699. *Immunopolysaccharides. Part VI.* The Isolation and Properties of the Dextransucrase of* Betacoccus arabinosaceous.

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The isolation and properties of a cell-free dextransucrase preparation from *Betacoccus arabinosaceous* (Birmingham strain) is described. This preparation, which contains pre-formed dextran, is shown to contain two enzymes which together synthesise a branched dextran. Partial destruction of one of the enzymes yields a preparation which will then only synthesise a relatively unbranched dextran.

In 1941, Hehre [1] described the isolation, from cultures of *Leuconostoc mesenteroides*, of a heat-labile cell-free extract, which synthesised from sucrose a polysaccharide indistinguishable from a dextran by certain chemical and serological tests. He postulated that the synthesis, catalysed by dextransucrase, proceeded according to the equation:

$$n \text{ Sucrose} \longrightarrow (\text{Glucose})_n + n \text{ Fructose}$$

Improvements in the method of isolation by precipitation with chloroform [2] and ammonium sulphate [3] were later reported. The use of ethanol and ammonium sulphate has also been mentioned,[4] without experimental details. The purpose of this and the succeeding communication is to report the progress of attempts to isolate, purify, and free from pre-formed dextran the enzymes responsible for the synthesis of the linear (α-1 : 6) and branched

* Part V, *J.*, 1955, 2096.

[1] Hehre, *Science*, 1941, **93**, 237.
[2] Hehre and Sugg, *J. Exp. Med.*, 1942, **75**, 339.
[3] Hehre, *J. Biol. Chem.*, 1946, **163**, 221.
[4] Koepsell, Tsuchiya, Hellmann, Kazenko, Hoffmann, Sharpe, and Jackson, *ibid.*, 1953, **200**, 793.

(α-1 : 3) portions of structures of dextrans elaborated by *Betacoccus arabinosaceous* (Birmingham strain).[5,6]

A standard method of isolating a highly active dextransucrase from the cell-free culture of *B. arabinosaceous* was devised using precipitation at 0° with 35% ethanol and reprecipitation at 0° with 25% ethanol. Although control experiments had shown that in protein-free solution no dextran fraction normally elaborated by *B. arabinosaceous* was precipitated with 25% ethanol (v/v) at 0°, the presence of dextransucrase appeared to cause the formation of a dextran–dextransucrase complex which was precipitable under these conditions. The dextran content of three freeze-dried enzyme preparations, isolated by this method, varied from 71·4 to 81·4%.

The assay of enzyme activity was carried out by a method similar to that used by Koepsell *et al.*[4] except that the digest was incubated at 25° instead of 30°. A tenfold increase in the amount of sucrose present in the digest caused an apparent doubling in the activity of the enzyme as measured by the total amount of fructose produced (Table 1, p. 3533). The enzyme preparation was free from any enzyme which attacked the fructose formed during dextran synthesis and its activity was not affected appreciably by the amount of acetate buffer (pH 5·0) used (Table 2). In determining enzyme activity prolonged incubation (>20 hr.) was avoided since in the later stages there was a decrease in the rate of fructose production due to the decreased amount of sucrose present and the appreciable inhibition of the enzyme reaction by large amounts of fructose (see below). The optimum pH of dextransucrase activity was *ca.* 5·5 and the optimum temperature *ca.* 29°; the enzyme exhibited little activity above pH 7·0 and 36°.

Many unsuccessful attempts were made to free the enzyme preparation from pre-formed dextran. Fractionation by ammonium sulphate always gave inactive preparations; paper chromatography at 0° in various concentrations of aqueous acetone or aqueous ethanol did not separate enzyme activity and pre-formed dextran. No absorption could be detected on cellulose powder suspended in a buffered solution (pH 6·0) of the enzyme. All activity was readily absorbed on charcoal from which, however, it could not be eluted by water, 0·1M-citrate buffer (pH 6·0) or 0·1M-acetate buffer (pH 6·0). Shaking with chloroform at 0° by a method similar to that of Hehre and Sugg [2] gave an inactive precipitate. Reprecipitation with ethanol, although giving an active enzyme, did not remove any appreciable proportion of dextran. To avoid carrying these experiments out on the larger scale, necessary for the determination of the amount of dextran present by acid hydrolysis, each enzyme fraction was tested in the presence and absence of added dextran. This was done in the belief that dextransucrase, free from pre-formed dextran, would, like muscle phosphorylase, only exhibit full activity in the presence of added primer (in this case dextran).

The standard dextransucrase preparation, when freshly freeze-dried, synthesised a highly branched dextran which showed high optical rotation ($[\alpha]_D$ +217°), was converted into glucose by acid hydrolysis to the extent of 101%, contained <0·1% of fructose, and showed an infrared spectrum similar to the highly branched dextran normally produced by *Betacoccus arabinosaceous.*[5] After storage at 0° for 2 months, the same enzyme preparation synthesised a dextran showing a lower optical rotation ($[\alpha]_D$ +190°) and although obtained in approximately the same degree of purity (% conversion into glucose, 96; fructose content <0·1%) gave an infrared spectrum similar to the modified relatively unbranched dextran elaborated by *Betacoccus arabinosaceous* in a magnesium-deficient medium.[6] These results were confirmed by periodate oxidation and the amounts of glucose detectable in the hydrolysates of the periodate-oxidised dextrans. It is concluded that the standard dextransucrase preparation contains two enzymes, one of which is responsible for the synthesis of α-1 : 6-links, and another which is responsible for the formation of the α-1 : 3-branch points.

[5] Barker, Bourne, Bruce, Neely, and Stacey, *J.*, 1954, 2395.
[6] Barker, Bourne, James, Neely, and Stacey, *J.*, 1955, 2096.

In 1953, Koepsell *et al.*[4] showed that certain simple sugars could act as chain-initiators when the dextransucrase of *Leuconostoc mesenteroides* (NRRL B-512) acted on sucrose. This observation was also found to apply to the dextransucrase of *Betacoccus arabinosaceous.*[7] Isomaltose, maltose, and methyl α-D-glucoside caused marked increases in the amounts of fructose produced by dextransucrase from sucrose owing to their ability to act as alternative acceptors. The actual increase, although constant for a given enzyme preparation, varied with each batch of enzyme, owing no doubt to the amount and nature of preformed dextran already present in the enzyme. D-Glucose and polygalitol exhibited a less marked effect on fructose production, while the effects of D-galactose, lactose, melibiose, and cellobiose although detectable varied with the batch of enzyme taken. Fructose caused a decrease in the amount of fructose produced whichever batch of enzyme was taken (see Table 6).

The presence of isomaltose, maltose, and D-glucose caused a marked decrease in the amount (and probably molecular size) of dextran produced and each led to the synthesis of a series of oligosaccharides. Some of these oligosaccharides (isomaltotriose, panose, isomaltose, etc.) were of known constitution and contained the added sugar incorporated at the reducing end, together with a series of glucose units in α-1 : 6-linkage at the non-reducing end. Cellobiose, lactose, D-galactose, and methyl α-D-glucoside also gave series of oligosaccharides. The structures of trisaccharides obtained from cellobiose and lactose have been recently described,[8,9] and both were found to be formed by the addition of a glucose unit in α-1 : 2-linkage to the reducing moiety of the disaccharide. It is believed that some of the sugars in Table 8 (*e.g.*, maltose more than glucose) have the ability to desorb the enzyme from the dextran–dextransucrase complex, making it more effective and causing an increase in fructose production. The sugars then act either as alternative acceptors (*e.g.*, maltose) or competitive inhibitors (*e.g.*, fructose). It is realised that one anomer, *e.g.*, α-D-glucopyranose, might differ in these properties from the other anomer, *e.g.*, β-D-glucopyranose, particularly in view of the totally different behaviour of maltose and cellobiose.

Although these initial experiments did not produce a dextransucrase free from dextran they did suggest a method of obtaining such an enzyme by growing the bacteria on sucrose in the presence of large amounts of maltose and so providing a source in which the enzyme is accompanied by only traces of the polysaccharide. They also provided evidence of the presence of at least two enzymes concerned in the synthesis of branched dextrans.

Experimental

Standard Method of Isolation of Dextransucrase.—An aqueous medium (4 l.), containing yeast extract (1%), $Na_2NH_4PO_4$ (0·5%), KH_2PO_4 (0·1%), $MgSO_4,7H_2O$ (0·05%), and sucrose (10%), was adjusted to pH 7 with sodium hydroxide and steam-sterilised at 15 lb./sq. in. for 30 min. After inoculation from an actively growing culture of *Betacoccus arabinosaceous* (Birmingham strain) it was incubated at 25° for 17 hr. The culture (pH 6·6) was cooled to 0° and ethanol (2160 ml.; final concentration 35% v/v) added slowly with stirring. Care was taken to maintain the temperature at 0—1° throughout this and all the following procedure. After 1 hr., a dark gummy precipitate was recovered by centrifuging (2000 r.p.m.), dissolved in 0·1M-citrate buffer (500 c.c.; pH 6·0), and centrifuged at 5000 r.p.m. for 30 min. to give a cell-free enzyme solution. 0·1M-Citrate buffer (2500 c.c.; pH 6·0) was then added to give a solution containing approximately 1·5 units of activity/c.c. The dextransucrase was isolated from this solution by dissolving in water (200 c.c.) the precipitate produced overnight by the addition of ethanol (1 l.; final concentration 25% v/v). After clarification by centrifuging at 5000 r.p.m. the supernatant solution was freeze-dried and stored at 0° as a brown powder (4·06 g.). Several batches of enzyme were prepared.

Standard Method for the Determination of Dextransucrase Activity.—The enzyme (100 mg.)

[7] Bailey, Barker, Bourne, and Stacey, *Nature*, 1955, **175**, 635.
[8] *Idem, ibid.*, **176**, 1164.
[9] Barker, Bourne, Grant, and Stacey, *ibid.*, 1956, **178**, 1221.

was dissolved in 0·05M-acetate buffer (30 c.c.; pH 5·0), and the solution centrifuged (5000 r.p.m.) for 30 min. A portion (2 c.c.) was pipetted into a 25 c.c. standard flask, and freshly prepared sucrose solution (300 mg. in 3 c.c. of 0·05M-acetate buffer, pH 5·0) added. This digest was incubated at 25° for 3 hr. together with two blank digests containing respectively (*a*) enzyme solution (2 c.c.) and 0·05M-acetate buffer solution, pH 5·0 (3 c.c.), and (*b*) sucrose (300 mg.) in 0·05M-acetate buffer, pH 5·0 (5 c.c.). Each solution was then neutralised by 0·25N-sodium hydroxide (phenol-red). Iodine solution (2 c.c.; iodine, 8·7 g., and potassium iodide, 16·7 g. per l.) was added followed by 0·175N-sodium hydroxide (1 c.c.). Each flask was shaken, stoppered, and left for 10 min. at room temperature. After acidification with 0·25N-sulphuric acid (1 c.c.), the excess of iodine was removed with 0·02—0·04N-sodium sulphite, starch glycollate being used as an indicator. Each solution was neutralised with 0·25N-sodium hydroxide and diluted to 25 c.c. The fructose present in an aliquot part (5 c.c.) of each solution was then determined by the copper reagent of Shaffer and Hartmann.[10] A unit of dextransucrase activity is that required for the complete conversion of 1 mg. of sucrose into dextran and fructose in 1 hr. at 25° provided that not more than one-half of the sucrose present has been utilised.

Effect of Sucrose Concentration on Dextransucrase Activity.—The activity of a freeze-dried dextransucrase preparation (2 c.c. portions) was measured by the standard method except that differing amounts of sucrose, each dissolved in 0·05M-acetate buffer (3 c.c.; pH 5·0), were used in a series of digests. The results are given in Table 1.

TABLE 1. *Effect of sucrose concentration on enzyme activity.*

Sucrose added (mg.)	30	50	70	100	300	500
Fructose produced (mg.)	2·21	2·38	2·86	3·26	4·53	4·60
Apparent activity (units/2 c.c.)	1·40	1·51	1·81	2·06	2·87	2·91

Effect of Acetate Buffer on Dextransucrase Activity.—The activity of portions (2 c.c.) of the enzyme was determined by the standard method except that the volume of the 0·05M-acetate buffer (pH 5·0) was varied as in Table 2.

TABLE 2. *Effect of acetate buffer on enzyme activity.*

Volume of buffer (c.c.)	5	8	11
Fructose produced (mg.)	4·70	4·70	4·65

Stability of Fructose in the Presence of Dextransucrase.—Fructose dissolved in 0·05M-acetate buffer (pH 5·0; 2 c.c.) was incubated with the enzyme (2 c.c.) for 20 hr. at 25°. Estimation showed the same amount of fructose (10 mg.) to be present as in a control containing fructose incubated in the absence of the enzyme for 20 hr. at 25°.

Effect of Time of Incubation on Enzyme Activity Determination.—The activity of portions (2 c.c.) of dextransucrase was determined by the standard method except that 10% sucrose–buffer solution (5 c.c.) was used and the digests were incubated for various times (Table 3).

TABLE 3. *Effect of incubation time on activity determination.*

Time of incubation (hr.)	0	3	15	23	48	93
Fructose produced (mg.)	0	6·15	29·75	41·75	51·50	73·50
Apparent activity (units/2 c.c.)	0	3·89	3·77	3·45	2·04	1·50

Effect of pH *on the Activity of Dextransucrase.*—Portions of dextransucrase (100 mg.) were dissolved in 0·05M-acetate buffer (30 c.c.) of various pH values. After being centrifuged for 30 min. at 5000 r.p.m., aliquot parts (2 c.c.) of each solution were mixed with sucrose (300 mg.), dissolved in 0·05M-acetate buffer (3 c.c.) of the same pH, and the enzyme activity measured by the standard method (see Table 4). Control digests omitting (*a*) the enzyme, and (*b*) the sucrose, were also run and gave normal blank titrations.

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

pH of acetate buffer	4·0	4·5	5·0	5·5	6·0	6·5	7·0
Apparent activity (units/2 c.c.)	0·47	1·39	1·75	2·19	1·78	1·08	0·58

Effect of Temperature on Dextransucrase Activity.—The activities of portions (2 c.c.) of dextransucrase solution were measured in a series of digests by the standard method except

[10] Shaffer and Hartmann, *J. Biol. Chem.*, 1921, **45**, 377.

that they were incubated at various temperatures. Those at 1° and 8° were incubated for 20 hr., the remainder were incubated for 3 hr. (see Table 5).

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

Temperature	1°	8°	19°	25°	29°	36°
Apparent activity (units in 2 c.c.)	0·30	0·40	1·09	1·75	1·85	0·56

Carbohydrate Content of Dextransucrase.—Freeze-dried enzyme (25 mg.) prepared by the standard method was hydrolysed with 2N-sulphuric acid (2 c.c.) at 100° for 4 hr. The hydrolysate was cooled, neutralised with 2N-sodium hydroxide and diluted to 50 c.c. The % conversion, estimated as glucose by the method of Shaffer and Hartmann,[10] corresponded to 71·4, 76·1, and 81·4% for three enzyme preparations.

Effect of Added Sugars on Dextransucrase Activity.—The activity of dextransucrase 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). Additional controls, omitting the sucrose or dextransucrase, were carried out in the presence of each added sugar. The digests were incubated for 20 hr. in order to obtain a suitable ratio of fructose to added sugar and after dilution to 25 c.c. the following aliquot parts were used for hypoiodite oxidation and fructose measurement: (i) added reducing monosaccharides, 1 c.c.; (ii) added reducing disaccharides, 2 c.c.; and (iii) added non-reducing sugars, 5 c.c. In the case of reducing sugars with a low priming effect a second fructose measurement was made on a larger aliquot portion (5 c.c.) and an increased amount of alkaline iodine to oxidise the added sugar. Controls showed that the extra iodine did not affect the fructose measurement. Except in the case of added fructose all the control digests gave thiosulphate titrations identical with the blank titration. Table 6 gives the effect of added sugars on two batches of enzyme, (A) containing 0·6 unit/mg. and (B) containing 0·3 unit/mg.

TABLE 6. *Effect of added sugars on dextransucrase activity.*

	Batch A		Batch B	
Added sugar	Fructose (mg.) produced	Change (%) in production	Fructose (mg.) produced	Change (%) in production
None	38·8	—	17·5	—
Isomaltose	86·3	+123	48·2	+175
Maltose	80·0	+107	43·8	+150
Methyl α-D-glucoside	54·3	+ 40	28·0	+ 60
D-Glucose	43·8	+ 13	21·3	+ 21
1-Deoxy-D-glucose	45·0	+ 16	—	—
D-Galactose	39·9	+ 3	18·3	+ 4
Lactose	31·3	− 19	18·5	+ 6
Melibiose	34·4	− 11	18·3	+ 4
Cellobiose	38·8	0	18·5	+ 6
D-Fructose	33·8	− 13	13·8	− 22

Other compounds tested with batch A enzyme and having a negligible effect (<5%) were α-D-glucose 1-phosphate, methyl β-D-glucoside, D-glucuronic acid, D-xylose, L-arabinose, D-mannose, D-glucitol, D-mannitol, inositol, αα-trehalose, laminaribiose, raffinose, and melezitose. Although all the reducing sugars, except isomaltose, were crystalline α- or β-anomers, they would, in solution, have been present through most of the incubation as mixtures of anomers.

Effect of Increasing Amounts of Maltose on the Activity of Dextransucrase.—The effect of varying amounts of maltose on the activity of batch A and batch B enzyme was investigated under the conditions outlined in the previous experiment (see Table 7).

Chromatographic Analysis of Standard Digests containing Added Sugars.—A series of standard dextransucrase digests containing added sugars (100 or 200 mg.) was prepared in the manner previously described and incubated at 25° for 20 hr. Without dilution or addition of hypoiodite, each digest was submitted to paper chromatography in the organic phase of a mixture of butanol (40%), ethanol (10%), water (49%), and ammonia (1%), the papers being sprayed severally with aniline hydrogen phthalate,[11] naphtharesorcinol,[12] and alkaline silver nitrate.[13]

[11] Partridge, *Nature*, 1949, **164**, 443.
[12] Forsyth, *ibid.*, 1948, **161**, 239.
[13] Trevelyan, Proctor, and Harrison, *ibid.*, 1950, **166**, 444.

For the separation of higher oligosaccharides a more aqueous solvent [14] containing butanol (40), water (20), ethanol (12), and ammonia (1) was used to irrigate the chromatograms. With sugars having reducing glucose or galactose groups the benzylamine–ninhydrin technique [14] was used. Additional evidence was obtained by paper ionophoresis [15] in borate buffer (pH 10). The additional components detected, other than the added sugar, sucrose, fructose, and traces of glucose, are listed in Table 8.

TABLE 7. *Effect of maltose concentration on dextransucrase activity.*

Batch A			Batch B		
Maltose (mg.) added	Fructose (mg.) produced	Increase (%) in fructose	Maltose (mg.) added	Fructose (mg.) produced	Increase (%) in fructose
0	38·8	—	0	17·5	—
10	42·5	10	12·5	22·4	28
20	49·9	29	25	27·0	55
30	55·9	44	50	34·4	97
50	62·1	60	75	39·8	127
100	78·6	103	100	43·8	150
200	90·9	135	200	45·5	160

TABLE 8. *Analysis of standard digests containing added sugars.*

Added sugar	Additional components and probable identities
Isomaltose (100 mg.)	Isomaltotriose, tetra- and penta-saccharides
Maltose (100 mg.)	Panose, tetra- and penta-saccharides
Cellobiose (100 mg.)	Trisaccharide (see ref. 9)
Lactose (200 mg.)	Trisaccharide (see ref. 8)
Melibiose (200 mg.)	None
Methyl α-D-glucoside (200 mg.)	Methyl bioside, methyl trioside
Galactose (200 mg.)	Disaccharide, trisaccharide
Glucose (100 mg.)	Isomaltose, isomaltotriose, isomaltotetraose
Fructose (200 mg.)	None
1-Deoxy-D-glucose (100 mg.)	None

Synthesis of Dextran with Dextransucrase.—Freshly freeze-dried enzyme (100 mg.) was extracted with 0·05M-acetate buffer (pH 5·0; 30 c.c.), and a portion (20 c.c.) mixed with sucrose (4 g.) in 0·05M-acetate buffer (pH 5·0; 40 c.c.). After 5 days at 25°, ethanol (120 c.c.) was slowly added with stirring and the mixture left at 0° overnight. The precipitated dextran was dissolved in water (200 c.c.), boiled for 10 min., then cooled, and the suspension centrifuged to remove coagulated protein. After dialysis against running water for 48 hr. the dextran (0·590 g.), obtained by freeze-drying, was dissolved in water (200 c.c.) and reprecipitated by addition of ethanol (200 c.c.) at 0°. After freeze-drying and drying over phosphoric oxide at 60° *in vacuo* 0·430 g. of dextran (I) was obtained. After the freeze-dried enzyme used above had been stored at 0° for 2 months, a second larger batch of dextran was synthesised under the same conditions, by using enzyme (400 mg.) and sucrose (24 g.) in 0·05M-acetate buffer (pH 5·0; 360 c.c.). The dextran obtained (3·813 g.) was also subjected to reprecipitation from water (600 c.c.) by the addition of ethanol (600 c.c.) at 0°, the yield obtained after drying being 3·553 g. of dextran (II).

Dextran (I) had $[\alpha]_D^{19}$ +217° (*c* 0·22 in N-NaOH) while dextran (II) showed $[\alpha]_D^{19}$ +190° (*c* 0·20 in N-NaOH). After hydrolysis of each dextran (20 mg.) with N-sulphuric acid (2 c.c.) at 100° for 2 hr., neutralisation, and paper chromatography of the hydrolysate, visual comparison of spot intensities with those of fructose subjected to the same treatment indicated that each dextran hydrolysate contained <0·1%.

The conversions of the dextrans into glucose after acid hydrolysis with 1·5N-sulphuric acid at 100° for 5 hr. were 101 and 96%, respectively.

Both dextran (I) and dextran (II) showed infrared absorption peaks at 917 and 768 cm.$^{-1}$ (α-1 : 6-linkages) and at 841 cm.$^{-1}$ (shown by α-anomers in the D-glucopyranose series). Dextran (I) showed an additional peak at *ca.* 794 cm.$^{-1}$ (α-1 : 3-linkages) (cf. Barker, Bourne, Stacey, and Whiffen [16]).

Both dextran (I) and dextran (II) were subjected to periodate oxidation. The procedure

[14] Bayly and Bourne, *ibid.*, 1953, **171**, 385.
[15] Foster, *Chem. and Ind.*, 1952, 828.
[16] Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171.

adopted was essentially that of Jeanes and Wilham [17] which incorporates Fleury and Lange's method [18] for the determination of the quantity of periodate consumed, and that of Halsall, Hirst, and Jones,[19] with certain modifications, for the determination of the quantity of formic acid produced. The number of mols. of sodium periodate consumed per mol. of anhydroglucose was: 48 hr., 1·80 (I), 1·85 (II); 72 hr., 1·85 (I), 1·97 (II); 96 hr., 1·86 (I), 2·02 (II). The corresponding figures for mols. of formic acid produced were: 48 hr., 0·69 (I), 0·88 (II); 72 hr., 0·84 (I), 0·90 (II); 96 hr., 0·84 (I), 0·90 (II).

The solutions remaining from the oxidations of the dextrans were treated with excess of ethylene glycol, concentrated *in vacuo*, made 2N with sulphuric acid, and hydrolysed for 4 hr. at 100°. After neutralisation with barium carbonate each hydrolysate was concentrated *in vacuo* to 2 c.c. and submitted to chromatographic analysis. Visual comparison with suitable standards showed that periodate-oxidised dextran (I) gave *ca.* 10% of glucose and periodate-oxidised dextran (II) less than 5% of glucose after acid-hydrolysis.

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Edgbaston, Birmingham, 15. [*Received, April 4th,* 1957.]

[17] Jeanes and Wilham, *J. Amer. Chem. Soc.*, 1950, **72**, 2655.
[18] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107, 196.
[19] Halsall, Hirst, and Jones, *J.*, 1947, 1427.