : 49 : 1), and showed $[\alpha]_D^{19}$ +169° (*c* 0·848 in H_2O) (Found: OMe, 8·4. Calc. for $C_{13}H_{24}O_{11}$: OMe, 8·7%). Complete hydrolysis of the methyl α-bioside (6·44 mg.) with 1·5N-sulphuric acid for 4 hr. at 100° gave glucose as the only sugar (identified paper-chromatographically) equivalent to a 95·7% conversion. Paper chromatography of a partial hydrolysate (N-sulphuric acid for 1 hr. at 90°) of IV showed components with R_F values identical with those of methyl α-D-glucoside, glucose, methyl α-bioside, and isomaltose. Oxidation of IV (52·6 mg.) in aqueous solution (50 c.c.) with 0·25M-sodium periodate (10 c.c.) consumed, as mols. of periodate per mol. of IV: 3·42 (2 hr.), 3·75 (10 hr.), 3·94 (24 hr.). After 24 hr., 2·1 mols. of formic acid were produced per mol. of IV.

The methyl α-trioside V was non-reducing to Shaffer–Hartmann reagent,[4] had an $R_{Glucose}$ value of 0·24 in the organic phase of a mixture of butanol–ethanol–water–ammonia (40 : 10 : 49 : 1), and showed $[\alpha]_D^{19}$ +176·3° (*c* 1·12 in H_2O) (Found: OMe, 6·2. Calc. for $C_{19}H_{34}O_{16}$: OMe, 6·0%). Complete hydrolysis of V (8·89 mg.) with 1·5N-sulphuric acid for 4 hr. at 100° gave glucose as the only sugar (identified paper-chromatographically) equivalent to a 98·1% conversion. Paper chromatography of a partial hydrolysate (N-sulphuric acid for 1 hr. at 90°) of V showed components with R_F values identical with those of methyl α-D-glucoside, glucose, methyl α-bioside, isomaltose, isomaltotriose, and methyl α-trioside. Oxidation of V (44 mg.) with sodium periodate (as with IV) consumed as mols. of periodate per mol. of V: 3·05 (0·25 hr.), 4·67 (2 hr.), 5·18 (4 hr.), 5·73 (6 hr.), 5·80 (8 hr.) and 5·96 (23 hr.). After 23 hr. 2·64 mols. of formic acid were produced per mol. of V.

(e) *Other sugar–sucrose media.* Standard media, in which the maltose was replaced by D-glucose (10%, 20%) or D-galactose (25%, 30%), were inoculated with *Betacoccus arabinosaceous* and incubated at 25°. Analysis of aliquot parts removed during incubation showed (i) the production of isomaltose, isomaltotriose, and higher oligosaccharides in the presence of added glucose and (ii) di- and tri-saccharide production in the presence of added galactose. No new oligosaccharides could be detected in cultures containing added melibiose (10, 20, or 40%) or raffinose (40%) incubated under the same conditions.

One of us (R. W. B.) thanks the Colonial Products Research Council for the award of a scholarship.

CHEMISTRY DEPARTMENT, THE UNIVERSITY,
EDGBASTON, BIRMINGHAM, 15.

[*Received, April 12th*, 1957.]