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α-D-GLUCOPYRANOSYL FLUORIDE AS A D-GLUCOPYRANOSYL DONOR FOR A GLYCOSYLTRANSFERASE COMPLEX FROM *Streptococcus mutans* FA1

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ABSTRACT

α-D-Glucopyranosyl fluoride can serve as the D-glucopyranosyl donor for the glycosyltransferase from *Streptococcus mutans*. The products of the reaction are a D-glucan of high molecular weight and fluoride ion The rate of reaction was measured by an electrode specific for the fluoride ion The reaction was inhibited by the substrate at concentrations >30mM, but was not affected by fluoride ion There was little inhibition of the reaction by a series of monosaccharides, except for 2-amino-2-deoxy-D-glucose, D-mannitol, and 3-deoxy-D-*ribo*-hexose Maltose, isomaltose, isomaltotriose, and D-fructose all stimulated the release of fluoride from α-D-glucopyranosyl fluoride

INTRODUCTION

The capability of certain oral micro-organisms to elaborate extracellular polysaccharides when cultured in the presence of sucrose has been shown to be involved in the formation of dental plaque The enzyme(s) catalyzing the formation of either dextrans or levans from sucrose have been isolated and purified from several different subtypes of *Streptococcus mutans*[1–5] and from *Streptococcus salivarius*[6,7]. Several of these enzyme preparations from *S mutans* also contain either invertase[8] or levansucrase[1] activity, in addition to the dextransucrase activity. In general, the enzymic activity has been measured by the release of D-glucose, the increase of total reducing sugar, or the formation of polymer The presence of several sucrase activities in a partially purified preparation complicates the analysis of any one of these competing reactions We now describe the use of α-D-glucopyranosyl fluoride as the α-D-glucopyranosyl donor in D-glucan formation by the dextran-sucrase [(1→6)-α-D-glucan D-fructose 2-glucosyltransferase, EC 2 4 1 5] from *S mutans* FA1 The general assay employs an electrode specific for the fluoride ion to measure the release of fluoride during D-glucan synthesis

MATERIALS AND METHODS

Preparation of the glycosyltransferase. — The glycosyltransferase complex, which contains both dextransucrase and levansucrase, was isolated from[1] the culture broth of *Streptococcus mutans* FA1. The enzyme preparation used in this study was purified by ammonium sulfate precipitation, and gel filtration on a column of Bio-Gel P-60. As the purified enzyme aggregates when the preparation is concentrated, the P-60 purified enzyme was not concentrated beyond 100 μg ml^{-1} The ammonium sulfate precipitate, used in several studies, will be referred to as the crude enzyme preparation (CEP) The CEP had a specific activity of 0 26 U mg^{-1}, and the material purified on Bio-Gel P-60, an activity of 0 45 U.mg^{-1}. One unit of activity (U) releases one μmole of fluoride per minute from α-D-glucopyranosyl fluoride at 25° in phosphate buffer (pH 6 0, 50mM)

Synthesis of sugar derivatives. — D-Allose[9], 3-deoxy-D-*ribo*-hexose[10], and 6-deoxy-D-glucose[11] were prepared by published procedures. Trifluoroacetic acid was routinely used to remove the isopropylidene groups in these series of reactions The α-D-glucopyranosyl fluoride[12], α-D-allopyranosyl fluoride[12], 6-deoxy-α-D-glucopyranosyl fluoride, and 2-deoxy-α-D-*arabino*-hexopyranosyl fluoride[13] were prepared by treatment of the corresponding per-*O*-acetylated hexosides with liquid hydrogen fluoride, followed by deacetylation with sodium methoxide[14]. The n m r. spectrum of tri-*O*-acetyl-6-deoxy-D-glucopyranosyl fluoride in chloroform-*d* had the following characteristics: for H-1, τ 4 25 Hz ($J_{1,2}$ 3.5 Hz, $J_{1,F}$ 56 Hz); for H-6, τ 8.75 Hz, and, for acetyl H, τ 7 90–8 10 Hz The other acetylated glycosyl fluorides showed n m r - spectral values comparable to published values[12,13]

The deacetylated glycosyl fluorides contained ~5 to 10% of free sugar, and were used without purification, as neither the free sugars nor fluoride inhibited the enzymic reaction The degree of purity of each glycosyl fluoride was determined by the difference between acid-labile fluoride (10 min at 100°) in 50mM sulfuric acid and the free fluoride in the preparation. The amount of D-glucose was confirmed by D-glucose oxidase–peroxidase analysis, and by paper chromatography with 4:1 5 (v/v) butanol–ethanol–water In all cases, the glycosyl fluoride migrated farther than the free sugar The R_F values of the fluorides on silica gel G with 4:1 5 (v/v) butanol–ethanol–water are as follows. α-D-glucopyranosyl fluoride, 0 52, 2-deoxy-α-D-*arabino*-hexopyranosyl fluoride, 0 72, α-D-allopyranosyl fluoride, 0.40; and 6-deoxy-α-D-glucopyranosyl fluoride, 0 74

Measurement of fluoride. — The release of fluoride was measured with a fluoride-specific electrode (Orion Instrument Co , Cambridge, Mass) coupled to a recording pH-meter Routinely, the P-60-purified enzyme (450 μl, 0 045 U) and α-D-glucopyranosyl fluoride (50 μl, 50mM) were mixed in a 5-ml polyethylene beaker and the electrode was inserted About 20 sec was required for equilibration of the electrode, due to the free fluoride in the substrate preparation The reaction was monitored for 15 min, and the initial activity was determined The fluoride release was usually linear during the first 5–15 min of the reaction.

Analytical methods. — Total carbohydrate (D-glucan) was measured by the phenol–sulfuric acid method on dialyzed samples[15] D-Glucose was measured by the D-glucose oxidase–peroxidase method[16] Protein concentrations were determined by the Lowry method[17], using bovine serum albumin as the standard

Thin-layer chromatography. — Thin-layer chromatography (t.l.c.) was performed on silica gel G plates (Eastman Chemical Co.) developed with 4:1:5 (v/v) butanol–ethanol–water. Aniline–diphenylamine–phosphoric acid spray-reagent was used to detect the sugars[18].

Serological methods — New Zealand white rabbits were immunized by intravenous injection of heat-killed, whole cells of *S. mutans* grown in trypticase soy-broth supplemented with 5% of sucrose The animals were bled by cardiac puncture The reactivity of the sera was determined by standard, quantitative, precipitin reactions[19] Reactions of the soluble D-glucan were compared to those of the dextran from *Leuconostoc mesenteroides* B512 and from *S. mutans* cultured in the presence of sucrose.

EXPERIMENTAL

Characterization of the reaction of α-D-glucopyranosyl fluoride with the glycosyltransferase. — The rate of reaction was determined during 10 to 15 min at the various concentrations of enzyme. The release of fluoride was found to be linearly dependent on enzyme concentration (see Fig 1) The dependence of the reaction on α-D-glucopyranosyl fluoride (1–100mM) was measured at a constant concentration of enzyme

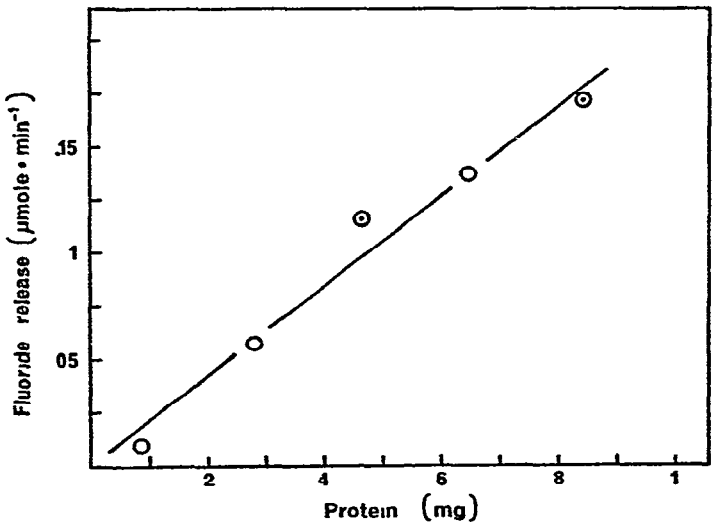

Fig 1 Dependence of the rate of release of fluoride from α-D-glucopyranosyl fluoride (5mM) on the amount of glycosyltransferase in the crude enzyme-preparation (0 20 U) (Identical results were obtained with the P-60 purified material)

(see Fig. 2) At a concentration of α-D-glucopyranosyl fluoride >30mM, there was a marked inhibition of the reaction The double reciprocal plot of the data (see Fig 3) yields a K_m of 11 1mM and a V_{max} of 1 3 μmole min^{-1}

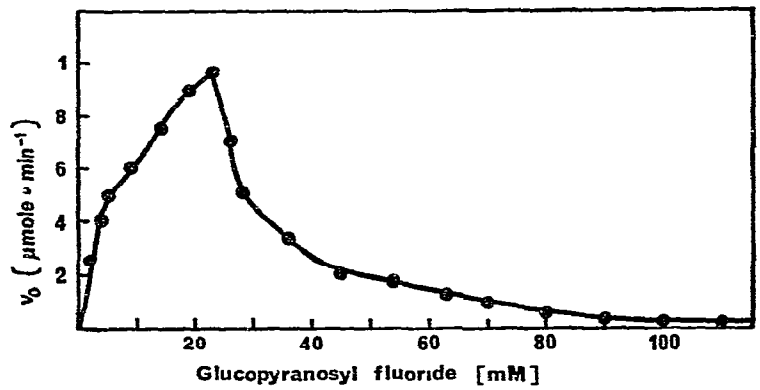

Fig 2 The effect of the concentration of α-D-glucopyranosyl fluoride on the rate of fluoride release, with a constant amount of P-60-purified glycosyltransferase (0 045 U)

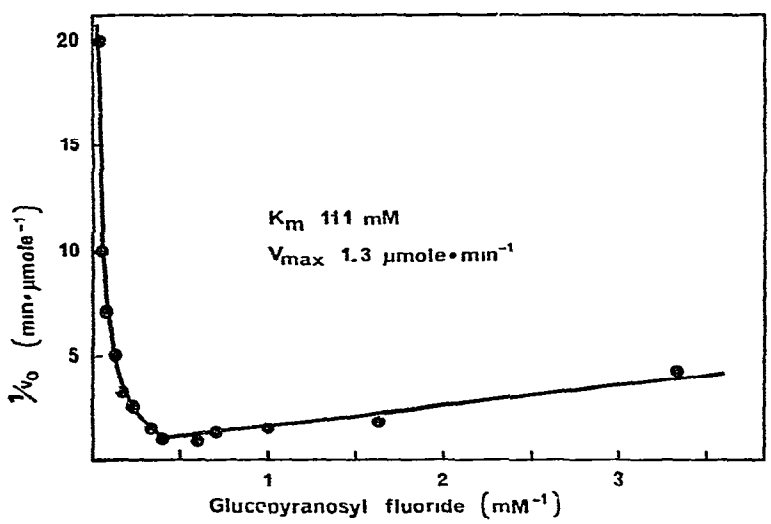

Fig 3 Double reciprocal plot of the rate of release of fluoride (μmole min^{-1}) at various concentrations of α-D-glucopyranosyl fluoride (Data from Fig 2)

The products of the reaction from α-D-glucopyranosyl fluoride were studied as follows A reaction mixture (40 ml) containing α-D-glucopyranosyl fluoride (5mM) and glycosyltransferase (1 62 U) was incubated at 25° Aliquots (1 ml) were assayed for D-glucose by the D-glucose oxidase method, for glucan by the phenol–sulfuric acid assay (following exhaustive dialysis), and for fluoride by the ion-specific

electrode (see Fig 4) D-Glucan was synthesized during the first 90 min of incubation However, fluoride was released in an amount greater than that corresponding to glucan synthesis, and its release continued after the glucan synthesis had stopped The enzyme appears to have the ability to hydrolyze the α-D-glucopyranosyl fluoride When the enzyme was denatured, there was little release of fluoride. The amount of fluoride released during the first 90 min was about equal to the amount of free D-glucose and D-glucan After this, the amount of fluoride released was greater than could be accounted for as corresponding to D-glucose release and D-glucan synthesis; this may be the result of formation of oligosaccharides, which would not be detected after dialysis

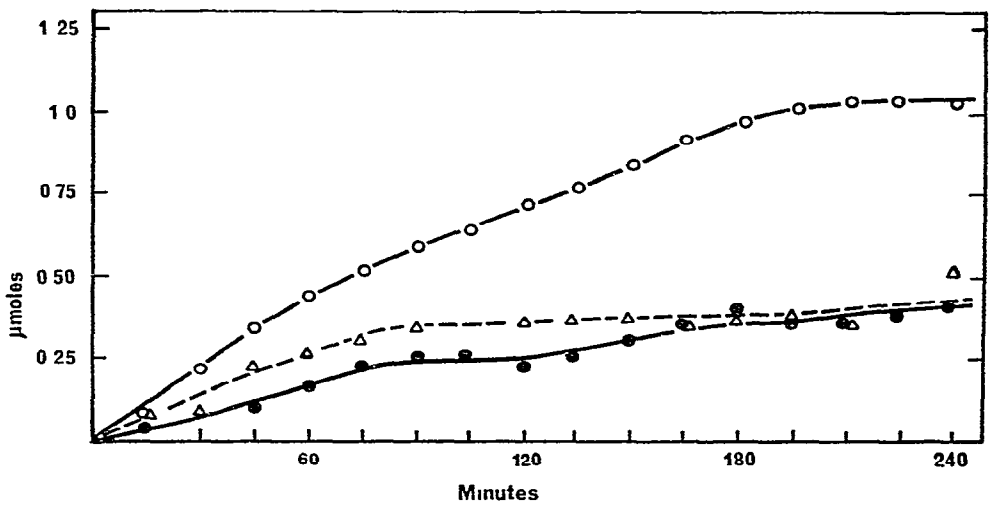

Fig 4 The liberation of fluoride and D-glucose, and formation of D-glucan, during the incubation of α-D-glucopyranosyl fluoride (5mM) and glycosyltransferase (1 62 U) in phosphate buffer (pH 6 0, 50mM) (Fluoride release, —○—, D-glucose release --△--, nondialyzaole glucan —●—)

Characterization of the glucan formed — An incubation mixture containing α-D-glucopyranosyl fluoride (5mM) and glycosyltransferase was incubated for 24 h, during which, the mixture became opalescent, indicating polymer formation. This was followed by formation of insoluble material, probably a glucan The glucan was precipitated from the mixture with ethanol (3 vol), and then collected by centrifugation and dried with absolute ethanol The insoluble material was soluble in alkali, a property characteristic of "insoluble" dextran The glucan was analyzed by (*a*) the phenol–sulfuric acid method, (*b*) D-glucose oxidase assay following acid hydrolysis for 5 h at 100° with 2M hydrochloric acid, and (*c*) the Lowry protein assay Based on the dry weight of the material, 83% was carbohydrate and 17% was protein. The protein most likely was from the enzyme preparation. T.l c of the hydrolyzate showed that glucose was the only carbohydrate and the D-glucose

oxidase assay revealed this to be D-glucose. The D-glucan was precipitated by anti-sera directed against (1→6)-α-D-glucans, suggesting that it contained α-D-(1→6)-linkages, as would be expected for a dextran. The isolated glucan and the dextran isolated from the culture broth of *S. mutans* FA1 gave identical, quantitative-precipitin curves with the anti-sera.

Use of various sugars as possible inhibitors of the glycosyltransferase. — As dextran has been associated with dental caries, a number of sugars were added to the assay system to determine if any of them would inhibit dextran synthesis from α-D-glucopyranosyl fluoride The effect of increasing sucrose concentration (0–50mM) on the release of fluoride from α-D-glucopyranosyl fluoride (30mM) is shown in Fig. 5. Competition between the two substrates was apparent from the data, which show a decrease in fluoride released as the sucrose concentration was increased; this might be expected if both were substrates for the enzyme. Addition of sodium fluoride (0–100mM, final concentration) to the standard reaction-mixture was without effect on the reaction

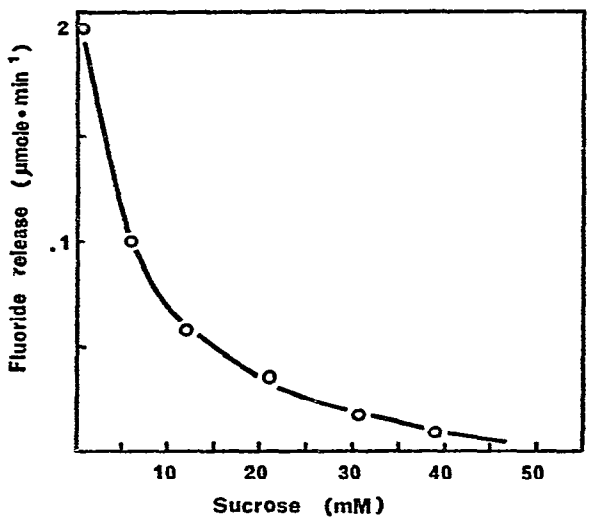

Fig 5. The effect of sucrose on the release by the glycosyltransferase of fluoride from α-D-glucopyranosyl fluoride (5mM)

A selection of sugars and sugar derivatives (25 and 50mM, final concentration) were separately added to the α-D-glucopyranosyl fluoride–glycosyltransferase mixture, and the initial rate of reaction was determined Most of the sugars and their alditol relatives had little inhibitory effect on the rate of fluoride release (see Table I) Of the sugars tested, 2-amino-2-deoxy-D-glucose, D-mannitol, and 3-deoxy-D-*ribo*-hexose had the greatest inhibitory effect Neither 6-deoxy-D-glucose (75mM) nor 6,6′-dideoxysucrose had any effect on the release of fluoride, but 6,6′-dideoxysucrose appeared to protect the enzyme from inactivation by acid. However, several sugars

TABLE I

EFFECT OF VARIOUS CARBOHYDRATES ON THE RATE OF RELEASE OF FLUORIDE FROM α-D-GLUCOPYRANOSYL FLUORIDE (5mM) BY THE GLYCOSYLTRANSFERASE FROM *S mutans* FA1

Compound added	*Percentage of control*	
	*25*mM	*50*mM
D-Galactose	100	100
D-Glucose	100	101
D-Tagatose	100	100
D-Xylose	100	107
D-Glucitol	100	101
Galactitol	100	89
α-Cyclodextrin	100	101
β-Cyclodextrin	100	100
D-Ribose	85	85
2-Amino-2-deoxy-D-glucose	68	79
D-Mannitol	74	76
2-Deoxy-D-*arabino*-hexose	85	88
3-Deoxy-D-*ribo*-hexose	57	54
L-Sorbose	110	124
D-Mannose	157	121
D-Arabinose	128	138
D-Allose	116	125
D-Fructose	285	293
L-Rhamnose	176	224
Maltose	290	290
Isomaltose	390	425
Isomaltotriose	415	440

greatly stimulated the release of fluoride, *viz* D-fructose, maltose, isomaltose, isomaltotriose, and L-rhamnose When 2-deoxy-D-*arabino*-hexopyranosyl fluoride, D-allopyranosyl fluoride, and 6-deoxy-D-glucopyranosyl fluoride (0 5–250mM) were tested for their reactivity as glycosyl donors, there was no release of fluoride None of these glycosyl fluorides acted as an inhibitor of α-D-glucopyranosyl fluoride

DISCUSSION

The presence of levansucrase and invertase in addition to dextransucrase in the purified enzyme-complex from *S. mutans* has complicated the routine analyses of the enzymic reaction[1] Hehre initiated the use of α-D-glucopyranosyl fluoride as the D-glucopyranosyl donor with dextransucrases from *Leuconostoc* and *Streptoccus*[20] and with an amylosucrase[21] In both enzyme-systems, a glucan was synthesized and fluoride was liberated. α-D-Glucosyl α-L-sorboside has been enzymically synthesized by sucrose phosphorylase from L-sorbose plus α-D-glucopyranosyl fluoride[22]. The use of α-D-glucopyranosyl fluoride has permitted us to monitor glucan synthesis, even in crude preparations of the glycosyltransferase complex, without interference

by the levansucrase The use of the fluoride-specific electrode simplified the measurement of fluoride release, and allowed ready determination of the initial velocities of the reaction Moreover, this assay now provides an easy test for the possible effects of reducing sugars, of sugars that might interfere with the D-glucose oxidase assay, and of primer glucans However, high concentrations of certain ions that are known to influence the electrode[23] must be avoided.

The incubation mixture of the glycosyltransferase and the α-D-glycopyranosyl fluoride became opalescent after 30 min, and insoluble glucan was formed after longer periods of incubation The soluble glucan was excluded from a column of Bio-Gel P-60, indicating a molecular weight >60,000 Acid hydrolysis showed the glucan to contain glucose (by t l c), and this was shown to be D-glucose by the D-glucose oxidase assay The D-glucan reacted with antisera against dextran, indicating that the D-glucan contains α-D-(1→6)-linkages The presence of (1→3)-branches in this enzymically synthesized D-glucan has not been established The α-D-glucopyranosyl fluoride was also hydrolyzed by the enzyme preparation, as free D-glucose was detected during the reaction (see Fig 4) The possibility that oligosaccharides are formed during the reaction has not been discounted, as the amount of fluoride released after 90 min is greater than that corresponding to the D-glucose released and the D-glucan synthesized Oligosaccharides would have been lost during the dialysis, prior to glucan analysis

The enzymic activity was inhibited by substrate concentrations >30mM Genghof and Hehre[20] described a similar phenomenon for the *Leconostoc* dextransucrase, but at somewhat higher concentrations of α-D-glucopyranosyl fluoride The Michaelis–Menten constant for the glycosyltransferase acting on sucrose is[1] 55mM; it was determined to be 11 1mM for α-D-glucopyranosyl fluoride Gold and Osher[24] reported a lower K_m value for sucrose phosphorylase acting on α-D-glucopyranosyl fluoride than on sucrose The amount of glucan synthesized by the glycosyltransferase from α-D-glucopyranosyl fluoride (5mM) was found to be ~6% and ~31% of that synthesized from sucrose at 146mM and 3mM, respectively It has been observed that the amount of glucan synthesized decreases as the concentration of sucrose decreases[1]

The general lack of inhibition of the enzyme by the various hexoses was not anticipated Competitive inhibition by D-glucose, 6-deoxy-D-glucose, D-mannose, D-arabinose, D-xylose, D-galactose, and 6,6′-dideoxysucrose might have been expected, owing to their general structural similarities to the D-glucopyranosyl moiety of sucrose The inhibition exhibited by 3-deoxy-D-*ribo*-hexose may reflect interference with the branching process, which then slows down the linear propagation of the chain The dextran from *S. mutans* E49 contains about 20% of (1→3) branch-points[25] The stimulation of the release of fluoride by D-fructose may be due to formation of sucrose or leucrose rather than of glucan Genghof and Hehre[20] showed that sucrose and leucrose [α-D-glucopyranosyl-(1→5)-D-fructopyranose] are synthesized by the *Leuconostoc* dextransucrase when D-fructose is added to a reaction mixture containing α-D-glucopyranosyl fluoride. Concentrations of D-fructose may occupy part of the enzymic site of the dextransucrase, and accept the D-glucopyranosyl

group bound on the enzyme Maltose, isomaltose, and isomaltotriose may serve as primers for the formation of the glucan or oligosaccharide(s), and stimulate the release of fluoride.

Similar stimulation of streptococcal dextransucrases by isomaltose has been reported with sucrose as the D-glucosyl donor[26 27] Dextrans having molecular weights ranging from 20,000 to 2×10^6 in this study did not stimulate the release of fluoride Similar enzyme preparations from other subtypes of *S mutans* are stimulated by the addition of dextrans[27 28]

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