Aggregation of human salivary Ca-proteinates in the presence of simple carbohydrates in vitro

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Abstract – The effect of 8 polyols and 14 aldoses or ketoses on the spontaneous aggregation of Ca-proteinates was followed spectrophotometrically in supernatants and filtrates of human mixed saliva. Each carbohydrate was added to the saliva samples at 37°C and the precipitated material was analyzed for protein, total carbohydrate and Ca. Based on their effect on aggregation, the carbohydrates could be divided into three groups: 1) those that showed no effect on aggregation: D-xylose, D-ribose and *i*-erythritol, 2) those that inhibited aggregation strongly: xylitol, D-sorbitol and D-mannitol, and 3) those that inhibited aggregation moderately: glucose, fructose and sucrose. The inhibitory effect of the above polyols on the aggregation of Ca-proteinates varied greatly among the saliva donors, and correlated positively with the turbidity of the saliva and its protein content more than with the Ca-concentration or the pH of the saliva sample. It is suggested that inhibition of aggregation shown the most clearly for xylitol, sorbitol and mannitol manifests itself as a retardation of the final, irreversible aggregation of those glycoproteins that already exist in a precipitated form and which are responsible for the turbidity of saliva.

Key words: Ca-proteinates; carbohydrates; polyols; saliva.

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The protein components of saliva participate in the formation of acquired pellicle (1–4) and dental plaque (5, 6) and in the clearance of bacteria from the oral cavity (7, 8). Comparative analyses have suggested a close analogy between the salivary proteins and those of the acellular plaque matrix (1, 9). According to JENKINS (10), Ca^{2+} precipitates salivary glycoproteins which adhere to the enamel and also adsorb to the developing plaque. Acids produced by plaque bacteria liberate Ca^{2+} which in turn may participate in the precipitation of new Ca-proteinates. The composition of the material precipitated depends on the pH; at low pH values the precipitate is high in glycoprotein and low

in calcium and phosphate. At higher pH values, however, the ratio is the opposite (11).

The favorable influence of xylitol on oral health has partly been attributed to the non-fermentability of this polyol and to its effects on the amount and composition of dental plaque (12–14). Apart from a few studies (15, 16), the direct physicochemical effects of polyols on saliva have not been investigated. Consequently, the aim of this study was to compare the influence of several simple sugars and polyols on the spontaneous aggregation which takes place in saliva, and if possible to relate these effects to the structure of the carbohydrates and the composition of saliva. A parallel study investigated Ca^{2+} complex formation with a number of common carbohydrates (16). This study suggested that the ability of xylitol and other polyols to complex Ca^{2+} may play a role in the remineralization process of caries lesions, as well as in other reactions involving Ca^{2+} in the oral cavity.

Material and methods

The sugars and polyols examined were D-glucose from BDH (Poole, England); *i*-erythritol, D-arabitol, L-arabitol, ribitol and D-mannose from Sigma (St. Louis, MO, USA); D-xylose, glycerol, D-fructose, xylitol, D-sorbitol and D-mannitol from Merck (Darmstadt, Germany); D-ribose, D-arabinose, L-xylose and L-sorbose from Fluka (Buchs, Switzerland); DL-glyceraldehyde, L-arabinose, L-lyxose, D-lyxose and D-allose from Serva (Heidelberg, Germany) and galactose from Gurr (London, England).

Paraffin-stimulated mixed saliva was collected after regular oral hygiene procedures between 9 a.m. and 10 a.m. from men and women (aged 25–35 yr) who had refrained from eating and drinking for at least 2 h prior to saliva collection. The collection of saliva from each subject was completed in 10 min. The individual or pooled samples were centrifuged at $3000 \times g$, $6000 \times g$ or $12100 \times g$ for 5 min at 4°C or filtered at 25°C (Millipore HA, 0.45 μm) before addition of the solid carbohydrates at 37°C. The aggregation of Ca-proteinates was followed at this temperature for 30 min spectrophotometrically at 540 nm in the supernatant, or at 700 nm in the filtrate of saliva in the presence of added carbohydrates. The volume of the incubation mixture was 0.5 ml or 1.0 ml. The change in turbidity was compared both to the turbidity recorded before incubation and to that of control mixtures incubated without added carbohydrate. All incubations were performed in 7 ml capped plastic tubes. In some experiments the rate of aggregation was deliberately increased by addition of 20 μl aliquots of concentrated $CaCl_2$ solutions made in distilled water to 1 ml of the salivary supernatants or filtrates of saliva.

For analysis of the precipitated material, centrifugation for 60 min at $50\,000 \times g$ was used. The pellet was suspended in saline (+4°C) by 30 s sonication (MSE disintegrator, amplitude 6 μ, end diameter of the probe 3 mm) and the homogeneous suspension was divided into two equal aliquots. For analysis of total proteins (17), one aliquot was boiled for 1 h in 1.0 M NaOH, whereas the other aliquot was used for the determination of calcium by atomic absorption spectrophotometry (Perkin-Elmer 460 Atomic Absorption Spectrophotometer, Norwalk, CT, USA).

Phosphate was analyzed by the malachite-green method (18), total sialic acids by the thiobarbiturate method (19), total carbohydrates with the Anthrone reagent (20), and Na by atomic absorption spectrophotometry. Neuraminidase activity was determined according to the Worthington Manual (Worthington Biochemical Corporation, Freehold, NJ, USA). The pH values were measured electrometrically with a combination electrode and the buffering capacity of saliva was measured by the Dentobuff system (Orion Diagnostica, Espoo, Finland)

Results

Pooled samples of paraffin-stimulated mixed saliva of two donors were used in a series of preliminary experiments for the assessment of optimal experimental conditions for studying the effect of D-xylose, D-arabinose, D-arabitol, D-glucose, D-sorbitol and xylitol on the aggregation of salivary proteins. Incubation at 37°C of pooled saliva supernatant

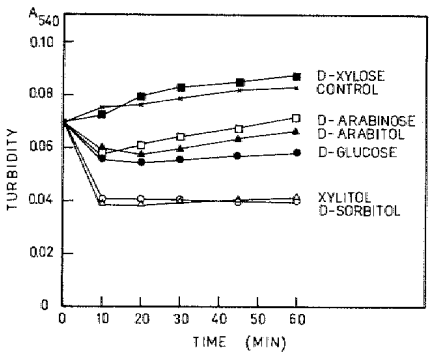

Fig. 1. Time dependence of aggregation of Ca-proteinates in supernatant fluid of mixed saliva ($3000 \times g$). Degree of aggregation was recorded at A_{540} in the presence of different carbohydrates at 0.5 M and 37°C.

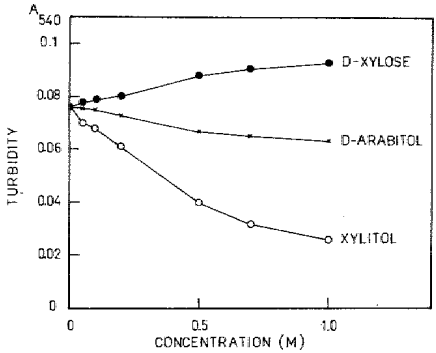

Fig. 2. Dependence of degree of aggregation of Ca-proteinates on concentration of carbohydrate in supernatant fluid of mixed saliva ($3000 \times g$). Turbidities (A_{540}) were recorded after 30 min incubation at 37°C.

($3000 \times g$) from these subjects showed strong inhibition of aggregation with xylitol and D-sorbitol, weak inhibition with D-arabinose, L-arabitol and D-glucose, and practically no effect on aggregation with D-xylose as compared to the control (Fig. 1). The concentration dependence of the effect of xylitol, D-arabitol and D-xylose on precipitate formation is shown in Fig. 2. The influence of other experimental conditions on aggregation was studied more closely with xylitol and D-sorbitol. The effect of pH on aggregation was studied in aliquots of a salivary supernatant (original pH 7.2). The pH was adjusted with lactic acid to pH 4.0. The inhibition caused by the above polyols was of similar degree at both pH 7.2 and 4.0. An experiment with pooled supernatants centrifuged at 3000, 6000 and $12\,100 \times g$ showed that the inhibition percentages for D-sorbitol and xylitol were of the same magnitude in all three supernatants. The turbidity changes were highest in the $3000 \times g$ supernatant. Addition of $CaCl_2$ (final concentration 0.025 M) to the supernatant fluids increased the turbidity but the inhibitions obtained with both polyols were again of the same degree as without added $CaCl_2$. Based on these preliminary experiments, precipitation of saliva in the presence of various 0.5 M carbohydrates was studied at 37°C using the $3000 \times g$ supernatants and 30 min reaction time without adjustment of the pH or Ca-concentration. The results are summarized in Fig. 3.

Based on their effects on aggregation the carbohydrates could be divided into three groups: those without effects, moderate inhibitors and strong inhibitors. When the same experiment was performed with saliva samples from six other donors, individual differences were observed in the effect of some carbohydrates: L-arabitol had no effect on aggregation in some samples, and the degree of inhibition obtained with D-glucose, DL-glyceraldehyde, D-allose and glycerol also varied individually. 0.5 M sucrose showed slight inhibition of aggregation.

The individual variation that occurred in the degree of inhibition of aggregation in the presence of xylitol and D-sorbitol was studied with eight donors of saliva, chosen to represent different flow rates, pH and buffering capacity of saliva. Xylitol and D-

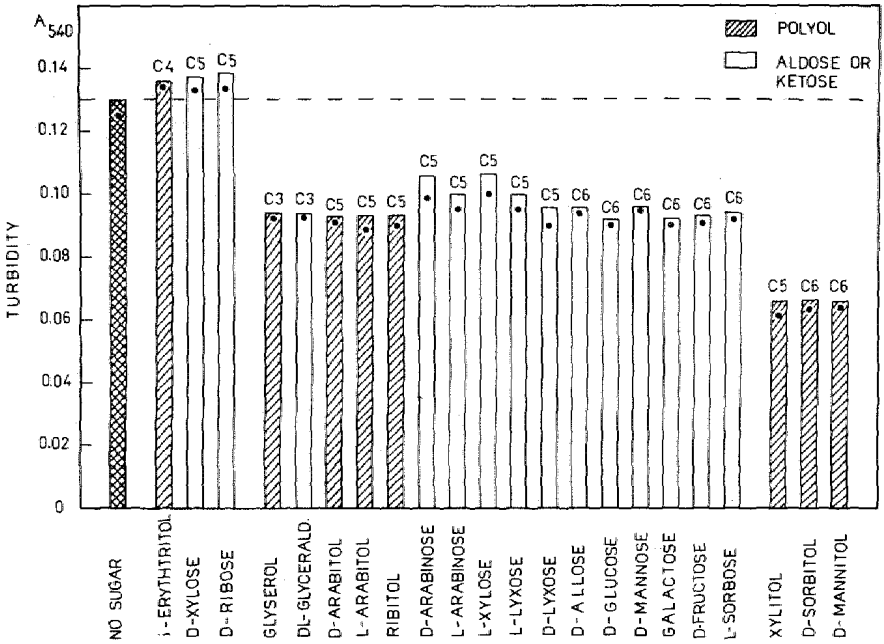

Fig. 3. Effect of various carbohydrates (0.5 M) on degree of aggregation of Ca-proteinates in supernatant fluids of mixed saliva ($3000 \times g$). Turbidities (A_{540}) were recorded after 30 min incubation time. ▨ Polyol added; □ aldose or ketose added; ▩ no carbohydrate added. Columns show means of two determinations and dots give range of determinations.

sorbitol caused an inhibition of the same magnitude. However, the individual variance in the inhibition percentage ranged from 14% to 39%. The only salivary parameters that showed correlation with the degree of inhibition were the initial turbidities of the salivary supernatants ($r=0.75$), total sugar ($r=0.78$) and total protein ($r=0.72$). No correlation was observed with salivary pH and buffering capacity, the content of Na, Ca, inorganic phosphate and total sialic acids, and neuraminidase activity. Repetition of these tests with the same subjects on the following day showed that the degree of inhibition induced by xylitol and D-sorbitol varied also for one and the same individual, and was associated with variations in the turbidities of the saliva samples. However, only the degree of inhibition varied individually, and no qualitative differences were detected between the various carbohydrates.

Chemical analysis of the material precipitated in the presence of 0.5 M xylitol and D-sorbitol compared to that without added polyol revealed no differences between the ratios of Ca or total carbohydrates to protein, these ratios being 51.0–58.7 μg/mg and 220–235 μg/mg ($n=3$), respectively. Thus, the effect of the polyols on aggregation appeared to be quantitative rather than qualitative. The analysis also justifies the use of the term Ca-proteinate to describe the precipitated material.

The importance of the turbidity of the salivary supernatants for the inhibitory effect

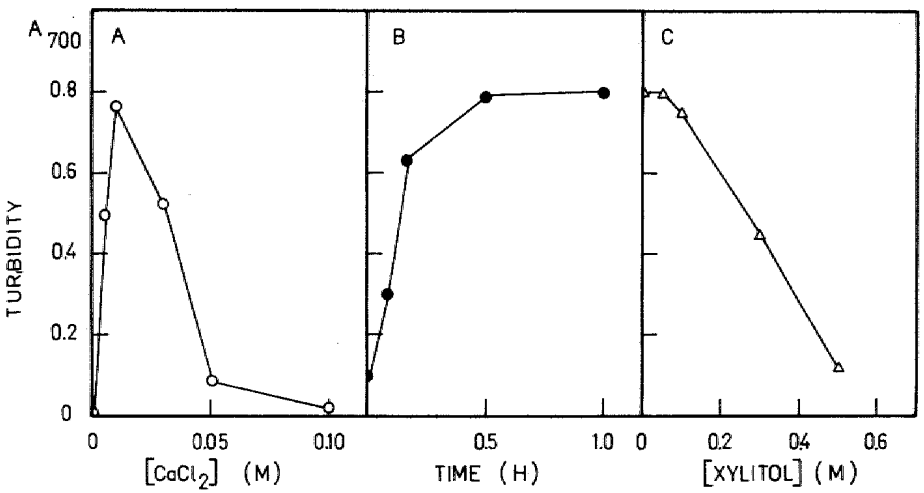

Fig. 4. Precipitation of Ca-proteinates in millipore-filtered supernatant fluid of mixed saliva. A, effect of Ca concentration on degree of precipitation (A_{700}) of Ca-proteinates after 30 min incubation time. B, dependence of degree precipitation on time in presence of 0.02 M $CaCl_2$. C, effect of xylitol concentration on degree of precipitation in presence of 0.02 M $CaCl_2$ determined after 30 min incubation.

of polyols was also studied using millipore-filtered saliva, collected from the two donors mentioned above. An optimal concentration of $CaCl_2$ (0.02 M; Fig. 4A) and incubation time (0.5 h; Fig. 4B) were used in this experiment to study the effect of 0.05–0.5 M xylitol on Ca-induced precipitation. Xylitol markedly inhibited the aggregation (Fig. 4C), but an identical effect was also obtained with the three other carbohydrates studied: L-xylose, D-sorbitol and D-glucose, chosen to represent strong and weak inhibitors of aggregation.

Discussion

The substances causing the turbidity of saliva are very heterogeneous. However, the inhibition of aggregation caused by xylitol and D-sorbitol was detected in both the most turbid $3000 \times g$ and least turbid $12100 \times g$ supernatants. This suggests that the inhibition was directed to the aggregation of proteins produced by the salivary glands and not, for example, to the agglutination of cellular and other debris present in saliva. The high calcium content of the precipitated material, as well as the result of the experiment performed in the presence of Ca^{2+} and without added calcium, also supported the idea that the polyols inhibited calcium-induced aggregation of *salivary* proteins. Thus, the inhibition of aggregation could be attributed either to Ca-precipitable glycoproteins of saliva or specific salivary proteins controlling the precipitation of minerals, such as statherin and acidic proline-rich proteins (21, 22). No difference, however, could be detected between the effects of aldoses (L-xylose, D-glucose) and polyols (xylitol, D-sorbitol) on the precipitation of Ca-proteinates in the millipore-filtered saliva. This result, as well as the time-dependence of the aggregation inhibition (Fig. 1), suggests that the aggregation inhibition caused by xylitol and D-sorbitol seems to manifest itself as a retardation of the aggregation of such pro-

teins as already exist in a precipitated form and which are responsible for the turbidity of saliva. Extracellular enzymes produced by oral bacteria deplete the carbohydrate moieties of salivary glycoproteins and cause the precipitation of the residual proteins (23). With the strong inhibitors of aggregation, D-sorbitol and xylitol, the degree of inhibition stayed, however, on the same level during the 1-h experiment (Fig. 1). Only with the carbohydrates without effect or the moderate inhibitors of precipitation was a slight time-dependent increase in the degree of precipitation observed (Fig. 1). Thus the role of hydrolytic enzymes in the phenomenon described in the present paper appears to be small.

The strong inhibition of the precipitation of soluble salivary proteins induced by addition of Ca^{2+} to millipore-filtered saliva is interesting, although the effects of the two aldoses and polyols were similar. It is interesting that a xylitol concentration of 5%, which would be feasible in foodstuffs, caused an inhibition of about 50% (Fig. 4). The local concentration of nonfermentable or slowly fermented carbohydrates in the oral cavity can be expected to remain higher for a longer period compared to that of fermentable ones. This fact may further prolong the inhibitory effect of xylitol and sorbitol in vivo.

As to the mechanism of the inhibition of the aggregation effects of carbohydrates on salivary proteins themselves as well as calcium-carbohydrate interactions should be considered. It has been shown in several studies that sugars and especially polyols protect protein structures (24–26). The protein stabilization by polyols has been attributed to polyol-induced strengthening of the hydrophobic interactions in protein molecules. This phenomenon alone, however, does not explain the present results statisfactorily, since the effect of polyols on the hydrophobic interactions should be determined by the molecular weight of linear polyols. Although the interaction between polyols and proteins may be considered nonspecific (26), the number of polar and hydrophobic regions of a protein will determine the effect of polyols on this protein. Thus the effect of polyols may be expected to be different for acidic proline-rich phosphoproteins or the tyrosine-rich statherin on one hand, and salivary glycoproteins on the other. The ability of polyols to complex calcium and delay precipitation of calcium phosphate in vitro most likely plays some role in this phenomenon as well (16). However, no simple correlation between the effect of carbohydrates on aggregation of salivary Ca-proteinates and the structure or chemical properties of these carbohydrates could be revealed. Results of LEACH *et al.* (15) support the idea that both calcium-carbohydrate and protein-carbohydrate interactions are involved. The above study demonstrated that some sugar substitutes, used as solutions with the same concentration percentage, decreased the turbidities of both suspensions of dental plaque and acellular mixed saliva. With saliva the sugar substitutes decreased the turbidities in the following order: sucrose ~ maltose < glucose < lycasin < xylitol < sorbitol << maltitol. Carbohydrate-induced hydrophobic interactions of proteins were considered to explain the results. However, our study only partly supports this idea. Firstly, equimolar solutions of xylitol and sorbitol would most likely have exerted an identical effect on the turbidity of saliva. Secondly, the superiority of maltitol in decreasing the turbidities as compared to other sugar substitutes included in the study (15) may also at least partly result from calcium-carbohydrate interactions. Our previous study showed that maltitol was by far the most effective retarder of precipitation of calcium phosphate in vitro (16).

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