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IN-VITRO INHIBITION OF GLUCOSYLTRANSFERASE FROM THE DENTAL PLAQUE BACTERIUM *STREPTOCOCCUS MUTANS* BY COMMON BEVERAGES AND FOOD EXTRACTS

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Summary—Some fruit juices and beverages inhibit the glucosyltransferases of *Streptococcus mutans*. Inhibition by cocoa, coffee and tea was due partly to gelatin-precipitable tannins and partly to components that exhibited properties of monomeric polyphenols. Charcoal treatment removed all inhibitory activity. Catechin, a known constituent of these beverages, was an effective inhibitor of the enzymes. The effects of the fruit juices were attributable mainly to the inhibition of the glucosyltransferases by the endogenous fructose and glucose. The findings show that naturally-occurring constituents of foods can inhibit extracellular polysaccharide formation from sucrose. Such constituents may play a role in regulating dental plaque formation *in vivo* and, thereby, may have long-term effects on the development of dental caries.

INTRODUCTION

Dietary intake of fermentable carbohydrates is a major factor in the complex system that leads to dental caries. However, certain foods and food constituents reduce the cariogenicity of high-sugar diets, or are associated with less than expected caries in experimental animal systems (Gustafsson *et al.*, 1954; Bibby, 1970; Mandel, 1970; Krasse, 1978; Navia and Lopez, 1983). Stralfors (1967) reported that cocoa in the diet led to a reduction in hamster caries; Grenby (1974) demonstrated, in short-term experiments in man, that a high chocolate diet reduced plaque accumulation compared to that in volunteers on a normal diet. Rosen *et al.* (1984) showed an anti-caries effect of tea in rats.

There is considerable evidence that *Streptococcus mutans* is causally associated with caries, and that cariogenicity is related to the ability of these micro-organisms to convert sucrose to extracellular polysaccharides. We showed that cocoa extracts inhibit the formation of extracellular polysaccharides by several strains of *Strep. mutans* and other oral micro-organisms (Paolino and Kashket, 1985). Palenik *et al.* (1977) reported similar results. We have proposed that the inhibition of glucosyltransferases by cocoa extracts accounts, at least in part, for the reported effects of cocoa and cocoa products on plaque accumulation and caries prevalence. Indeed, Palenik *et al.* (1979) showed that water extracts of cocoa powder reduced adherent growth of *Strep. mutans*. Furthermore, our preliminary evidence suggested that the tannins of cocoa are responsible for the effects on the enzymes. As it is known that tea, coffee and many fruit juices contain tannins, we have now examined the effects of these food extracts on the biosynthesis of extracellular polysaccharide.

MATERIALS AND METHODS

Bacterial cultures

Strep. mutans strain 6715 was obtained from the culture collection of Forsyth Dental Centre. Cultures were grown in brain heart infusion (BHI; Difco Laboratories, Detroit, Michigan, U.S.A.) at 37°C under N_2–H_2–CO_2 (80:10:10). Glucosyltransferase (GTF) was prepared by centrifuging an 18 h culture at 10,000 *g* for 10 min and retaining the supernatant. The enzyme was stored at −20°C.

Food extracts

Extracts of tea (tea L, T. J. Lipton, Incorporated, Englewood Cliffs, New Jersey, U.S.A. or tea S, Salada Foods, Incorporated, Little Falls, New York, U.S.A.) were prepared by immersing from one to five tea bags (average dry weight 2.1 g/bag) in 100 ml distilled water at 100°C for 3 min. The tea bags were removed, and the liquid was cooled and stored at −20°C. Instant coffee (Nescafe, The Nestle Company, White Plains, New York, U.S.A) was prepared by dissolving 1 tablespoon in 200 ml boiling water. Cocoa (Hershey's Cocoa, Hershey Foods Corporation, Hershey, Pennsylvania, U.S.A.) was prepared by extracting 30 g cocoa powder into 100 ml of distilled water at 100°C for 1 h. The mixture was cooled and undissolved particles were removed by centrifugation at 10,000 *g* for 10 min. The supernatant fluid was retained and stored at −20°C. Fruit juices were obtained from local retail stores. Black cherry (Hanson's Foods, La Mirada, California, U.S.A.), apple (J. Coutts and Son, Boxboro, Massachusetts, U.S.A.), cranberry (Knudsen and Son, Chico, California, U.S.A.), prune (Eden Foods, Ann Arbor, Michigan, U.S.A.), and grape (Welch Foods, Incorporated, Concord, Massachusetts, U.S.A.) juices were obtained as commercial preparations that were stated to contain only the labelled juice and water. After opening, the pH of each of the juices was adjusted to neutrality with NaOH and the juices were stored at −20°C. Extracts of peanuts, bananas, almonds and Swiss cheese were prepared by first blending each in a minimal quantity of water. The resulting paste was processed in a tissue homogenizer, diluted to 1 g/10 ml, filtered and stored at −20°C.

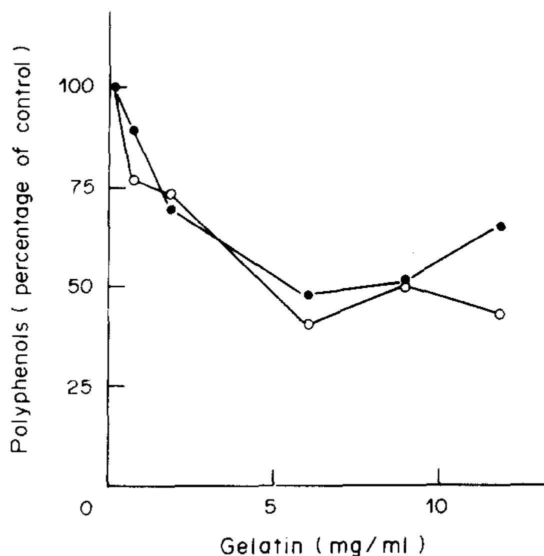

Fig. 1. Precipitation of tea tannins by gelatin. Tea extracts were mixed with equal volumes of solutions containing increasing concentrations of calf-skin gelatin in saturated NaCl. The resulting precipitates were removed by centrifugation and the polyphenol contents of the supernatant fluids were determined. Tea L (●); tea S (○).

Removal of tannins

Condensed tannins (polymeric polyphenols, or condensed proanthocyanidins; Haslam, 1979) were removed from the extracts by mixing the samples with an equal volume of gelatin in saturated NaCl, and removing the resulting precipitates by centrifugation (modified from Association of Official Agricultural Chemists, Official Methods of Analysis). Control samples of extract were diluted with an equal volume of saturated NaCl. All samples were further diluted 5-fold with H_2O before assay, in order to achieve about 50 per cent inhibition of GTF. Tannin concentrations were calculated as the differences between the total polyphenols and the polyphenols remaining in solutions after gelatin treatment. Preliminary experiments showed that precipitation of tannins increased with the concentration of added gelatin, with a maximum at about 5 mg of gelatin/ml (Fig. 1). In subsequent experiments, gelatin was added in excess (12 mg/ml) of that needed to effect maximal tannin removal.

Chemical assays

Dry weight was determined by drying at 80°C for 18 h. Total carbohydrate was determined with the phenol-sulphuric acid method (Dubois *et al.*, 1956). Glucose was determined with a glucose oxidase-based analyser (Yellow Springs Instrument Company, Yellow Springs, Ohio, U.S.A.). Sucrose was determined following enzymic hydrolysis with invertase and subsequent analysis of glucose as described. Fructose was determined with hexokinase. Calcium analysis was carried out with a flame-emission spectrophotometer and inorganic phosphate was determined with the method of Penniall (1966). Fluoride content was determined with a fluoride-specific electrode (Orion Research, Incorporated, Cambridge, Massachusetts, U.S.A.).

Total polyphenols were measured with the Folin–Ciocalteau reagent according to Hoff and Singleton (1966). Tannic acid from gall nuts were used as the standard. Tannic acid, calf-skin gelatin (type IV), and (+)catechin were obtained from the Sigma Chemical Company, St Louis, Missouri, U.S.A.

Extracellular polysaccharide (EPS) biosynthesis

Glucosyltransferase activity was determined by following the rate of incorporation of label from [^{14}C]-sucrose into polysaccharide using a modification of the method of Montville, Cooney and Sinskey (1977). The assay system included 10 μl of 1 per cent dextran T-10 (Pharmacia Fine Chemicals, Piscataway, New Jersey, U.S.A.), 10 μl of 0.35 M NaF, 50 μl of 1 M sodium acetate buffer, pH 5.5, 200 μl of cell-free supernatant, 10 μl of 0.5 M sucrose and 50 μl (1 μCi) of [^{14}C-U]-sucrose (New England Nuclear, Boston, Massachusetts; 400–700 Ci/mol), and up to 500 μl of extract in a total volume of 1.0 ml. The extracts behaved as weak buffers, but did not affect the final pH of the complete system. Incubation was at 37°C. At 0 and 100 min, 6 replicate samples of 50 μl each were withdrawn and applied to Whatman 3MM discs (2.5 cm). Total-radioactive polysaccharide was determined after washing three of the discs in absolute methanol. Water-insoluble polysaccharide was determined on the three remaining discs by washing once with methanol, twice with water and finally with methanol. Water-soluble polysaccharide was calculated as the total minus the water-insoluble products. Reaction rates were shown to be constant for at least 100 min. Radioactivity was measured in a liquid scintillation system. The coefficient of variation of the assay was 4.5 per cent.

Preliminary experiments were carried out in which enzyme activity was followed by measuring the release of glucose (with glucose oxidase) and total reducing sugars from unlabelled sucrose. The data indicated that the bulk of the reducing sugar was fructose and that at least 80 per cent of total EPS formation was attributable to GTF activity. Low levels of dextranase were detected in the enzyme preparations.

RESULTS

Tables 1 and 2 present the analyses of the various food extracts. Fruit juices contained high concentrations of sugars whereas cocoa, tea, coffee, banana, nuts and cheese contained little detectable carbohydrate. Glucose, fructose and sucrose (measured enzymically) made up about 30 per cent more than the chemically-determined total carbohydrate, and accounted for over 90 per cent of the dry weight of the juices. The juices also contained high concentrations of calcium and inorganic phosphate, but low levels of fluoride. Cocoa, tea and coffee contained no calcium and, within this group, only cocoa contained moderate amounts of phosphate. The teas contained 3–4 μg/ml of fluoride, more than any of the other extracts. Total polyphenols and tannins varied over a narrow range (0.3–4.0 and 0.1–2.0 mg/ml, respectively), with the more coloured juices and beverages containing the highest concentrations of these substances. Extracts of banana, nuts and cheese contained low levels of polyphenols and barely-detectable levels of tannins.

Table 1. Composition of food extracts

Extract	Dry weight (mg/ml)	Total carbohydrate (mg/ml)	Calcium (μg/ml)	Inorganic phosphate (μg/ml)	Fluoride (μg/ml)
Apple	115	97	42	64	0.2
Black cherry	132	103	145	144	0.9
Cranberry	49	29	82	21	1.2
Grape	141	127	154	87	0.7
Prune	176	100	111	168	0.6
Cocoa	15	5	<1	155	0.1
Coffee	11	5	<1	11	1.3
Tea (L)	3	<1	<1	9	3.1
Tea (S)	3	<1	<1	9	3.6
Almond	51	4	—	86	0.1
Banana	60	<1	—	33	0.5
Peanut	86	5	—	66	0.2
Swiss cheese	25	—	—	75	0.1

Inhibition of the biosynthesis of insoluble EPS by the juices (Table 3) was about 90 per cent; the extracts and beverages that contained low concentrations of carbohydrate gave inhibitions that ranged from 15 to almost 100 per cent. Soluble EPS formation was affected similarly, but the degree of inhibition was smaller than that obtained for the synthesis of insoluble polysaccharide. Gall-nut tannic acid (5–10 mg/ml) completely inhibited both soluble and insoluble EPS biosynthesis. Calcium, inorganic phos-

Table 2. Sugar and polyphenol content

Extract	Glucose (mg/ml)	Fructose (mg/ml)	Sucrose (mg/ml)	Total polyphenols (mg/ml)	Tannins (mg/ml)
Apple	43	26	38	0.4	0.1
Black cherry	55	22	46	2.3	0.3
Cranberry	19	9	17	1.6	1.3
Grape	61	33	58	3.1	1.7
Prune	64	20	56	2.4	0.5
Cocoa	11	<1	2	4.0	2.0
Coffee	<1	<1	<1	2.0	0.4
Tea (L)	<1	<1	<1	1.0	0.2
Tea (S)	<1	<1	<1	0.9	0.4
Almond	<1	<1	<1	0.2	—
Banana	<1	1	<1	0.1	0
Peanut	2	<1	<1	1.5	0.1
Swiss cheese	<1	<1	<1	0.5	0

Table 3. Inhibition of EPS biosynthesis by food extracts

		Glucosyltransferase activity			
		Insoluble EPS		Soluble EPS	
Addition	μl	cpm/50 μl	Percentage inhibition	cpm/50 μl	Percentage inhibition
None	—	4313	—	3507	—
Apple	150	636	85.2	1204	65.7
Black cherry	150	468	89.1	1662	52.6
Cranberry	150	1768	59.0	3048	13.1
Grape	150	289	93.3	1266	63.9
Prune	150	633	85.3	2050	41.5
None	—	9111	—	7203	—
Tea L	500	498	94.4	3898	45.9
Tea S	500	516	94.3	4411	38.8
None	—	3455	—	3124	—
Almond	500	1874	45.8	2095	32.9
Banana	500	454	86.9	1525	51.2
Cocoa	250	2070	40.1	2290	26.7
Coffee	250	2553	26.1	2512	19.6
Peanut	250	1801	47.9	2109	32.5
Swiss cheese	500	2920	15.5	3097	0.8

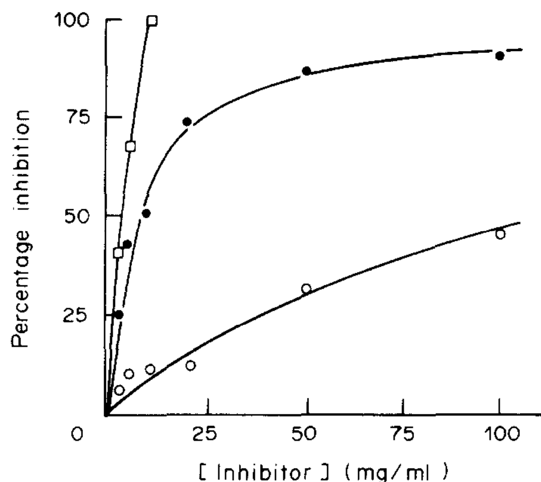

Fig. 2. Inhibitory effects of added hexoses. Glucosyltransferase from *Strep. mutans* 6715 was assayed with increasing concentrations of fructose (●) or glucose (○), or with increasing concentrations of gall-nut tannic acid (□).

phate and fluoride, at the concentrations found in the extracts, did not inhibit EPS formation.

Effects of endogenous sugars

An investigation was undertaken to determine the effects of the individual, measured components of the extracts on GTF activity. Hexoses inhibit GTF (Gibbons and Nygaard, 1968; Chludzinki, Germaine and Schachtele, 1976; Newbrun, Finzen and Sharma, 1977). Figure 2 shows the curves of inhibition that were obtained when glucose or fructose were examined as inhibitors of EPS biosynthesis. Concentrations were chosen to span those found in the extracts. Inhibition was determined also for increasing concentrations of gall-nut tannic acid. All three additives inhibited enzymic activity. Inhibition was greatest with tannic acid, less with fructose and least with glucose. The ratio of the molar activities (i.e. the millimolar concentrations needed to produce 25 per cent inhibition, and assuming only a moderate degree of purity of the tannic acid) was approx. 1:10:140.

In the assays of inhibition by the juices, the juices were diluted so that, for example, grape juice contributed about 4 mg glucose, 2 mg fructose and 3.5 mg sucrose/ml of assay mixture. From the data (Fig. 2), it can be calculated that the hexoses in grape juice would inhibit EPS biosynthesis by about 20 per cent. The endogenous sucrose, on the other hand, acted as a diluent of the [^{14}C]-sucrose in the assay system and gave an apparent inhibition of polysaccharide formation. Studies of the degree of apparent inhbition, with increasing concentrations of added sucrose, showed that the endogenous sucrose in grape juice would inhibit enzyme activity by about 30 per cent. The total observed inhibition, therefore, would be about 50 per cent. Actual inhibition by the juice was 46 per cent (Table 4). Calculations for the other juices gave similar results. Sugar concentrations of the extracts of cocoa, tea, coffee, banana, nuts and cheese were too low (Tables 1 and 2) to account for the observed inhibitions by these preparations.

Polyphenols

Tannins interact with proteins to give insoluble precipitates (Hagerman and Butler, 1978). Accordingly, cocoa, tea and coffee extracts were treated with gelatin to remove the tannins and the resulting supernatant fluids were assayed for inhibitory activity (Table 4). About 50 per cent of the total polyphenols were removed from the beverages as gelatin-precipitable tannins. The inhibitory activities of teas L and S were reduced by 43 and 51 per cent, respectively, by the treatment, but the cocoa and coffee were not affected and all of the inhibitor remained in the gelatin-soluble fraction.

Treatment of either the original beverages or the gelatin-soluble supernatants with charcoal (150 μg Norit A/ml) removed nearly all the polyphenols and all the inhibitory activity. (+)-Catechin, a monomeric polyphenol known to occur in the juices and beverages was an effective inhibitor of enzymic activity, giving 25 per cent inhibition with 2.5 mg catechin/ml. Gallic acid and other monomeric polyphenols were without effect on enzyme activity.

The removal of tannins from the juices affected inhibition only slightly. Thus, treatment of grape and black-cherry juices with gelatin reduced inhibition of GTF by 0 and 15 per cent, respectively (Table 4). Inhibitory effects of cranberry and prune juices were

Table 4. Effect of gelatin treatment of extracts on GTF inhibition

	Untreated extract		Gelatin-soluble supernatant		Gelatin-precipitable tannins	
Extract	Percentage inhibition	Polyphenols (mg/ml)	Percentage inhibition	Polyphenols (mg/ml)	Percentage inhibition	Polyphenols (mg/ml)
Tea (L)	58	1.2	33	0.6	25 (43)*	0.6 (50)
Tea (S)	43	0.5	21	0.2	22 (51)	0.3 (60)
Cocoa	33	0.6	31	0.3	2 (6)	0.3 (50)
Coffee	61	3.2	63	1.7	−2 (0)	1.5 (47)
Grape juice	46	0.7	46	0.4	0 (0)	0.3 (43)
Black-cherry juice	40	0.6	34	0.4	6 (15)	0.2 (33)

*Values in parentheses indicate the percentage of inhibition relative to the control, gelatin-untreated extract.

reduced by 5 and 10 per cent, respectively, but apple juice was not affected. Further treatment of the gelatin-soluble fractions from grape and black-cherry juices with charcoal did not change the inhibitory activities of the preparations. The inhibitory activities of the extracts of banana, nuts and cheese were not affected by treatment with gelatin.

DISCUSSION

The accumulation of cariogenic microorganisms such as *Strep. mutans* is enhanced by the formation of extracellular polysaccharide from sucrose (Gibbons, 1983). Many foods contain sufficient levels of sucrose to permit plaque organisms to form such polysaccharides, and it seems that any food constituents that can interfere with polysaccharide formation could affect the long-term survival of cariogenic organisms within the mouth. Such substances, therefore, may be important determinants of cariogenicity.

The present findings demonstrate that foods possess effective inhibitors of streptococcal glucosyltransferases; at least two major classes of inhibitors exist in fruit juices and beverages, one being the polyphenols and the other the free hexoses in the juices. We (Paolino and Kashket, 1985) demonstrated that the inhibitor in cocoa extract brings about a non-competitive inhibition of the enzyme and subsequent experiments showed that inhibition by cocoa extract is irreversible (data not presented). The effects of the exposure of plaque organisms to such extracts, therefore, may persist for some time. Inhibition by fructose is of the competitive type (Chludzinski *et al.*, 1976), the hexose presumably acting as alternate acceptor for the enzyme-coupled glucosyl moiety, in place of the growing polysaccharide chain. Regardless of the details of the mechanism, inhibition by hexoses took place at concentrations that were within the range of those existing within the beverages. The findings suggest that it may be important to consider the potential of foods to affect polysaccharide formation when assessing their cariogenicity.

The data with the teas indicate that the tannins can be inhibitory, but it is clear that the greater part of the inhibition by tea, and the entire inhibition by cocoa and coffee extracts, was due to substances that remained in solution following treatment with gelatin. The absorption to charcoal of the residual inhibitors, concurrent with the removal of all measurable polyphenols, strongly points to an inhibition by monomeric polyphenols. In support of this view, added catechin was an effective inhibitor of polysaccharide formation; furthermore Iio *et al.* (1984) showed that naringenin and other flavanoids inhibit the enzyme activity. Polyphenols occur widely in nature in the monomeric form and often co-exist in plants with the higher-molecular-weight tannins. Furthermore, processing of beans and tea leaves can lead to oxidation and polymerization of the monomeric polyphenols (Haslam, 1979). Clearly, the exact nature of the gelatin-soluble inhibitors in the beverages needs to be determined before a better understanding of the roles of the various polyphenols in the control of polysaccharide biosynthesis is achieved.

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