Effect of Dextran and Ammonium Sulphate on the Reaction Catalysed by a Glucosyltransferase Complex from *Streptococcus mutans*

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(Received 12 April 1979; revised 25 January 1980)

The highly aggregated proteins precipitated by $(\text{NH}_4)_2\text{SO}_4$ from the culture fluid of three strains of *Streptococcus mutans* gradually released less aggregated glucosyltransferase activities – dextranucrase and mutansucrase – which catalysed the synthesis of water-soluble and insoluble glucans from sucrose. Mutansucrase was eluted from a column of Sepharose 6B before dextranucrase. This activity was lost during subsequent dialysis and gel filtration, but there was a corresponding increase in dextranucrase activity which catalysed the formation of soluble glucan when incubated with sucrose alone, and insoluble glucan when incubated with sucrose and 1.55 M- $(\text{NH}_4)_2\text{SO}_4$. Relative rates of synthesis of soluble and insoluble glucan in the presence of 1.55 M- $(\text{NH}_4)_2\text{SO}_4$ were dependent upon the enzyme concentration: high concentrations favoured insoluble glucan synthesis. Insoluble glucans synthesized by mutansucrase or by dextranucrase in the presence of 1.55 M- $(\text{NH}_4)_2\text{SO}_4$ were more sensitive to hydrolysis by mutanase than by dextranase, but soluble glucans were more extensively hydrolysed by dextranase than by mutanase. Partially purified dextranucrase sedimented through glycerol density gradients as a single symmetrical peak with an apparent molecular weight in the range 100000 to 110000. In the presence of 1.55 M- $(\text{NH}_4)_2\text{SO}_4$, part of the activity sedimented rapidly as a high molecular weight aggregate. The results strongly suggest that soluble and insoluble glucans are synthesized by interconvertible forms of the same glucosyltransferase. The aggregated form, mutansucrase, preferentially catalyses (1→3)- α bond formation but dissociates during gel filtration to the dextranucrase form which catalyses (1→6)- α bond formation.

INTRODUCTION

Streptococcus mutans is a major causative organism of human dental caries (de Stoppelaar *et al.*, 1968; Sherr, 1971) and a major determinant of a strain's pathogenicity is its ability to synthesize an insoluble glucan from sucrose (Gibbons & Nygaard, 1968; Koga & Inoue, 1978). This insoluble glucan, called mutan, is a sticky polymer which promotes the retention of bacteria to, and inhibits the diffusion of acidic fermentation products from, the tooth surface.

Enzymes which catalyse glucan synthesis are collectively known as glucosyltransferases. Extracellular proteins from cultures of *S. mutans* catalyse the synthesis of both water-soluble and insoluble glucans from sucrose. The major type of glycosidic bond in the soluble glucan is (1→6)- α so, although other bond types are invariably present, the glucosyl-

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transferase involved in its synthesis is called dextransucrase (McCabe & Smith, 1973). In contrast, the glucosyl residues of mutan are linked predominantly by (1→3)- α bonds with a minority of (1→6)- α and other linkages (Lewicki *et al.*, 1971; Baird *et al.*, 1973; Ebisu *et al.*, 1974). The name dextransucrase has previously been used by some authors to refer to glucosyltransferases which catalyse the synthesis of both soluble and insoluble glucans and it has thereby been implied that a single protein is involved in both processes (McCabe & Smith, 1973; Robyt & Corrigan, 1977). An alternative possibility is that mutan is synthesized by two or more proteins, including a dextransucrase which inserts (1→6)- α -linked glucosyl residues into the growing polysaccharide and a second enzyme that catalyses the formation of (1→3)- α linkages (Guggenheim & Newbrun, 1969; Cole, 1977). As the number of proteins required for mutan synthesis is still unknown, in this paper this type of glucosyltransferase activity will be referred to as mutansucrase.

Attempts to purify mutansucrase have yielded conflicting results, especially with respect to the molecular weight of the proteins (Fukui *et al.*, 1974; Schachtele *et al.*, 1976) and their ability to use preformed dextran as a primer or precursor of mutan synthesis (compare Germaine *et al.*, 1977, with Robyt & Corrigan, 1977). Highly purified samples of glucosyltransferases have been prepared in several laboratories, but samples which enter the gel matrix during polyacrylamide gel electrophoresis invariably catalyse the synthesis of soluble glucans in which (1→6)- α glycosidic bonds predominate (Fukui *et al.*, 1974; Kuramitsu, 1975). We have previously reported that little mutansucrase activity was detected in proteins which redissolved in 0.1 M-acetate buffer after precipitation from culture filtrates by 70 % saturated $(\text{NH}_4)_2\text{SO}_4$ (Mohan *et al.*, 1979). Mutan synthesis was stimulated, however, when these redissolved proteins were incubated with sucrose and an equal volume of the 70 % saturated $(\text{NH}_4)_2\text{SO}_4$ supernatant fraction, or with $(\text{NH}_4)_2\text{SO}_4$ itself. This final concentration of $(\text{NH}_4)_2\text{SO}_4$ (1.55 M or 35 % saturation) is sufficient to aggregate and precipitate some proteins from solution, so it was suggested that mutansucrase and dextransucrase activities might be due to interconvertible forms of the same enzyme. The aims of the present work were to examine the possible release of dextransucrase activity from a mutansucrase aggregate, and whether dextransucrase can recombine with other protein fractions or reaggregate in the presence of $(\text{NH}_4)_2\text{SO}_4$ and catalyse mutan synthesis.

Ellwood *et al.* (1976) have documented the changes in the rates of glucosyltransferase synthesis that have occurred during the maintenance of clinical isolates of *S. mutans* on conventional laboratory media. The relevance to the disease process of many of the earlier glucosyltransferase studies is therefore in doubt. To avoid this criticism, many of our experiments with the well-characterized laboratory strain, C67-1, have been repeated with a fresh clinical isolate, strain 3209.

METHODS

Organisms, growth conditions and concentration of extracellular proteins. *Streptococcus mutans* strain 3209 was isolated in December 1977 by Miss D. A. Sidaway from the dentine of a carious fissure in a permanent tooth of a patient in the Birmingham Dental Hospital. It was maintained on horse blood agar and subcultured every 14 d by transferring bacteria from an area of confluent growth on to a fresh blood agar plate. The serotype, c, was determined by Dr R. R. B. Russell, The Royal College of Surgeons of England, Downe, Kent. Strains C67-1 and C67-25 were obtained as freeze-dried cultures from Dr J. M. Hardie, The London Hospital Medical College, Turner Street, London E1 2AD.

Bacteria were grown in continuous culture with excess glucose at a dilution rate of $0.12 \pm 0.01 \text{ h}^{-1}$ and pH 6.55 ± 0.1 , as described by Wenham *et al.* (1979). Extracellular proteins from the spent growth medium were concentrated by precipitation with 70 % saturated $(\text{NH}_4)_2\text{SO}_4$ as described by Mohan *et al.* (1979). The pellet was homogenized with 10 ml 0.1 M-sodium acetate/acetic acid buffer, pH 6.0 ('acetate buffer') for 10 min at 4 °C and immediately collected by centrifugation for 10 min at 30000 g. The pellet was immediately extracted three more times by homogenization with acetate buffer and centrifugation after 30 min at 4 °C. The final pellet was homogenized with 6 to 10 ml acetate buffer.

Enzyme assays. To assay glucosyl- and fructosyltransferase activities, 1 ml of culture supernatant, or 0.1 ml of concentrated protein solution or column fraction, was incubated for 0.5 to 2 h at 37 °C with 0.5 or 1.4 ml acetate buffer and 0.5 ml 0.29 M-sucrose, with or without 60 µg primer dextran ml⁻¹ (mol. wt range 60000 to 90000; BDH). In some experiments, insoluble glucans were collected by centrifugation after addition of 0.2 ml 10 % (w/v) trichloroacetic acid to stop the reaction. The pellet was resuspended in water and recollected by centrifugation and the washings were combined with the original supernatant fluid. Methanol at room temperature was then added to 80 % (v/v) and tubes were cooled at 4 °C for at least 50 min to precipitate water-soluble polysaccharides. The quantities of polysaccharide and ketohexose formed were then determined as described by Mohan *et al.* (1979).

The phenol-sulphuric acid reagent used to assay glucans also reacts with fructans which may be synthesized from sucrose (Dubois *et al.*, 1956). For this reason it was necessary to determine the relative activities of glucosyl- and fructosyltransferases in each type of experiment, as described by Wenham *et al.* (1979). Furthermore, the dextranase activity of a serotype *c* strain of *S. mutans* was almost entirely dextran-dependent (Wenham *et al.*, 1979), so without appropriate controls it is impossible to conclude whether (NH₄)₂SO₄ or dextran are altering rates of synthesis of soluble glucan, insoluble glucan or fructan. Tubes which were not incubated, together with those from which sucrose or enzyme were omitted, were included as controls in each set of assays. The quantities of ketohexose or total polysaccharide in these tubes were subtracted from corresponding quantities in assay tubes before the sucrose-dependent rates of fructan and glucan synthesis were calculated (Wenham *et al.*, 1979; Mohan *et al.*, 1979). These corrections were negligible for assays with glucosyltransferase which had been filtered through Sepharose 6B and were, at most, 20 % of the quantity of glucan or fructan formed when crude enzyme preparations were incubated with sucrose. Wenham (1979) has documented the validity of these procedures.

Gel filtration. A column containing Sepharose 6B (60 to 65 cm long; 2.3 cm diam.) was equilibrated with 2 M-NaCl in acetate buffer. Enzyme samples were concentrated by dialysis against polyethylene glycol 6000, then dialysed against several changes of 2 M-NaCl in acetate buffer at 4 °C, and applied to and eluted from the Sepharose column in 2 M-NaCl in acetate buffer at 18 to 20 °C. The flow rate was 18 ml h⁻¹ and approximately 5 ml fractions were collected.

Sedimentation of glucosyltransferase through glycerol gradients. The pooled fractions containing dextranase activity from the Sepharose 6B column were concentrated by dialysis against polyethylene glycol and dialysed against acetate buffer in the presence or absence of 1.55 M-(NH₄)₂SO₄. Samples (0.3 ml) were loaded on to 12.6 ml linear 10 to 30 % (v/v) glycerol gradients which were centrifuged and fractionated as described by Coleman *et al.* (1978). Fumarate hydratase (*s*_{20,w} = 9 S; mol. wt 194000) and catalase (*s*_{20,w} = 11.3 S; mol. wt 250000) were used as molecular weight markers in some gradients.

Sucrose was not used to prepare density gradients because it is a substrate for glucosyltransferase and is converted to products which might substantially alter both the shape and the molecular weight of the enzyme. Glycerol was not an ideal substitute because at 30 % (v/v) it inhibited the rate of glucan synthesis by 30 %. This inhibition was completely reversible by dialysis, so it is unlikely that glycerol promotes a major alteration in the structure of glucosyltransferase.

Polyacrylamide gel electrophoresis. Proteins were separated by electrophoresis in the presence of sodium dodecyl sulphate (SDS), as described by Laemmli (1970), with a 5.5 % (w/v) stacking gel and a 9 % resolving gel. Concentrated samples were diluted to 0.1 to 1.0 mg protein ml⁻¹ with 20 % (v/v) glycerol, 10 % (v/v) mercaptoethanol and 6 % (w/v) SDS in 0.12 M-Tris/HCl, pH 6.8, and boiled for 5 min. Bromophenol Blue [1 vol. of a 0.05 % (w/v) solution in water to 7 vol. of sample] was added and solutions containing 10 to 15 µg sample protein or 50 µg of the mixed marker proteins were applied to the gel. After electrophoresis, protein was stained with Coomassie Brilliant Blue R250, and gels were destained with 10 % (v/v) acetic acid. Alternatively, some gels were incubated for 48 h at 37 °C with acetate buffer containing 10 % (w/v) sucrose or raffinose, 1 % (w/v) Lubrol and 120 µg dextran ml⁻¹.

Proteins used as molecular weight markers were transferrin (78000), bovine serum albumin (69000), bovine glutamate dehydrogenase (53000), lactate dehydrogenase (36000) and egg white lysozyme (14300).

Chemicals and chemical analyses. Protein, total carbohydrate and ketohexose were determined as described by Mohan *et al.* (1979). The fructosyltransferase activity of the glucosyltransferase pool eluted from Sepharose 6B was only 2 to 10 % of the total rate of polysaccharide synthesis. Errors in soluble glucan determinations due to irreproducibility in the colour intensity contributed by fructans in the phenol-sulphuric acid assay were therefore extremely small. All assays were duplicated, and replicates rarely differed from the mean by as much as 2 %.

Samples of water-soluble and insoluble glucans were incubated with dextranase or mutanase and the susceptibility to hydrolysis of glycosidic bonds was determined from the increase in concentration of reducing sugar (Hodge & Hofreiter, 1962). Both dextranase and mutanase released reducing equivalents during incubation in the absence of added glucan, so conditions optimal for detecting glucan hydrolysis were

Table 1. *Solubility of glucosyl- and fructosyltransferase activities in acetate buffer after precipitation by ammonium sulphate*

Proteins precipitated from 3.7 l of strain 3209 culture supernatant were resuspended four times in acetate buffer, and redissolved proteins were collected by centrifugation for 10 min at 30000 g. Enzyme activities are expressed as mg glucose or fructose from sucrose incorporated into polysaccharide h⁻¹ ml⁻¹.

Enzyme sample (vol.)	Protein (mg ml ⁻¹)	Carbohydrate (mg ml ⁻¹)	Dextran in assay	Fructosyl- transferase activity	Glucosyltransferase activity		
					Soluble glucan -(NH ₄) ₂ SO ₄	Insoluble glucan -(NH ₄) ₂ SO ₄ + (NH ₄) ₂ SO ₄	
Extract 1 (10 ml)	5.7	17.8	— +	0.40	0.16	0.45	0.70
				0.25	0.44	0.72	—
Extract 2 (13 ml)	14.6	14.7	— +	5.3	5.8	0.84	6.7
				4.9	9.1	0.74	—
Extract 3 (8 ml)	20.0	8.8	— +	11.8	35.6	3.4	20.2
				11.6	39.2	4.1	—
Extract 4 (7 ml)	24.3	8.0	— +	10.3	20.0	2.0	15.5
				9.5	23.5	3.9	—
Final pellet resuspended in acetate buffer (6 ml)	14.5	10.3	— +	7.8	1.5	13.7	12.2
				7.8	3.0	13.3	—

determined. Subsequently, a freshly prepared solution of dextranase or mutanase (1.0 mg ml⁻¹) was incubated with polymer (0.9 to 2.0 mg ml⁻¹) for 2 h at 37 °C. After 2 min in a boiling water bath, 0.1 to 0.5 ml samples were diluted to 1.0 ml with distilled water, mixed with 1.0 ml Cu²⁺ reagent, heated to 100 °C for 20 min and cooled. The arsenomolybdate reagent (1.0 ml) was then added, followed by 7.0 ml distilled water. The *A*₅₇₀ of the samples were compared with the *A*₅₇₀ produced by standard glucose solutions (0 to 100 µg).

The types of glycosidic linkage in glucan polymers were determined by methylation, methanolysis and gas-liquid chromatographic techniques described in detail by Baird *et al.* (1973). At least 96 % of the bonds in the dextran sample were (1→6)-α and 90 % of the bonds in the insoluble glucan were (1→3)-α.

Raffinose, dextran and polyethylene glycol were obtained from BDH, and Lubrol sample 12A9 was a gift from ICI. The standard (1→3)-α-glucan was purified from *Peptobovus betulinus* and was a gift from D. C. Ellwood.

RESULTS

Sequential solubilization of extracellular proteins precipitated by ammonium sulphate

Little enzyme activity redissolved during the initial extraction with acetate buffer of proteins precipitated from culture supernatant of strain 3209 by 70 % saturated (NH₄)₂SO₄ (Table 1), but the trace of dextranase activity was stimulated by dextran. Most of the fructosyltransferase and dextranase activities redissolved during the third and fourth extractions, but 70 % of the mutanase activity was recovered in the final insoluble pellet. None of these activities was greatly stimulated by dextran, but each sample was heavily contaminated with polysaccharide (Table 1). Virtually identical results were obtained when extracellular proteins from strain C67-1 were precipitated by 70 % saturated (NH₄)₂SO₄.

Rates of insoluble glucan synthesis by the redissolved proteins were stimulated 6- to 8-fold by 1.55 M-(NH₄)₂SO₄, but mutan synthesis by the final pellet was slightly inhibited. The increased rate of insoluble glucan synthesis therefore appears to be due to an effect of (NH₄)₂SO₄ on dextranase activity rather than on mutanase. To test this suggestion convincingly, glucosyltransferase was first partially purified to separate it from the contaminant fructosyltransferase activity.

Fig. 1. Elution of various glucosyltransferase samples (strain 3209) from Sepharose 6B. The samples and the columns were equilibrated with 2.0 M-NaCl in 0.1 M-sodium acetate, pH 6.0. Each sample (7 to 10 ml; 10 to 20 mg protein ml⁻¹) was re-equilibrated with the column buffer and loaded on to the column. The flow rate was 18 ml h⁻¹ and 4.8 to 5.0 ml fractions were collected. The samples were (a) pooled proteins which redissolved in extracts 2, 3 and 4 from the initial 70 % saturated (NH₄)₂SO₄ pellet (Table 1); (b) the mutansucrase pool (fractions 29 to 33) from (a). ○, Mutansucrase activity [without (NH₄)₂SO₄ in the assay]; ●, soluble glucan synthesis; △, fructosyltransferase activity; ▲, A₂₈₀. The arrow in (b) indicates the expected position for elution of mutansucrase activity.

Elution of glucosyltransferase activity from Sepharose 6B

Precipitated proteins from strain 3209 which redissolved during the second, third and fourth extractions with acetate buffer were concentrated, dialysed against 2 M-NaCl in acetate buffer and filtered through Sepharose 6B. Most of the mutansucrase activity eluted well after the void volume, immediately before dextransucrase activity (Fig. 1a). Fructosyltransferase was eluted predominantly between the void volume and mutansucrase but some activity was also detected in subsequent tubes. Virtually no fructosyltransferase activity was lost during gel filtration and the specific enzymic activity of the most active fractions was increased 8-fold.

Fractions (29 to 33) with mutansucrase activity from the first column were pooled and dialysed for 72 h at 4 °C against four changes (each 4 l) of distilled water. Only 22 % of the mutansucrase activity was retained during dialysis, but this loss was largely compensated by a 1280 % increase in dextransucrase activity (Table 2, line 8). The dialysate was concentrated,

Table 2. *Specific activities and yields of glucosyl- and fructosyltransferases after filtration through Sepharose 6B*

Activities refer to the enzyme preparations from strain 3209 used for the experiments shown in Fig. 1.

Sample	Enzyme activities [mg glucose or fructose polymerized h ⁻¹ (mg protein) ⁻¹]		
	Fructosyltransferase	Mutansucrase	Dextransucrase
Redissolved (NH ₄) ₂ SO ₄ precipitate applied to 1st Sepharose column	3.86	4.39	7.67
Dextransucrase pool (fractions 35 to 48) from 1st Sepharose column	1.40	0.10	12.90
Mutansucrase (fractions 29 to 33) from 1st Sepharose column	5.11	24.5	0.84
Mutansucrase applied to 2nd Sepharose column after dialysis	4.48	5.46	10.00
Fructosyltransferase pool (fractions 30 to 34) from 2nd Sepharose column	33.2	0.05	0.2
Leading edge of glucosyltransferase pool (fractions 39 to 44) from 2nd Sepharose column	2.52	0.05	35.1
Trailing edge of glucosyltransferase pool (fractions 45 to 50) from 2nd Sepharose column	0.70	0.05	38.3
Activity retained during dialysis of pooled fractions 29 to 33 from 1st Sepharose column	88 %	22 %	1283 %
Activity recovered from 2nd Sepharose column	89 %	0 %	108 %

re-equilibrated with 2 M-NaCl in acetate buffer and again filtered through Sepharose 6B (Fig. 1*b*). No mutansucrase activity was detected in the eluate, but more dextransucrase activity was eluted than was applied to the column (Table 2, line 9). When fractions with dextransucrase activity were incubated with sucrose in the presence of 1.55 M-(NH₄)₂SO₄, the glucan formed was predominantly insoluble in water. Carbohydrate was readily detected associated with the eluted enzyme, so it is possible that this carbohydrate interacted with the column matrix and that the elution of dextransucrase activity was thereby retarded.

Glucosyltransferase preparations from strain C67-1 differed from those from strain 3209 in that only a trace of mutansucrase activity was detected in the eluate from the first Sepharose 6B column. More dextransucrase activity was again eluted than was applied to the column but a mixture of water-insoluble and soluble glucans were formed when active fractions were incubated with sucrose in the presence of 1.55 M-(NH₄)₂SO₄.

Polyacrylamide gel electrophoresis of glucosyl- and fructosyltransferases eluted from Sepharose 6B

Proteins in the sample applied to the first Sepharose column and in the pools of eluted fructosyl- and glucosyltransferase activity (Fig. 1*b*) were separated by SDS-polyacrylamide gel electrophoresis. Although the sample applied to the first Sepharose column (Fig. 1*a*) was extremely heterogeneous (Fig. 2, track 1), essentially only three protein species of high molecular weight were detected in samples corresponding to the trailing edge of the peak of dextransucrase activity illustrated in Fig. 1*b*): the lowest molecular weight component of the triplet was the dominant species (Fig. 2, track 2). The leading edge of the dextransucrase peak was more heterogeneous and was relatively enriched with the highest molecular weight component of the triplet (Fig. 2, track 3). The fructosyltransferase pool was extremely heterogeneous, but the two most prominent protein bands were absent from the dextransucrase pools (Fig. 2, track 4). After electrophoresis, some gels were incubated with sucrose, Lubrol and dextran. White bands of polysaccharide appeared after 24 to 48 h at positions corresponding to the three high molecular weight proteins in the dextransucrase pool,

Fig. 2. SDS-polyacrylamide gel electrophoresis of glucosyl- and fructosyltransferase samples. Track 1: sample applied to the 1st Sepharose 6B column (Fig. 1*a*). Tracks 2 to 4 were loaded with samples eluted from the 2nd Sepharose column (Fig. 1*b*): track 2, glucosyltransferase fractions 44 to 50; track 3, glucosyltransferase fractions 39 to 43; track 4, fructosyltransferase fractions 30 to 33. Track 5: protein standards. S and R indicate where white bands of polymer formed when gels were incubated with sucrose (S) or raffinose (R).

and with some samples two or three bands of polymer formation were associated with proteins of lower molecular weight. When gels were incubated with raffinose [a substrate for fructosyl- but not for glucosyltransferase (Russell, 1979)], the only protein species to form polysaccharide rapidly was one of the dominant species in the fructosyltransferase pool (Fig. 2, track 4). After 48 h, faint deposits of polysaccharide were also visible at positions corresponding to the three high molecular weight species in the dextransucrase pools. It is assumed, therefore, that the protein which readily becomes visible when incubated with raffinose is fructosyltransferase, and that the proteins which form polysaccharides from sucrose are predominantly glucosyltransferases. Although it is surprising that glucosyl- and fructosyltransferase activities had been retained by samples heated with SDS, similar phenomena were reported previously by Scales *et al.* (1975) and by Russell (1979). No residual activity was detected after electrophoresis if samples were heated for more than 5 min, but the pattern of stained proteins was unchanged.

Effect of enzyme concentration on soluble and insoluble glucan synthesis

The loss of mutansucrase activity during dialysis and gel filtration and the differences in protein composition of the leading and trailing edges of the peak of dextransucrase activity were consistent with the possibility that both dextransucrase and another protein are required to catalyse insoluble glucan synthesis. Samples from column fractions were therefore recombined and assayed for insoluble polymer formation when incubated with

Fig. 3. Effect of enzyme concentration on the rates of synthesis of soluble ($\square$), insoluble ($\blacksquare$) and total ($\circ$) glucan in the presence of $1.55\text{ M}-(\text{NH}_4)_2\text{SO}_4$. In (a), rates are expressed as mg glucan formed in the assay tube h^{-1} ; in (b), rates are expressed as mg glucan formed $\text{h}^{-1} (\text{ml enzyme})^{-1}$. The enzyme used for this experiment was fraction 39, Fig. 1(b).

sucrose and $(\text{NH}_4)_2\text{SO}_4$. Enzyme (0.1 ml) from fractions 39 and 47 (Fig. 1b) formed 0 and $75\text{ }\mu\text{g}$ insoluble glucan h^{-1} and 51 and $16\text{ }\mu\text{g}$ soluble glucan h^{-1} when incubated with sucrose alone; in contrast, $170\text{ }\mu\text{g}$ insoluble glucan h^{-1} and only $34\text{ }\mu\text{g}$ soluble glucan h^{-1} were formed when 0.1 ml samples of these fractions were incubated together. Thus insoluble glucan synthesis was apparently stimulated 2.3-fold but soluble glucan synthesis was inhibited 2-fold. Similar results were obtained with three independent enzyme samples from strain 3209 and with glucosyltransferase from strain C67-1. However, stimulations of the same order of magnitude were also obtained when samples of enzyme from adjacent fractions were combined. This suggests that the stimulatory effects were due to a non-linear dependence of the rate of insoluble glucan synthesis on the enzyme concentration rather than to fractions such as 39 and 47 (Fig. 1b) being relatively enriched in different proteins required for mutan synthesis. This was confirmed in subsequent experiments designed to determine the optimal conditions for insoluble glucan synthesis by partially purified dextransucrase.

At the lowest enzyme concentrations only soluble glucan was formed even in the presence of $1.55\text{ M}-(\text{NH}_4)_2\text{SO}_4$, but with higher concentrations most of the product was water-insoluble (Fig. 3a). When the data were expressed as specific activities, it became apparent that the sum of the rates of soluble and insoluble glucan synthesis was constant, but that as the enzyme concentration was increased, insoluble glucan was progressively formed instead of soluble glucan (Fig. 3b).

Effect of the concentration of ammonium sulphate and other salts on soluble and insoluble glucan synthesis

The rate of insoluble glucan synthesis in the presence of various concentrations of $(\text{NH}_4)_2\text{SO}_4$ was determined. The optimal concentration was 1.55 M ; at higher concentrations insoluble glucan synthesis was inhibited. Ammonium citrate also stimulated insoluble glucan synthesis, the optimum concentration being 1 M . No insoluble glucan was formed when partially purified dextransucrase was incubated with sucrose and 1.0 , 2.0 or $3.0\text{ M}-\text{Na}_2\text{SO}_4$, NaCl or KCl or with $0.6\text{ M}-\text{K}_2\text{SO}_4$.

Fig. 4. Sedimentation of partially purified glucosyltransferase through a glycerol density gradient in the presence or absence of 1.55 M-(NH₄)₂SO₄. Glucosyltransferase from strain C67-1 was filtered through Sepharose 6B, concentrated by dialysis against polyethylene glycol and then against acetate buffer at 4 °C. Samples (0.3 ml) mixed with marker proteins were loaded on to 12.6 ml linear 10 to 30% (v/v) glycerol gradients with (○) or without (●) 1.55 M-(NH₄)₂SO₄. Tubes were centrifuged and fractionated as described in Methods. The glucosyltransferase activity of each 0.8 ml fraction was assayed by incubating 0.25 ml with 2.25 ml sucrose, 1.55 M-(NH₄)₂SO₄ and acetate buffer for 6.5 h at 37 °C.

Apparent molecular weight of glucosyltransferase during glycerol gradient centrifugation

In the absence of (NH₄)₂SO₄, glucosyltransferase sedimented through a linear glycerol gradient as a symmetrical peak of activity with an apparent molecular weight in the range 100000 to 110000 (Fig. 4). Most of the activity sedimented slightly less rapidly in the presence of 1.55 M-(NH₄)₂SO₄, but 30% was recovered as a pellet at the bottom of the tube (Fig. 4). Thus, part of the sample applied to the gradient had been aggregated (or precipitated) by 1.55 M-(NH₄)₂SO₄. Each active fraction catalysed the synthesis of only soluble glucan when incubated with sucrose alone but predominantly formed insoluble glucans when incubated with sucrose and 1.55 M-(NH₄)₂SO₄. Graphs of the two types of activity plotted against fraction number gave coincident peaks.

Effect of dextran on rates of soluble and insoluble glucan synthesis

One major source of confusion in the glucosyltransferase literature concerns the effect of dextran on glucan synthesis (compare Germaine *et al.*, 1977, with Robyt & Corrigan, 1977). In general, dextran stimulates the rate of soluble glucan synthesis, but inhibits insoluble glucan synthesis (Montville *et al.*, 1977).

Even very low concentrations of dextran apparently inhibited the rate of formation of insoluble glucan by the supernatant fluid from cultures of strain C67-1, but soluble glucan synthesis was stimulated (Fig. 5). In several, but not all, experiments, the increased rate of soluble glucan synthesis was greater than the inhibition of insoluble glucan synthesis, so the total glucosyltransferase activity increased when dextran was added. Essentially similar results were obtained with unpurified enzyme from strain 3209, but the concentrations of dextran used were far below those used by Germaine *et al.* (1974) to stimulate dextran synthesis by glucosyltransferase from the serotype *d* strain, 6715.

Dextran slightly stimulated the total rate of glucan synthesis by partially purified glucosyltransferase from strain 3209 in the absence of (NH₄)₂SO₄, but the product was entirely water-soluble. Although the total amount of glucan formed was unaffected by (NH₄)₂SO₄, the relative proportions of soluble and insoluble products depended on both the incubation period and the presence of dextran (compare Figs 6*a* and 6*b*). In the absence of dextran the initial product was entirely water-soluble, but after 0.4 h almost all of the glucan formed was insoluble (Fig. 6*a*). When dextran was added, insoluble glucan was synthesized only after a lag of 1.5 h and the appearance of the first insoluble product coincided with a

Fig. 5. Effect of dextran on glucosyl- and fructosyltransferase activities. Strain C67-1 was grown in continuous culture with excess glucose. Culture supernatant (1.0 ml) was incubated for 2 h at 37 °C with 1.0 ml 0.29 M-sucrose and various concentrations of dextran (mol. wt 60000 to 90000), and the quantities of accumulated glucan and fructan were determined. The error bars indicate the range of enzyme activities calculated from two independent assays.

decrease in the concentration of soluble glucan (Fig. 6*b*). The subsequent rate of insoluble product formation was sufficiently greater when dextran was added to compensate for the loss of water-soluble product (Fig. 6*b*). These results are entirely consistent with the suggestion that glucosyltransferase first forms a water-soluble precursor (a 'dextran') which is subsequently converted into the insoluble glucan (Germaine *et al.*, 1977).

Ammonium sulphate-dependent synthesis of insoluble glucan by glucosyltransferase from a non-cariogenic mutant

Mutant C67-25, derived from strain C67-1, differs from its parent in that it is unable to induce carious lesions when experimental animals are fed a sucrose-rich diet (de Stoppelaar *et al.*, 1971; Wenham, 1979). During growth in continuous culture, the mutant secretes approximately 40 % of the glucosyltransferase activity of its parent, but the glucan product is entirely water-soluble (Wenham *et al.*, 1978). Enzyme from 4 l of culture supernatant was precipitated with 70 % saturated $(\text{NH}_4)_2\text{SO}_4$, redissolved in acetate buffer, equilibrated with 2 M-NaCl in acetate buffer and filtered through a column of Sepharose 6B. A single peak of glucosyltransferase activity was eluted well after the void volume. When incubated with sucrose alone, active fractions catalysed the synthesis of only soluble glucans, but both soluble and insoluble glucans were formed during incubation with sucrose plus 1.55 M- $(\text{NH}_4)_2\text{SO}_4$. Thus, even glucosyltransferase from a non-cariogenic mutant strain will catalyse insoluble glucan synthesis under these incubation conditions.

Susceptibility of glucans to hydrolysis by dextranase and mutanase

The soluble glucans synthesized by partially purified glucosyltransferases from *S. mutans* strains 3209, C67-1 and C67-25 in the absence of $(\text{NH}_4)_2\text{SO}_4$ were resistant to hydrolysis by mutanase but were degraded by dextranase (Table 3). In contrast, the insoluble glucans made with samples of the same enzyme preparations in the presence of 1.55 M- $(\text{NH}_4)_2\text{SO}_4$

Fig. 6. Effect of dextran on the time course of soluble (□) and insoluble (■) glucan synthesis. The partially purified dextranase from strain 3209 (0.1 ml of fraction 48, Fig. 1b) was incubated with 0.5 ml 0.29 M-sucrose and 0.1 M-sodium acetate, pH 6.0 (final vol. 2.0 ml) without (a) or with (b) 60 µg dextran ml⁻¹.

Table 3. *Hydrolysis of glucans by mutanase and dextranase*

Polymers were incubated for 2 h at 37°C with dextranase or mutanase (1 mg ml⁻¹). The concentration of reducing sugar after incubation was corrected for the concentration of reducing sugar in control tubes with enzyme alone and with glucan alone and was then expressed as a percentage of the total concentration of glucose equivalents originally added.

Glucan	Glucan concn (mg ml ⁻¹)	Strain of <i>S. mutans</i>	Percentage hydrolysed by	
			Mutanase	Dextranase
Standard (1→3)-α-glucan	1.0	—	35	4
Standard (1→6)-α-glucan	1.0	—	3	37
Soluble glucan synthesized by partially purified glucosyltransferase	0.84	3209	2	20
		C67-1	0	17
		C67-25	3	23
Insoluble glucan synthesized by partially purified glucosyltransferase in the presence of 1.55 M-(NH ₄) ₂ SO ₄	0.73	3209	14	13
		C67-1	31	12
		C67-25	18	13
Mutan synthesized by the culture filtrate	1.17	3209	21	6
		C67-1	23	6

were hydrolysed by both mutanase and dextranase. As expected, mutans synthesized by culture filtrates of strains 3209 and C67-1 in the absence of 1.55 M-(NH₄)₂SO₄ were far more extensively hydrolysed by mutanase than by dextranase.

Commercial dextran with predominantly (1→6)-α glycosidic bonds was extensively hydrolysed by dextranase but was a poor substrate for mutanase (Table 3). Conversely, the (1→3)-α-glucan from *Peptobovus betulinus* was extensively hydrolysed by mutanase but not by dextranase. We can conclude, therefore, that the insoluble glucans made by dextranase in the presence of 1.55 M-(NH₄)₂SO₄ are structurally different to the soluble glucans made by the same enzyme: full structural analyses of these polymers will be required to confirm that they contain a high proportion of (1→3)-α bonds which are absent from the soluble product of the dextranase reaction.

DISCUSSION

Results of previous experiments in this laboratory led to the suggestion that dextran-sucrase and mutansucrase activities result from interconvertible forms of a single glucosyltransferase protein (Mohan *et al.*, 1979). Ebert & Schenk (1968) also proposed that soluble and insoluble glucans were synthesized by a single enzyme and a plausible mechanism was subsequently proposed by Robyt (1979).

Our results provide an experimental basis for Robyt's model and confirm that glucosyltransferase occurs in two forms which differ in their degree of aggregation. Evidence that the aggregated form catalyses insoluble glucan synthesis includes the retention of mutansucrase activity in proteins which failed to redissolve after precipitation by 70% saturated $(\text{NH}_4)_2\text{SO}_4$ (Table 1) and the elution of mutansucrase before dextran-sucrase from Sepharose 6B (Fig. 1*a*, Table 2). In contrast, dextran-sucrase activity redissolved more readily after precipitation with $(\text{NH}_4)_2\text{SO}_4$ (Table 1), eluted from Sepharose 6B after mutansucrase activity and sedimented slowly through a glycerol density gradient, with an apparent molecular weight in the range 100000 to 110000 (Fig. 4).

The total glucosyltransferase activity (i.e. the sum of the dextran-sucrase and mutansucrase activities) was almost quantitatively recovered after dialysis and gel filtration through two columns of Sepharose 6B, but mutansucrase was gradually lost and there was a corresponding increase in dextran-sucrase activity (Table 1, Fig. 1*b*). This demonstrates that dextran-sucrase activity can be released from a mutansucrase aggregate. Conversely, dextran-sucrase preparations which were devoid of mutansucrase activity catalysed the synthesis of insoluble glucan when incubated with sucrose and 1.55 M- $(\text{NH}_4)_2\text{SO}_4$ and were partially reaggregated to a form that sedimented rapidly through a glycerol density gradient (Table 1, Fig. 4). Most of the dextran-sucrase activity sedimented less rapidly in the presence of 1.55 M- $(\text{NH}_4)_2\text{SO}_4$ because this high concentration of salt increases the density of the gradient; glycerol partially inhibits glucan synthesis, so we assume that this interaction prevented the complete aggregation of dextran-sucrase. The concentrated dextran-sucrase applied to electrophoresis gels migrated slowly and catalysed insoluble glucan synthesis on incubation with sucrose after electrophoresis (Fig. 2). These observations and the enzyme concentration dependence of soluble and insoluble glucan synthesis (Fig. 3) provide further evidence that the disaggregated dextran-sucrase can be converted back to an aggregated form which catalyses mutan synthesis. Until dextran-sucrase has been purified to homogeneity, it will be impossible to state whether other proteins or a polysaccharide are also required for the disaggregated enzyme to catalyse mutan synthesis, or for the aggregation process itself. Mutant C67-25 could be non-cariogenic because its glucosyltransferase is unable to aggregate naturally to the form which catalyses mutan synthesis, the molecular defect being in either the primary structure of glucosyltransferase itself, or in the hypothetical association factor.

One possible explanation of the effect of dextran is that it preferentially binds to disaggregated glucosyltransferase. If so, disaggregation would be promoted either because dextran increases the solubility of the disaggregated form, or because it competes with other glucosyltransferase molecules or association factors for binding sites. This suggestion is similar to that of Montville *et al.* (1977): it implies that dextran inhibits the rate of (1→3)- α bond formation. Alternatively, dextran could simply compete with precursors of insoluble glucans as substrates into which new (1→3)- α bonds are inserted. Dextran is extremely soluble in water: a considerable number of (1→3)- α branch points would therefore need to be incorporated before an exogenous dextran precursor became insoluble and therefore detectable in the mutan assay. This second explanation, which is more consistent with observations in Fig. 6(*a, b*), predicts that dextran would have no effect on the rate of (1→3)- α bond formation, that it would delay the first appearance of insoluble glucan during incubation with sucrose, but that after this delay, the subsequent rate of mutan accumulation would

be enhanced as the precursor glucans were enlarged to their solubility limit. The second explanation is also consistent with reports that the magnitude of the dextran effects are independent of the molecular weight of the added primer, and are observed even when the terminal, non-reducing hydroxyl groups of the dextran have been blocked with tripsyl chloride (Germaine & Schachte, 1976; Robyt & Corrigan, 1977).

Our model is also compatible with the mechanism for glucan synthesis proposed by Robyt (1979), namely that there are two equivalent nucleophilic sites on glucosyltransferase at which either sucrose or a polysaccharide can be bound. When preformed glucan is unavailable, glucosyltransferase binds sucrose at both sites with the release of fructose and formation of a (1→6)- α bond by an insertion mechanism at C-1. A third sucrose molecule can then bind to the vacated site, fructose is again released and the glucosyl moiety is the acceptor in the formation of a second (1→6)- α glycosidic bond. Robyt (1979) proposed that the same protein can catalyse (1→3)- α bond formation when a preformed glucan is the glucosyl- or dextranosyl-acceptor in the absence of sucrose. If exogenous glucan must bind to one of the active sites before a (1→3)- α bond is formed, enzyme aggregation will both decrease the accessibility of the second site to sucrose and will also increase the chance of dextranosyl-transfer between different enzyme molecules. Either effect would result in an increased rate of (1→3)- α bond formation but a decreased rate of (1→6)- α bond formation by the aggregated enzyme. We suggest that glucosyltransferase aggregation is an important factor in determining the rate of mutan synthesis by, and therefore the cariogenicity of, *S. mutans* strains.

We are grateful to Dr R. R. B. Russell for many helpful suggestions, for confirming the identity of the extracellular enzymes secreted by *S. mutans* and for communicating his results to us prior to publication. The research was supported by grants from The Medical Research Council to B.M.N. and J.A.C.

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